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PROGRAMA DE BIOMEDICINA

TESIS DOCTORAL

LEUCEMIA LINFOBLÁSTICA AGUDA PRO-B DEL LACTANTE: IMPACTO FUNCIONAL DEL ONCOGÉN MLL-AF4 EN CÉLULAS PROGENITORAS HEMATOPOYÉTICAS DE CORDÓN UMBILICAL Y EN CÉLULAS TRONCALES EMBRIONARIAS HUMANAS.

Memoria presentada por ROSA Mª MONTES LORENZO para optar al grado de DOCTOR.

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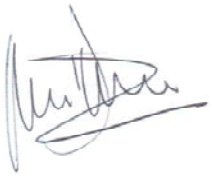
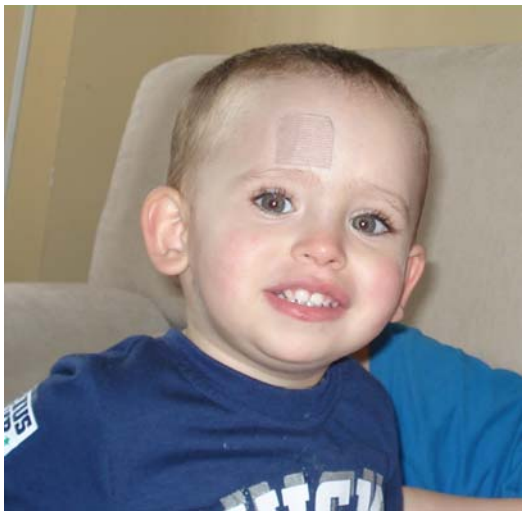


Doctorando

ROSA Mª MONTES LORENZO

Fdo.

Dedicado a Lorenzo,
con todo mi amor.



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Me gustaría, finalmente, tener un recuerdo muy especial para las personas enfermas de cáncer y sus familias. Con ellos, todo nuestro afecto, apoyo y cariño.

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NOTA

A lo largo del texto se utilizarán las abreviaturas de varios términos en inglés. Esto es debido al uso extendido del inglés en publicaciones científicas y al uso de abreviaturas en inglés de manera cotidiana en el lenguaje científico. En el glosario de abreviaturas, al final de esta memoria, se detalla la descripción en inglés y en castellano de cada abreviatura. Así mismo, en este glosario se recogen las abreviaturas de los términos usados en castellano.

INTRODUCCIÓN

1.1 HEMATOPOYESIS: CONCEPTO Y JERARQUÍA.

La hematopoyesis es el proceso fisiológico que conduce a la formación de las células de la sangre. Es un proceso continuo en el que la destrucción diaria de millones de células se compensa con la formación permanente y ajustada de nuevas células.

El sistema hematopoyético deriva del mesodermo. En el ser humano, la hematopoyesis tiene diferentes localizaciones anatómicas a lo largo del desarrollo embrionario y postnatal. En el desarrollo embrionario, la hematopoyesis comienza principalmente a nivel de saco vitelino a partir de los precursores hemogénicos con capacidad de diferenciación hematopoyética y endotelial (Zambidis et al. 2006). Estos precursores hemogénicos derivan de mesodermo extraembrionario y son el origen de la vasculogénesis y de la formación de sangre primitiva, no definitiva, en los islotes hematopoyéticos (conocidos como *blood islands*) del saco vitelino, restringiéndose a la producción de células eritroides nucleadas con hemoglobina embrionaria. En la literatura, y según se refiera al mamífero en general, ratón o humano, se entremezcla el concepto de precursor hemogénico con endotelio hemogénico. En ambos casos, las células hematopoyéticas más inmaduras (en muchos casos embrionarias; hematopoyesis no definitiva) se originan a partir de células con fenotipo endotelial ($CD45^-$, $CD31^+$, VE-Cad^+, $CD34^+$, c-kit^+, etc.). Posteriormente, la hematopoyesis es intraembrionaria, en la esplectopleura paraaórtica, y luego en la región aorta-gónada-mesonephros (AGM) (Ivanovs et al. 2011). Entre el segundo y séptimo mes de gestación es el hígado, y en menor medida el bazo, ganglios linfáticos y timo, los lugares de actividad hematopoyética, para colonizar definitivamente la médula ósea (MO) y tejidos linfáticos periféricos a partir del séptimo mes de embarazo (Dzierzak and Speck 2008). En el adulto, es la MO el órgano principal de generación de células hematopoyéticas en condiciones fisiológicas y patológicas.

La hematopoyesis es un sistema jerárquico en el que los elementos más inmaduros van proliferando y diferenciándose hacia las formas maduras y funcionales en base a las necesidades fisiológicas. Se reconocen a nivel funcional y fenotípico diversos tipos celulares que podemos agrupar en células troncales hematopoyéticas (HSCs), células progenitoras hematopoyéticas (HPCs), células precursoras comprometidas a un linaje y células maduras. Las HSCs tienen la máxima capacidad de autorenovación y diferenciación hacia linajes linfoide y mieloide. La autorenovación puede ser simétrica o asimétrica. En el primer caso, tras la división celular, las dos células hijas resultantes son idénticas a las HSC y se mantienen indiferenciadas. En el caso de la división asimétrica, la HSC origina otra HSC y una célula ya comprometida/diferenciada a un linaje. Las HSCs son multipotentes, las únicas responsables de regenerar a largo plazo el sistema hematopoyético del receptor post trasplante, por lo que se denominan LT-HSCs (*Long Term Hematopoietic Stem Cells*). Representan menos del 0,01% del total de células nucleadas de la MO.

Las HPCs tienen ya una capacidad de diferenciación más restringida y comprometida hacia el linaje linfoide o hacia el linaje mieloide. Por ello, no pueden reconstituir el sistema hematopoyético a largo plazo

pero si juegan un papel fundamental en dicha reconstitución en las tres primeras semanas post trasplante, en la recuperación de neutrófilos y plaquetas (Menendez et al. 2002). El proceso de diferenciación a línea mieloide o linfoide no se conoce en detalle pero no es estocástico o aleatorio, ya que las necesidades fisiológicas, las condiciones locales del nicho, las concentraciones de factores de crecimiento hematopoyético, y las señales directas emitidas por los componentes celulares del microambiente, pueden favorecer la diferenciación hacia una línea determinada. Las células precursoras son progenitores monopotenciales y tienen restringida su diferenciación hacia una línea celular específica, como puede ser la eritroide, gránulo-monocítica, megacariocítica, etc. Las células precursoras son fenotípicamente y morfológicamente reconocibles (mieloblastos, promonocitos, eritroblastos, megacariocitos, etc.). Las células maduras no tienen capacidad de división y son las funcionalmente activas (leucocitos, hematíes y plaquetas). Son las encargadas de mantener una fisiología normal y compensada que dá respuesta a las necesidades fisiológicas diarias.

En todo este proceso, mientras que la capacidad de diferenciación va disminuyendo, la actividad proliferativa aumenta. Así, las HSCs más inmaduras están en su mayoría en estado quiescente o con baja tasa de proliferación, mientras que las células en estadios de precursor tienen una alta capacidad proliferativa y de amplificación, que cesa en los estadios más maduros. Es importante comprender que el estado quiescente de las HSCs corresponde a un estricto control fisiológico natural para evitar introducir mutaciones durante las divisiones. La proliferación descontrolada de HSCs y HPCs se asocia al desarrollo de leucemias agudas.

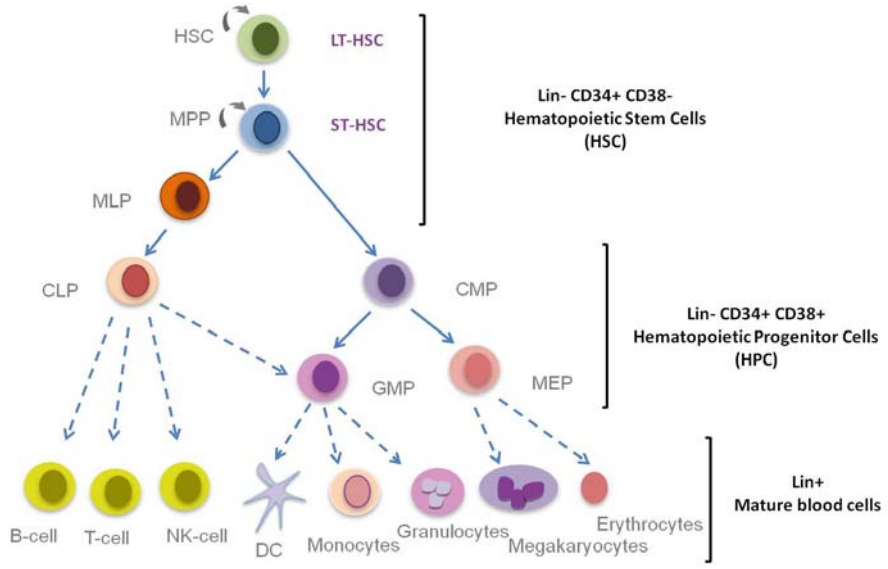

Figura 1. Esquema representativo de la hematopoyesis humana. Las HSCs, que tienen capacidad de autorenovación (Lin-CD34+CD38-), comprenden tanto las células troncales con capacidad de repoblación hematopoyética a largo plazo (LT-HSC) como los progenitores multipotentes (MPPs) con capacidad de reconstitución hematopoyética a corto plazo (ST-HSC). Las HPCs (Lin-CD34+CD38+) tienen ya comprometida su capacidad de diferenciación hacia linaje linfoide (CLP) o linaje mieloide (CMP). Finalmente, estos progenitores hematopoyéticos se diferencian hacia las células maduras con marcadores de linaje específicos (Lin+); células B, células T, células NK (*natural killer*), células dendríticas (DC), monocitos, granulocitos, megacariocitos y eritrocitos. Imagen cedida por Lourdes López-Onieva (Dominique Bonet Lab. Cancer Research UK. London, UK).

Diferenciación mieloide o mielopoyesis

La mielopoyesis comienza con la diferenciación de las HSCs a progenitores mieloides, que tienen alta capacidad proliferativa y se identifican por el inmunofenotipo $CD34^{+}CD38^{+}CD90^{-}CD45RA^{-}FLT3^{+}CD33^{+}CD13^{+}CD117^{+}$. Este progenitor mieloide da lugar al progenitor eritrode-megacariocítico y al progenitor gránulo-monocítico.

El progenitor eritroide-megacariocítico dará lugar a su vez a los precursores eritroides y a los precursores megacariocíticos. Los precursores eritroides más inmaduros son los BFU-E, que aún mantienen alto potencial de proliferación, y que dan lugar a los CFU-E, con potencial de proliferación limitado. La diferenciación de estos precursores continúa dando lugar a formas intermedias (pro-eritroblastos, eritroblastos basófilos, eritroblastos policromatófilos, eritroblastos ortocromáticos y reticulocitos) que temina con la formación de eritrocitos tras un proceso de enucleación. Durante esta ruta de diferenciación actúan diferentes citocinas reguladoras claves como son eritropoyetina (EPO), interleucina (IL)-3, el ligando de FLT3 y SCF (del inglés *stem cell factor*).

Los precursores megacariocíticos derivados del progenitor eritroide-megacariocítico, corresponden con los Meg-BFU, que dan lugar a los Meg-CFU. A partir de los mismos se generan los precursores poliploides, como son los megacariocitos inmaduros y los maduros, que darán lugar a las plaquetas mediante un proceso de digestión citoplasmático. Las citocinas reguladoras de este proceso son la trombopoyetina (TPO), IL-3, IL-6 e IL-11.

La diferenciación gránulo-monocítica comienza con los precursores GM-GFU, que dan lugar a las unidades formadoras de colonias granulocíticas (G-GFU) y a las unidades formadoras de colonias monocíticas (M-GFU). Las G-CFUs se diferencian a precursores tipo mieloblastos, pro-mielocitos, mielocitos, metamielocitos y células maduras (neutrófilos, eosinófilos y basófilos). Las M-CFUs se diferencian a monoblastos, promonocitos, mononocitos y finalmente macrófagos y células dendríticas. En la diferenciación gránulo-monocítica intervienen los siguientes factores de crecimiento GM-CSF, G-CSF, M-CSF, así como IL-3, IL-6 y el SCF.

Diferenciación linfoide o linfopoyesis.

Consiste en el proceso de proliferación y diferenciación de los progenitores linfoides que culmina con la producción de linfocitos B, linfocitos T, células NK (*natural killer*) y células dendríticas. Las primeras fases de la diferenciación linfoide se desarrolla en la MO, aunque la maduración de los linfocitos T y B se produce en timo y bazo, respectivamente.

La linfopoyesis de linfocitos T en el timo es mantenida por la importación periódica de progenitores hematopoyéticos desde la MO a través del torrente sanguíneo, ya que el timo no contiene ni produce progenitores. Este proceso de maduración T consiste en la producción y presentación en la membrana del receptor de célula T (TCR), necesario para la activación de los linfocitos T. Tras la producción del

TCR, los linfocitos T sufren un proceso de selección relacionada con la afinidad por el Complejo Mayor de Histocompatibilidad (MHC). De este modo, los linfocitos T afines al MHC de clase I maduran hacia linfocitos T CD8+ (llamados citotóxicos), mientras que los linfocitos T con afinidad al MHC de clase II maduran hacia linfocitos T CD4+ (llamados *helper* o colaboradores). En el desarrollo de los linfocitos T a partir de HSCs, la vía de Notch y el eje IL7-IL7R juegan un papel clave que está bien documentado (Gonzalez-Garcia et al. 2012).

La linfopoyesis de linfocitos B tiene lugar en la MO y su desarrollo consta de dos fases; una fase independiente de antígeno y otra fase dependiente de antígeno (López-Larrea C. 1995). La primera fase de diferenciación se caracteriza por la producción de Inmunoglobulina M (IgM) citoplasmática y su presentación en la membrana celular, tras lo que se produce la selección de los linfocitos idóneos en bazo (células B maduras). La fase dependiente de antígeno, sucede cuando los linfocitos presentes en los órganos linfoides entran en contacto con antígeno, generando células B activadas, que darán lugar a células plasmáticas (secretoras de anticuerpos) y células de memoria. La selección de linfocitos B se produce en el bazo, donde entran en contacto con una serie de linfocitos T que presentan a los linfocitos B antígenos propios del organismo. Así, los linfocitos B que respondan a antígenos propios serán eliminados, mientras que el resto sobrevivirán y madurarán en el proceso dependiente de antígeno referido anteriormente. En la Figura 2 se muestra el inmunofenotipo de las distintas etapas del proceso de diferenciación de linfocitos B en los diferentes órganos hematopoyéticos donde tiene lugar (Perez-Andres et al. 2010). La IL-7 es una de las citocinas más importantes en la diferenciación de linfocitos a partir de HSC y HPC dado que aumenta la supervivencia y proliferación de los distintos estadios B (Gonzalez-Garcia et al. 2012). Además, en el desarrollo de los linfocitos B, los reordenamientos VDJH de las inmunoglobulinas es crucial, representando uno de los mecanismos moleculares más precisos pero complejos que ocurren en el sistema linfo-hematopoyético (Schatz and Ji 2011).

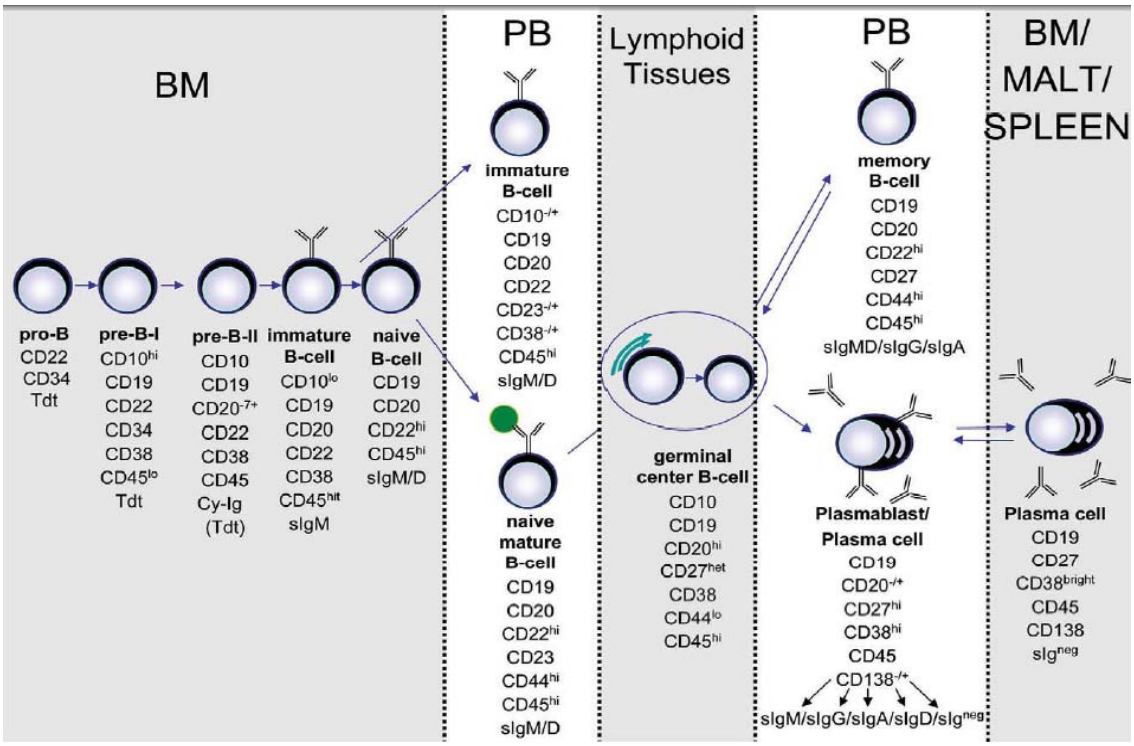

Figura 2. Diferenciación linfoide B: estadios celulares durante la diferenciación de los linfocitos B en MO (BM), sangre periférica (PB) y tejidos linfoides y mucosa-asociados (MALT), según sus características fenotípicas e isotipo (Perez-Andres et al. 2010).

Todo el proceso de formación de las células hematopoyéticas está regido por un delicado balance entre proliferación, diferenciación, maduración y apoptosis, en el que juegan un papel tanto factores intrínsecos como extrínsecos. Los factores intrínsecos incluyen reguladores del ciclo celular, factores de trascripción, microRNAs y elementos epigenéticos. Los elementos epigenéticos de regulación génica incluyen mecanismos como acetilación o metilación de histonas, en las que tienen un papel importante las proteínas metiltrasferasas, entre las que se incluye MLL. Los factores extrínsecos se corresponden con las señales proporcionadas por el microambiente o nicho donde se diferencian, proliferan y maduran (Lo Celso and Scadden 2011). Dentro de los factores extrínsecos se encuentran factores solubles (Sachs 1996), citocinas, quimiocinas, factores de crecimiento y diferenciación, e interacciones intercelulares. De esta manera, las HSCs responden de manera eficiente a condiciones de estrés, tales como pérdidas de sangre, infecciones o exposición a agentes citotóxicos, via expansión de las HSCs y demás progenitores sin depleción del compartimento de las HSCs.

El estudio de la funcionalidad de las HSPCs radica en ensayos *in vivo* e *in vitro*. El ensayo *in vivo* de reconstitución hematopoyética a largo plazo es el único que evalúa con certeza el potencial multipotente de las células troncales hematopoyéticas (Dick et al. 1997; Cavazzana-Calvo et al. 2011). Este ensayo se realiza mediante trasplante de células de interés en ratones inmunodeprimidos o inmunodeficientes. Cuando las células trasplantadas son de origen humano, este sistema se denomina xenotrasplante, y los ratones utilizados han de ser inmunodeficientes para minimizar una reacción inmunológica frente al

injerto. Así también, estos ratones son previamente irradiados o acondicionados para provocar, además, una aplasia medular que facilite espacio en la MO para el injerto. Inicialmente se han usado los ratones SCID (del inglés *severe-combined immunodeficiency*), NOD/SCID (del inglés *nonobese diabetic/severe-combined immunodeficiency*), y más recientemente se vienen utilizando otras cepas desarrolladas a partir de los ratones NOD/SCID como las NOD/SCID β2m$^{-/-}$ (Christianson et al. 1997) y NOD/SCID IL-2Rγ$^{-/-}$ (Shultz et al. 2005; Shultz et al. 2007). Los ratones NOD/SCID β2m$^{-/-}$ (Levac et al. 2005) no tienen expresión de MHC de clase I por lo que la función de los linfocitos NK está aún más atenuada. Los ratones NOD/SCID IL-2Rγ$^{-/-}$ (también conocidos como NOG o NSG) presentan deficiencias en la cadena gamma del receptor de la IL-2 lo que conduce a un mayor déficit en la inmunidad innata (Andre et al. 2010). Estos ratones, aún siendo inmunodeprimidos, son condicionados mediante irradiación para permitir la repoblación de células humanas. El injerto humano puede evaluarse mediante citometría de flujo en la médula ósea, bazo, sangre periférica y otros órganos hematopoyéticos. La cepa NOD/SCID IL-2Rγ$^{-/-}$ es la más adecuada para estudios de leucemogénesis dado que viven más de 16 meses, no desarrollan linfoma tímico y son más sensibles a la reconstitución hematopoyética (Shultz et al. 2005).

Entre los ensayos *in vitro*, cabe mencionar los co-cultivos, los ensayos de clonogenicidad y los cultivos a largo plazo. En los co-cultivos, las HSCs se cultivan sobre una capa de células estromales generalmente murinas (MS5, S17, OP9) que dan soporte a la mielopoyesis y a la linfopoyesis (Coulombel 2004; Kouro et al. 2005). Los ensayos clonogénicos se realizan en medios semisólidos (metilcelulosa) y sirven para identificar progenitores comprometidos a algún linaje (Miller and Lai 2005). Tras 14 días en cultivo en metilcelulosa, estos progenitores dan lugar a colonias que pueden ser clasificadas en base a su morfología, tamaño, color y composición. Hasta la fecha, no han podido estandarizarse ensayos de clonogenicidad para células linfoides humanas. Por último, el ensayo de células iniciadoras de cultivos a largo plazo (LTC-IC) sirve para identificar las HSCs, que tras 5 semanas en co-cultivo con células estromales, son capaces de producir progenitores comprometidos detectables por ensayos de clonogenicidad (Prosper et al. 1997).

1.2. CÉLULAS TRONCALES: DEFINICIÓN Y TIPOS.

La definición de célula troncal debe hacerse de acuerdo a criterios funcionales, ya que no poseen características morfológicas únicas y específicas. Son células inmaduras, no diferenciadas, con una alta capacidad de autorenovación y que pueden diferenciarse en múltiples tipos de células especializadas con funciones específicas en el organismo. Su versatilidad funcional, tanto *in vivo* como *in vitro*, y la capacidad de cultivarlas *ex vivo*, han motivado su uso en terapia celular y medicina regenerativa, y están ayudando a entender procesos biológicos clave como diferenciación celular, desarrollo y cáncer.

A lo largo del desarrollo se generan diversos tipos de células troncales con diferente potencial de autorenovación y de diferenciación. La célula con mayor capacidad de autorenovación y diferenciación es el cigoto, llamada célula *troncal totipotencial*, capaz de producir todos los tejidos del embrión así como las estructuras extraembrionarias (placenta, saco vitelino y cordón umbilical). Le siguen el estadio de mórula (cuyas células también se consideran totipotentes) y de blastocisto, en el que cada una de las células que forma parte de la masa celular interna es capaz de formar (bajo condiciones de cultivo adecuadas) células de cualquier tejido del organismo, pero son incapaces de dar origen a estructuras extraembrionarias, por lo que se consideran *células troncales pluripotentes*. El blastocisto representa el primer fenómeno de diferenciación que ocurre en el mamífero dado que las células procedentes de la mórula originan la masa celular interna (pluripotentes) y trofoectodermo (tejido diferenciado). Las células *troncales multipotentes*, por otro lado, son capaces de generar todos los tipos celulares presentes en un mismo tejido (Rodriguez et al. 2012). Es el caso de las células troncales hematopoyéticas que originan todo el sistema sanguíneo, y las células troncales mesenquimales que originan múltiples tejidos derivados del mesodermo como grasa, músculo, hueso y cartílago.

En el estudio desarrollado en esta tesis doctoral se han utilizado dos tipos de células troncales correspondientes a dos estadios ontogénicos diferentes como herramientas para estudiar el efecto del oncogén de fusión MLL-AF4 (relacionado con la leucemia linfoblástica aguda pro-B del lactante), que son células troncales embrionarias humanas (hESCs) como estadio prenatal, y células troncales/progenitoras hematopoyéticas (HSPCs) procedentes de sangre de cordón umbilical como estadio neonatal. Es por esto, que a continuación se detallan algunos aspectos relativos a estos dos tipos de células troncales. Así mismo, y debido al creciente interés que están teniendo las células troncales pluripotentes inducidas (iPSCs), del que también participa nuestro laboratorio con el desarrollo de varias líneas de investigación, también se introducen este tipo de células troncales.

Células troncales embrionarias humanas.

Las hESCs son células troncales pluripotentes, con capacidad de proliferación ilimitada y potencial de generar todos los tejidos y estructuras propias del embrión. Thomson *y cols*. derivaron por primera vez hESCs a partir de células de la masa celular interna de un embrión humano en estado de blastocisto (Thomson et al. 1998). El potencial uso de las hESCs en medicina regenerativa, o como herramienta de estudio de múltiples aspectos relacionados con biología del desarrollo y enfermedad, han provocado un gran interés en caracterizar a fondo las hESCs, así como en la estandarización de sus condiciones de cultivo (Draper et al. 2004). La capacidad pluripotente de las hESCs se define *in vivo* por la formación de teratomas en ratón e *in vitro* mediante la formación de cuerpos embrionarios (EBs, del término inglés *embryoid bodies*). La diferenciación de las hESCs en ambos sistemas dá lugar a las tres capas germinales (mesodermo, endodermo y ectodermo). Las hESCs presentan en cultivo una morfología

característica en colonias epiteliales, y positividad para los antígenos asociados a pluripotencia SSEA-3, SSEA-4, TRA1-60 y TRA1-81. Además expresan factores de trascripción relacionados con el estado indiferenciado de las hESCs, como son Oct3/4, Nanog, Rex-1 y Sox-2 (Nichols et al. 1998; Niwa et al. 2000; Chambers et al. 2003; Mitsui et al. 2003).

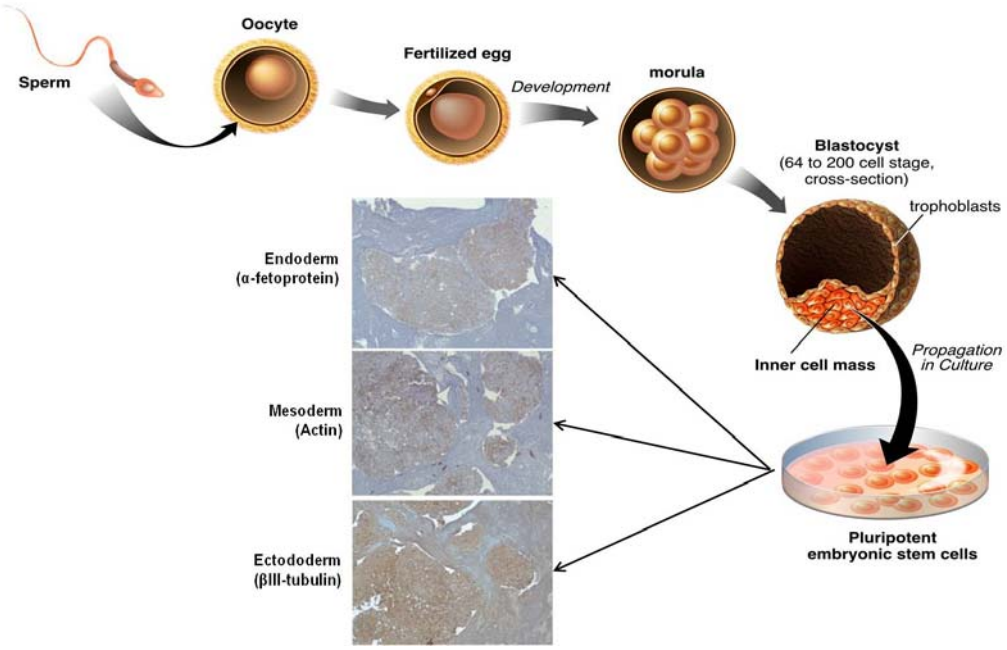

Figura 3. Imagen representativa de la obtención de hESCs a partir de la masa celular interna del blastocisto. La capacidad pluripotente de estas células permite la diferenciación hacia las tres capas germinales: endodermo, mesodermo y ectodermo (distinguibles por su positividad para α-fetoproteína, actina y βIII-tubulina, respectivamente) (Cortes et al. 2009). Modificado de http://www.stemcellsforhope.com.

Con respecto al mantenimiento de las hESCs, existen en la actualidad sistemas de cultivo basados en el uso de células de soporte o *feeders* en co-cultivo con las hESCs (Xu et al. 2005), y sistemas libres de dichas células (Hoffman and Carpenter 2005). Las *feeders* más utilizadas hasta la fecha han sido MEFs (*mouse embryonic fibroblasts*) (Thomson et al. 1998) y HFFs (*human foreskin fibroblasts*) (Hovatta et al. 2003; Xu et al. 2004; Wang et al. 2005a), y más recientemente hMSCs (*mesenquimal stem cells*) (Cortes et al. 2009). Los sistemas libres de *feeders* utilizan medio de cultivo previamente condicionando (CM - *conditioned media*) por MEFs y por HFFs (Wang et al. 2005a; Hovatta 2006). Sin embargo, el cultivo de hESCs con hMSC-CM no ha sido caracterizado. Por otro lado, con el fin de reducir componentes xenogénicos (que dificultan la posible utilización de hESCs en medicina regenerativa y biología), y de facilitar el cultivo de hESCs, el uso de MEFs y de MEF-CM se está viendo restringido. Además, se ha demostrado recientemente que los MEFs tienen un alto contenido de partículas virales, desaconsejando su uso (Cobo et al. 2008).

Células progenitoras hematopoyéticas derivadas de sangre de cordón umbilical.

El uso de hESCs, pese a su gran potencial, tiene como contrapunto implicaciones éticas (Cortes et al. 2007). Desde hace dos décadas se han venido utilizando otras fuentes de células troncales, que aunque con menor potencial de diferenciación (multipotentes) que las hESCs se utilizan para regenerar determinados tipos de tejidos. En este sentido la sangre de cordón umbilical (SCU) supone una fuente de fácil acceso de células progenitoras hematopoyéticas. La SCU corresponde a la cantidad de sangre que queda remanente en la placenta y el cordón umbilical tras el nacimiento del bebé. A pesar del bajo volumen obtenido de cada placenta (aproximadamente 150 ml.), la concentración de HPSCs es elevada, con un porcentaje de células CD34+ de 0.8-2.9% (Jetmore et al. 2002). Las HSCs derivadas de SCU son de origen fetal, siendo su origen mesodérmico, al igual que el resto de células hematopoyéticas. Las células de SCU presentan por su estadio fetal/neonatal una carga mutacional muy escasa lo que representa un aspecto biológico relevante para su uso en modelos de enfermedad. El trasplante de progenitores hematopoyéticos provenientes de SCU constituye una práctica clínica habitual de la que se benefician pacientes hematológicos pediátricos y también adultos (Ballen et al. 2001; Broxmeyer 2005; Liao et al. 2011; Park and Lee 2013). De hecho, las unidades de SCU son almacenadas en Bancos de SCU donde son tipificados, evaluados según estándares de calidad apropiados, y criopreservados (Gluckman and Rocha 2009; Gluckman 2011). Sin embargo, el trasplante de HSPCs está supeditado a algunos aspectos críticos, como el número de progenitores por kg. de peso del paciente. Es por esto que continuamente se están implementando mejoras en los protocolos de congelación/descongelación (Berz et al. 2007; Tijssen et al. 2008) y cultivo/expansión de progenitores hematopoyéticos de modo que la descongelación no resulte en una pérdida significativa de los progenitores y que su expansión *ex vivo* no conlleve pérdida de potencial diferenciación multilínea (Broxmeyer et al. 1992; Broxmeyer 1995; Broxmeyer et al. 2006; Broxmeyer 2010; Broxmeyer 2012b; Broxmeyer 2012a).

Células troncales pluripotentes inducidas.

Las iPSCs humanas son el resultado de la reprogramación epigenética de células somáticas adultas. Esta reprogramación se consigue mediante la expresión ectópica de factores de trascripción capaces de revertir el fenotipo somático a un fenotipo indiferenciado semejante al de hESCs. El conjunto de factores de trascripción utilizado, ha variado entre grupos de investigación, utilizándose tanto Oct4, Sox2, Nanog y Lin28 (Yu et al. 2007), como Oct3/4, Sox2, Klf4 y c-Myc (Takahashi et al. 2007), obteniéndose en ambos casos células con capacidad pluripotencial con características morfologicas, fenotípicas y funcionales similares a las hESCs. En la actualidad hay trabajos en los que se consigue reprogramar células con uno o dos factores en función del tipo celular a reprogramar y del estado celular de dicha célula. Hay evidencias que indican que cuanto más inmadura sea la célula a reprogramar, menor es el número de factores necesarios. Estos factores de trascripción, aunque necesarios para reprogramar estas células somáticas, deben ser silenciados posteriormente para que la capacidad de diferenciación de las iPSCs no

se vea restringida (Ramos-Mejia et al. 2012c). Estas células reprogramadas se pueden generar a partir de tejidos provenientes de donantes sanos, pero también se han utilizado tejidos provenientes de pacientes, e incluso de líneas celulares ya establecidas, constituyendo una herramienta de gran interés para el estudio de los mecanismos celulares y moleculares que subyacen a la trasformación oncogénica y las bases del desarrollo de otras patologías con especial interés en la asociación fenotipo-genotipo (Ramos-Mejia et al. 2012b).

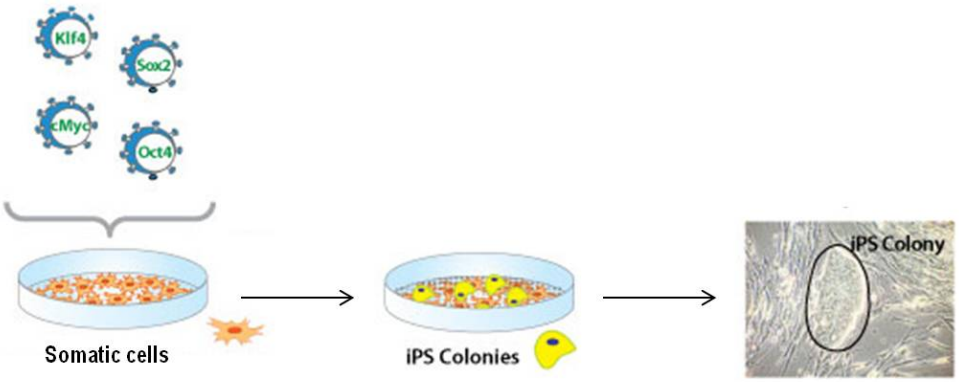

Figura 4. Imagen representativa de la obtención de iPSCs. La reprogramación de células somáticas se produce utilizando los factores de pluripotencia Oct3/4, Sox2, Klf4 y c-Myc (en el ejemplo mostrado, utilizando vectores virales). Modificado de www.biocat.com.

1.3. CÉLULAS TRONCALES, CÁNCER Y DESARROLLO.

La heterogeneidad tumoral es una característica constatable en términos de morfología, marcadores, progresión y respuesta a terapia, entre diferentes pacientes. Esta heterogeneidad también existe entre las células que forman parte de un tumor, a pesar de su origen clonal, esto es, iniciado a partir de una célula individual. Para explicar la heterogeneidad existente entre las células de un tumor, se han desarrollado dos modelos, excluyentes entre sí, que son el modelo estocástico y el modelo jerárquico (Dick 2008). Según el modelo estocástico las células de un tumor son biológicamente homogéneas y su comportamiento está influenciado por factores intrínsecos (como la expresión de factores de trascripción, vías de señalización,…) y extrínsecos (como el microambiente, respuesta inmune,…) (Wang and Dick 2005). En este modelo todas las células son igualmente influenciables por los factores mencionados y sus cambios pueden revertirse ya que no son estables. El modelo jerárquico, por el contrario, defiende que las células que conforman un tumor mantienen un sistema jerárquico (describiéndolo como caricatura de su tejido normal correspondiente). En este sistema jerárquico, al igual que en un sistema fisiológico, existen células troncales con capacidad de autorenovación y de mantenimiento de las demás células que componen la población tumoral.

Ambos modelos, estocástico y jerárquico, defienden la existencia de las células troncales tumorales (Dalerba et al. 2007). La diferencia es que para el estocástico cualquier célula tumoral puede llegar a

funcionar como una CSC (del inglés *cancer stem cell*) / CIC (del inglés *cancer initiating cell*), mientras que para el modelo jerárquico las CSCs constituyen una subpoblación determinada que aún siendo mínima en frecuencia es muy relevante biológicamente. Aunque el concepto de células troncales tumorales se ha extendido a tumores sólidos (Rubio et al. 2010; Rodriguez et al. 2012), se desarrolló inicialmente en leucemias mieloides por lo que se denominaron LSCs (del inglés *leukemic stem cell*) o LICs (del inglés *leukemic initiating cell*) como las responsables de originar y mantener la enfermedad (Bonnet and Dick 1997). Las LSCs comparten muchas características biológicas con las HSCs normales (Zhao et al. 2007).

Por otro lado, estudios recientes de secuenciación masiva han constatado que el número de mutaciones presentes en las células de un tumor puede variar desde decenas a cientos de miles (Stratton 2011), lo que ha permitido el desarrollo de un nuevo modelo llamado "diversidad subclonal del cáncer" (Greaves and Maley 2012). En este modelo, las células tumorales, aún teniendo procedencia clonal, están expuestas a un proceso de selección natural típico del modelo darwiniano por el que evolucionan de manera muy diversa en respuesta a esta presión selectiva, de tal forma que el estudio de células troncales tumorales fenotípicamente idénticas demuestra que cada célula es portadora de un número y tipo de mutaciones génicas distinto (Anderson et al. 2011; Notta et al. 2011).

Como se ha mencionado en el apartado anterior, las células troncales tienen capacidad de autorrenovarse, así como de proliferar y diferenciarse dando lugar a todas las células que conforman un tejido, es decir, mantienen la homeostasis del mismo. Dentro del tejido, son las que tienen una vida media mayor y por tanto más probabilidad para acumular mutaciones. Las células troncales tumorales, como células troncales que son, se mantienen generalmente en estado quiescente hasta su entrada en ciclo celular. Este estado quiescente contribuye a la resistencia frente a terapias antitumorales, que ejercen su acción sobre células en división. Por otro lado, los altos niveles de proteínas trasportadoras ABC y MDR contribuyen a que las células troncales tumorales sean refractarias a los regímenes terapéuticos actuales, dada su habilidad de expulsar citostáticos. Así, el estado quiescente y la alta expresión de proteínas trasportadoras son dos "causas" cuya "consecuencia" es que una célula troncal normal sea más propensa en convertirse en una CSC. Es más, según el modelo subclonal, los tratamientos terapéuticos pueden suponer un factor añadido de presión selectiva que favorezca la aparición de nuevas mutaciones, y por tanto de nuevas vías de escape a dichos tratamientos.

Otro aspecto importante a tener en cuenta en la conexión entre células troncales, cáncer y desarrollo, es el origen prenatal (en útero) de los eventos oncogénicos iniciadores y que tendrían su efecto en células troncales pluripotentes o en células troncales multipotentes. El origen prenatal ha quedado demostrado para leucemias infantiles en estudios de gemelos monocigóticos portadores ambos del mismo reordenamiento cromosómico, en los que se detectaron células pre-leucémicas con la traslocación TEL-AML1 (correspondiente a la traslocación t(12;21)(p13;q22)) en dos hermanos gemelos, aunque sólo uno de ellos había desarrollado la enfermedad (Hong et al. 2008). También se ha podido detectar TEL-AML1

de manera retrospectiva en muestras de sangre almacenadas tras el nacimiento en la llamada prueba del talón, indicando el origen prenatal de la alteración (Wiemels et al. 2002). El origen prenatal de las traslocaciones del gen *MLL* (muy frecuentes en leucemias agudas infantiles) ha podido determinarse también mediante estudios en gemelos monocigóticos con idéntica leucemia linfoblástica aguda (LLA) pro-B, así como con estudios retrospectivos en muestras de sangre recogidas en la prueba del talón. (Ford et al. 1993; Gale et al. 1997). Es importante reconocer qué factores de trascripción (MLL, TEL, AML1, BCR, PBX1 E2A, etc.) y vías de señalización (Notch, Wnt, SHH, TGF-β), que juegan un papel clave en la homeostasis y formación/desarrollo del tejido hematopoyético, están alterados en cáncer.

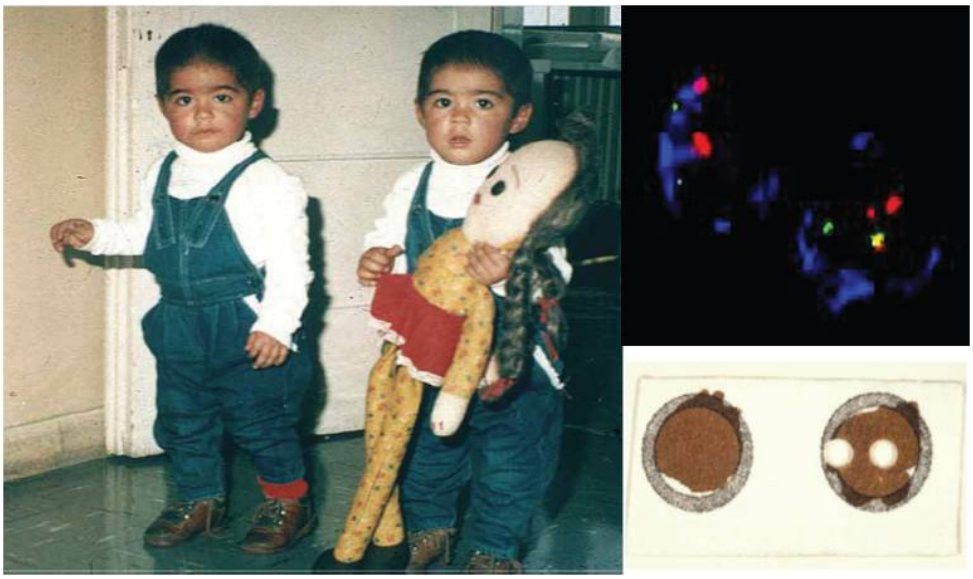

Figura 5. Gemelos idénticos (Santiago de Chile) diagnosticados con LLA pre-B, en los que pudo determinarse por primera vez el origen prenatal de la traslocación genética TEL-AML1. En la imagen superior derecha se visualizan los reordenamientos de los genes *TEL* y *AML1* por I-FISH en una muestra de SCU: un marcaje reconoce el tipo celular (azul), y mediante dos tipos de sondas se puede distinguir una célula B inmadura normal con dos copias de los dos genes (izquierda) (dos señales verdes y dos señales rojas) y una célula con la traslocación cromosómica (derecha), con una señal roja y una señal verde (correspondientes a los alelos normales) y una señal amarilla indicativa de la traslocación. También se muestra una imagen de la sangre recopilada en el momento del nacimiento, en la llamada prueba del talón (derecha, abajo). Modificado de (Greaves 2008), publicado por el autor con el permiso de los padres.

En el caso de la LLA infantil, a pesar de que la tasa de concordancia entre gemelos es aproximadamente del 100%, la tasa de aparición de la enfermedad es, aunque alta, sólo del 5% (Greaves 2002). Concretamente, en el caso de la leucemia TEL-AML1, el oncogén tiene un origen prenatal y aparece en el 1% de las muestras de SCU. Sin embargo, sólo el 1% de ellas (0.01%, es decir 1 en 10.000) desarrolla la enfermedad, sugiriendo que son necesarios otros eventos secundarios para el desarrollo del proceso leucémico. Esto es lo que sugiere el llamado "modelo de dos *hits*" para las leucemias infantiles (Greaves 1999). Según este modelo, en útero, en una célula troncal se produciría el reordenamiento génico generando un clon pre-leucémico asintomático. Tras el nacimiento, y un periodo más o menos largo de latencia, sobre este clon pre-leucémico se produce un segundo daño responsable del desencadenamiento del proceso leucémico (Greaves 2003). En el caso de las LLAs infantiles (que

presentan en un 80% de los casos reordenamiento del gen *MLL*), es posible que el segundo daño ocurra antes del nacimiento, debido a su corta latencia, ya que la leucemia se manifiesta antes del primer año de vida.

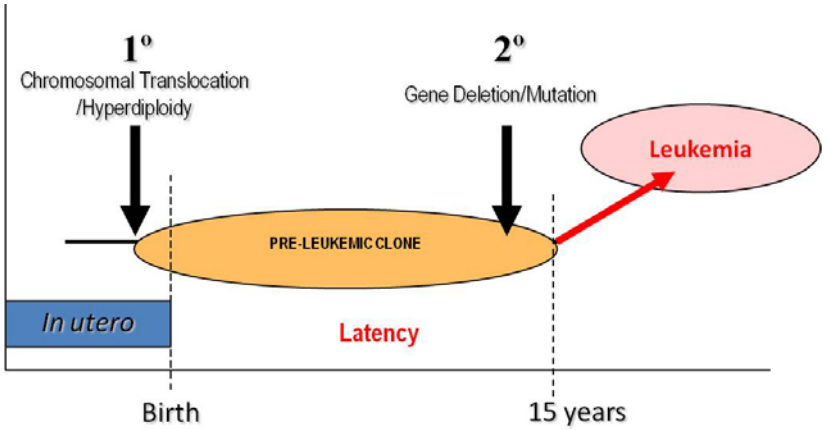

Figura 6. Esquema del "Modelo de dos *hits"* propuesto por Mel Greaves. Antes del nacimiento, *in utero*, sobre una célula troncal ocurriría un primer daño a nivel genético, consistente en una traslocación cromosómica, hiperdiploidía,…) originando el clon pre-leucémico (Greaves 2003). Posteriormente, y tras un periodo más o menos largo de latencia (que en el caso de la traslocación TEL-AML1 puede ser de 15 años), sobre el clon pre-leucémico ocurría un segundo daño genético (deleción o amplificación génica, mutación, SNVs/SNPs, etc.), responsable del desencadenamiento del proceso leucémico.

1.4. LEUCEMIA. DESCRIPCIÓN Y TIPOS.

La leucemia consiste en una trasformación maligna de las células hematopoyéticas, en las que un bloqueo de la diferenciación se acompaña de una proliferación descontrolada de las células leucémicas (Sachs 1996). Estas células leucémicas se acumulan en MO, tejidos linfoides, así como en la circulación sanguínea, y pueden infiltrar otros órganos y tejidos como bazo e hígado, provocando un aumento de tamaño de los mismos (conocidos como esplenomegalia y hepatomegalia, respectivamente). Por otro lado, y como consecuencia de la acumulación anormal de las células leucémicas, la producción de células hematopoyéticas sanas, maduras y funcionales se ve desplazada y comprometida, por lo que, según el estadio y linaje afectado, pueden aparecer una serie de síntomas como anemia (por ausencia de función de eritrocitos), falta de plaquetas (lo que puede conducir a defectos en coagulación o hemorragias), y afección del sistema inmune (con mayor riesgo a infecciones).

Las leucemias pueden clasificarse según el grado de diferenciación en leucemias agudas, en donde las células leucémicas son indiferenciadas (blastos), y leucemias crónicas, donde las células leucémicas corresponden a estadios diferenciados (Foon and Todd 1986; Moraleda-Jiménez 1996). En general, las leucemias agudas evolucionan con un curso clínico rápido, y las crónicas más lentamente, por lo que, tradicionalmente se atribuía a las leucemias agudas un peor pronóstico. Sin embargo, con los tratamientos actuales, una parte importante de las leucemias agudas pueden ser curadas definitivamente y otras incluso sobreviven más tiempo que las llamadas crónicas.

Las leucemias agudas y crónicas pueden clasificarse a su vez, en función del linaje hematopoyético que se ve afectado por un bloqueo en su diferenciación y que prolifera de manera descontrolada. Así, cuando se afecta el linaje linfoide tenemos una leucemia linfoide o linfoblástica, y leucemia mieloide o mieloblástica cuando se afecta a linaje mieloide. En el adulto son más frecuentes las leucemias crónicas, tanto mieloides como linfoides, mientras que en la infancia predominan las agudas, especialmente la LLA.

En esta tesis no entraremos a describir otro tipo de hematopatías malignas tipo linfomas, mielomas, síndromes mielodisplásicos, etc., por estar alejados del contexto de este trabajo.

1.4.1. LEUCEMIAS AGUDAS.

Las leucemias agudas son el resultado de una proliferación clonal de células inmaduras que sufren un bloqueo en la diferenciación. La célula dónde se produce la trasformación leucémica es un precursor que pierde la capacidad de diferenciación. Este precursor puede ser de origen mieloide (leucemia mieloblástica aguda-LMA) o linfoide (leucemia linfoblástica aguda-LLA). Mientras que a nivel global las leucemias agudas suponen el 10% de todos los cánceres, en la infancia es la neoplasia más frecuente (30%), correspondiendo el 80% de los casos a LLA y el 15% a LMA.

Las leucemias agudas comprenden un grupo heterogéneo que difiere tanto en sus características biológicas, como en la etiología, patogenia, historia natural y pronóstico, por lo que la clasificación de las mismas, no se apoya en un único parámetro, sino que tiene en cuenta criterios morfológicos, citoquímicos, inmunológicos, citogenéticos y de biología molecular. La clasificación más ampliamente utilizada ha sido la del Grupo Cooperativo Franco-Americano-Británico (*FAB*) (Bennett et al. 1976), basada en criterios morfológicos y citoquímicos fundamentalmente, pero más recientemente se ha propuesto la clasificación de la Organización Mundial de la Salud (OMS) que tiene en cuenta también criterios inmunofenotípicos y citogenéticos (Campo et al. 2011).

Para las LMAs, la clasificación *FAB* distingue siete subtipos, según el grado de diferenciación y maduración de las células predominantes hacia granulocitos, monocitos, eritrocitos o megacariocitos. Además de criterios morfológicos, también se usan tinciones citoquímicas para identificar los diferentes subtipos, como *mieloperoxidasa*, *Sudan negro* o *esterasas* específicas para detectar diferenciación granulocítica, y la tinción esterasas inespecíficas que caracteriza la línea monocítica. Así, la clasificación *FAB* distingue las siguientes variedades morfológicas de LMA: M0 (leucemias mieloides agudas indiferenciadas), M1 (leucemias mieloides agudas pobremente diferenciadas), M2 (leucemias mieloides agudas diferenciadas), M3 (leucemia promielocítica), M4 (leucemia mielo-monocítica aguda), M5 (leucemia monocítica), M6 (eritroleucemia) y M7 (leucemia megacarioblástica).

La clasificación de las LMAs de la OMS divide en cuatro categorías a la LMA, según (i) la recurrencia de anomalías citogenéticas (como por ejemplo, t(8;21), inv 16, t(15;17), y otras a nivel de cromosoma

11q23), (ii) aparición de displasia multilínea con al menos afectación de dos líneas celulares, (iii) relacionada con tratamientos previos a pacientes como agentes alquilantes o inhibidores de la topoisomerasa II, y (iv) otras no categorizadas anteriormente en la que se incluyen la mayoría de los subtipos descritos por el grupo *FAB* (Brunning 2003; Vardiman et al. 2009).

1.4.1.1. LEUCEMIAS LINFOBLÁSTICAS AGUDAS.

Como se ha mencionado anteriormente, la LLA es más frecuente en niños, especialmente menores de 15 años, aunque puede aparecer en edades posteriores e incluso en edad adulta. Se origina por la proliferación en MO y tejidos linfoides de células blásticas de origen linfoide de línea B o de línea T. Las LLAs de tipo B son las más frecuentes y constituyen el 80% de los casos. Las LLAs de tipo T representan el 25% de las LLAs en adulto y sólo el 15% en niños (Pui et al. 2004).

En cuanto a las anomalías citogenéticas, la mayoría de las leucemias presentan alteraciones cromosómicas clonales que se relacionan con el subtipo leucémico y con el pronóstico. Más del 80% de los pacientes con LLA tienen alteraciones de cariotipo, numéricas o estructurales. Las alteraciones numéricas de cariotipo corresponden con hipodiploidías (menos de 46 cromosomas) e hiperdiploidias (más de 50 cromosomas), teniendo las hiperdiploidías un pronóstico más favorable (Holmfeldt et al. 2012). Las alteraciones estructurales de cariotipo son habitualmente traslocaciones que suelen afectar a proto-oncogenes o genes supresores de tumores con papel clave en proceso leucémico, y que se asocian con importancia pronóstica (Moraleda-Jiménez 1996).

Alteración cromosómica	*Proteína de fusión*	*Fenotipo predominante*	*Clínica. Pronóstico.*
t(12;21)	TEL-AML1	Común/pre-B	Buen pronóstico.
Hiperdiploide		Común/pre-B	Buen pronóstico.
t(8;14)	Myc-IgH	LLA-B madura	Morfología L3 (*FAB*), tipo Burkitt. Infiltración extramedular. Mal pronóstico con tratamiento convencional.
t(9;22)	BCR-ABL	Línea B	Leucocitos altos. Muy mal pronóstico con quimioterapia convencional.
t(4;11)	MLL-AF4	LLA-pro B	Hiperleucocitosis. Recién nacidos. Muy mal pronóstico con cualquier tratamiento.
t(1;19)	E2A-PBX1	LLA-pre B	Leucocitosis, más frecuente en raza negra, infiltración SNC, mal pronóstico con tratamiento convencional.
t(11;14)	TTG2-TCRD	LLA-T	Hiperleucocitosis, enfermedad extramedular.
t(9;12)	PAX5-ETV6	Línea B	Buen pronóstico con tratamiento convencional.

Tabla 1. Alteraciones citogenéticas comunes relacionadas con LLA.

Con respecto a la morfología de los blastos, para las LLAs, el grupo cooperativo *FAB* distingue los subtipos:

L1: Células pequeñas de tamaño homogéneo con núcleo redondo y regular (sin nucléolo), y citoplasma escaso con ligera basofilia. A este subtipo pertenece el 85% de casos de LLA en niños. Pueden ser de línea B o de línea T.

L2: Células grandes y heterogéneas con núcleo irregular (con uno o más nucléolos), y citoplasma abundante de basofilia variable. Pueden ser de línea B o de línea T.

L3: Células grandes y homogéneas con núcleo redondo u ovalado (nucléolos prominentes), y citoplasma abundante de basofilia abundante y elevada actividad mitótica. Este subtipo suele corresponder con línea B madura o linfoma de Burkitt.

Por otro lado, el Grupo Europeo para la caracterización de las leucemias agudas (EGIL) (Bene et al. 1995) distingue diferentes subtipos de LLAs T y B en función de la expresión de diferentes marcadores inmunológicos (fenotipo) en las células blásticas. El grado de diferenciación de los precursores B y T tiene implicaciones clínicas, citogenéticas y pronósticas.

LLA de línea B: CD19+, CD22+, CD79a+	
Pro B	CD19+, CD22+, CD79a+, CD10-
Común	CD19+, CD22+, CD79a+, CD10+
Pre B	CD19+, CD22+, CD79a+, IgM citoplasmática+
Madura (tipo Burkitt)	CD19+, CD22+, CD79a+, inmunoglobulina de superficie+
LLA de línea T: CD3+ (citoplasmática o de membrana)	
Pro T	CD3+, CD7+
Pre T	CD3+, CD2+, CD5+, CD8+
Tímico-cortical	CD3+, CD1a+
Madura	CD3+ de membrana, CD1a-

Tabla 2. Clasificación inmunológica de las LLAs según *EGIL* (Bene et al. 1995).

1.4.1.2. LEUCEMIAS LINFOBLÁSTICAS AGUDAS PEDIÁTRICAS.

La LLA se origina a partir de un progenitor linfoide inmaduro pudiendo ser de línea B (LLA-B) o de línea T (LLA-T), siendo la LLA-B la más frecuente en niños (80%) (Pui et al. 2004). En las LLAs pediátricas son muy frecuentes las anomalías citogenéticas, tales como variaciones en el número de cromosomas y reordenamientos cromosómicos. Estas anomalías tienen además gran trascendencia en cuanto a la evolución de la enfermedad y sus implicaciones terapéuticas. En la figura 7 (Greaves 2002) se muestra la distribución en frecuencia de las anomalías genotípicas en LLAs pediátricas (mayor de 1 año de edad) y

las infantiles (menos de 1 año de edad). Como puede observarse, los reordenamientos del gen *MLL* suponen el 6% del total de los genotipos de las LLAs pediátricas, mientras que para las LLAs infantiles corresponden al subtipo más abundante (80% de los casos).

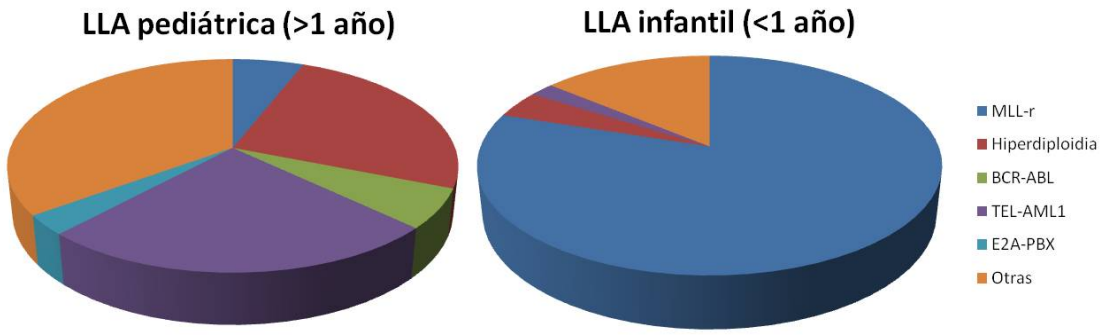

Figura 7. Principales subtipos en LLAs pediátricas e infantiles. Merece destacar que mientras que los reordenamientos de *MLL* (MLL-r) suponen aproximadamente el 6% de los casos en LLAs pediátricas, este porcentaje aumenta hasta el 80% en las LLAs infantiles. Modificado de Greaves *y col.* (Greaves 2002).

La clasificación de la OMS de las LLAs pediátricas de línea B relaciona inmunofenotipo, basado en los diferentes estados de maduración B, con anomalías genéticas (Schrappe M. 2004):

Inmunofenotipo	*CD19*	*CD10*	*Cadena μ citoplasmática*	*Cadena μ en superficie*	*Gen de fusión/Genotipo*	*Traslocación*
LLA pro B*	+	-	-	-	MLL-AF4 MLL-ENL	t(4;11) t(11;19)
LLA B común	+	+	-	-	TEL-AML1 BCR-ABL	t(12;21) t(9;22)
LLA pre B	+	+	+	-	E2A-PBX Hiperdiploide	t(1;19) (>50 cromo.)
LLA B madura	+	+	+	+	IgH-c-myc	t(8;14)

*En muchos casos pueden ser mixtas (pro-B/monocíticas): expresan marcadores B con CD15 y CD65.

En cuanto al tratamiento, los regímenes actuales han conseguido una supervivencia global mayor del 85% de los pacientes. El tratamiento quimioterápico incluye una combinación de diferentes compuestos como son glucocorticoides, vincistrina, antraciclinas, L-asparginasa, metotrexato y 6-mercaptopurina (Pui and Evans 2006). Sin embargo, el éxito del tratamiento varía entre los grupos citogenéticos, siendo los de mejor pronóstico las hiperdiploidías y la fusión TEL-AML1 (traslocación t(12;21)), mientras que las LLAs que presentan reordenamiento del gen *MLL* constituyen un subtipo especialmente agresivo (ver figura 8 correspondiente a (Pieters et al. 2007). De hecho, existe un grupo denominado de alto riesgo, al que

pertenecen el 10% de los pacientes, y cuya supervivencia global está en torno al 20% (Marshall et al. 2013).

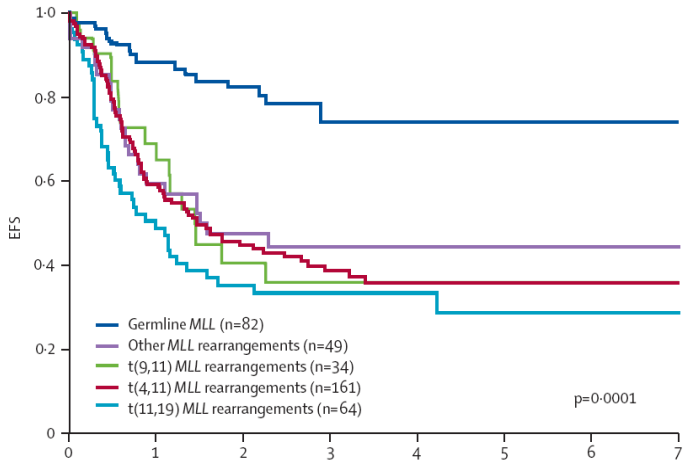

Figure 8. Supervivencia libre de enfermedad (*EFS*) entre diferentes grupos sin reordenamiento del gen *MLL* (*MLL germline*) y otros que presentan el gen *MLL* reordenado (Pieters et al. 2007).

1.4.1.3. LEUCEMIA LINFOBLÁSTICA AGUDA PRO-B INFANTIL.

Como se ha mencionado anteriormente, los reordenamientos del gen *MLL* constituyen el subtipo citogenético más abundante en pacientes con LLA infantil (Greaves 2002). Concretamente para la LLA pro-B del lactante, los reordenamientos de *MLL* constituyen el marcador genético más común (Eguchi et al. 2006). Esta enfermedad presenta un inmunofenotipo altamente inmaduro (CD34+, CD19+, CD10-) y co-expresa además marcadores de línea mieloide como CD15 y CD65 (Jansen et al. 2007). En la actualidad, aunque existe controversia, el antígeno neuro-glial 2 (NG2) reconocido por el anticuerpo 7.1, parece que se expresa de forma diferencial en leucemias con reordenamiento en *MLL*. Sin embargo, existen casos de leucemias con *MLL* reordenado sin expresión de NG2, y otros que en ausencia de *MLL* reordenado expresan NG2. Esto indica que el uso de NG2 (7.1) en paneles de diagnóstico por citometría de flujo sigue sin identificar el 100% de leucemias con reordenamiento de *MLL* (Smith et al. 1996), (Behm et al. 1996; Hilden et al. 1997; Wuchter et al. 2000; Schwartz et al. 2003; Zangrando et al. 2008). Esta LLA pro-B tiene una latencia extremadamente corta, manifestándose antes del primer año de vida, frecuentemente entre los 3 y 6 meses de edad. También se caracteriza por su agresividad ya que tiene una supervivencia libre de enfermedad y una supervivencia global a los 5 años inferior al 20% (Pui and Evans 2006).

La traslocación t(4;11) supone aproximadamente el 50% de los reordenamientos de *MLL* en las LLAs infantiles (Pui et al. 2012), dando lugar a la proteína de fusión MLL-AF4. Estudios realizados en gemelos monocigóticos con idéntica LLA pro-B junto con estudios retrospectivos en muestras neonatales (prueba

del talón) en lactantes con leucemia MLL-AF4+, revelaron el origen prenatal de la misma (Ford et al. 1993; Gale et al. 1997).

Con respecto a la etiología, se ha postulado que los reordenamientos de *MLL* pueden ser el resultado de la exposición trasplacental durante el desarrollo embrionario a sustancias que alteran la función de la enzima topoisomerasa II que se encarga de reparar rupturas en el DNA y que está altamente expresada durante el desarrollo embrionario y fetal (Zandvliet et al. 1996; Moneypenny et al. 2006; Libura et al. 2008). Entre dichas sustancias, el más conocido es el etopósido, utilizado en protocolos de quimioterapia y responsable de ciertas leucemias agudas secundarias a tratamiento (Blanco et al. 2001; Felix 2001). Bueno *y cols*. (Bueno et al. 2009) comprobaron que tanto las hESCs como HSCs CD34+ derivadas de SCU expuestas a dosis bajas de etopósido eran susceptibles a rupturas del gen *MLL* induciendo alta inestabilidad génica en dichas células. También hay evidencias epidemiológicas que sugieren que la LLA infantil podría iniciarse por una infección en el útero durante el embarazo (Gustafsson et al. 2007), detectándose DNA de adenovirus en muestras de sangre neonatales (prueba del talón). La traslocación MLL-AF4 ha sido detectada en células troncales mesenquimales de MO en pacientes con LLA pro-B infantil (Menendez et al. 2009), sugiriendo que esta traslocación ocurre en un precursor mesodérmico temprano, trasmitiéndose a los linajes mesenquimal y hematopoyético, pero teniendo efecto oncogénico únicamente en células hematopoyéticas. Por tanto, la célula diana en LLA pro-B podría ser una célula de origen embrionario o un célula hematopoyética temprana en desarrollo, pudiendo utilizarse como modelo para el estudio de la enfermedad hESCs (estadio prenatal) y HSPCs CD34+ de SCU (estadio neonatal).

Respecto al tratamiento, mientras que en las leucemias pediátricas se han conseguido avances con respecto a la tasa de supervivencia total y libre de enfermedad, para la LLA pro-B del lactante con reordenamiento MLL-AF4 el pronóstico sigue siendo fatal, mostrando resistencia a glucocorticoides y L-asparaginasa, que sí tienen efecto en otros pacientes pediátricos (Jansen et al. 2007). El uso de citarabina (citosina arabinosido o Ara-C) en dosis bajas y altas (protocolo denominado INTERFANT-99 (Pieters et al. 2007)), ha propiciado que se puedan alcanzar tasas de supervivencia aproximadamente del 40%, aún bajas ya que para más de la mitad de los pacientes no se consigue cura.

1.4.1.3.1. MODELOS DE ENFERMEDAD LLA PRO-B INFANTIL.

Disponer de modelos biológicos capaces de reproducir la LLA pro-B infantil MLL-AF4+ permitiría conocer los mecanismos de trasformación oncogénica que subyacen en este tipo de leucemia, así como desarrollar nuevas herramientas terapéuticas que permitiesen tratar a estos niños con mejores perspectivas de curación. Sin embargo, los intentos desarrollados hasta la fecha no han conseguido reproducir la enfermedad de modo fidedigno a como ocurre en los pacientes, ni en lo respectivo al fenotipo de la LLA pro-B, ni a la corta latencia. El laboratorio del Prof. Kersey desarrolló un modelo murino

con ratones *knock-in Mll-AF4* mediante recombinación homóloga. Tras una prolongada latencia de 22 meses, los ratones desarrollaron hiperplasia mixta linfoide/mieloide y linfoma de células B (Chen et al. 2006). El grupo del Prof. Rabbitts utilizó la técnica *invertor* para desarrollar un modelo murino *knock-in* condicional, en el que la acción de la recombinasa *Cre* permitía la expresión de *Mll-AF4* por recombinación intercromosómica (Metzler et al. 2006). La expresión de *Mll-AF4* era letal para embriones murinos, mientras que los ratones adultos desarrollaban linfomas de células B maduras, lo que dista del fenotipo observado en pacientes. El grupo del Prof. Armstrong generó otro modelo de expresión condicional de *Mll-AF4* en el contexto del locus endógeno de *Mll*. Este modelo consigue reducir la latencia, pero el fenotipo de la enfermedad tampoco se corresponde con el observado en humanos, siendo propio de LLA pre-B y de LMA (Krivtsov et al. 2008). Más recientemente, el modelo murino propuesto por el laboratorio del Prof. Marschalek ha logrado generar un fenotipo pro-B (Bursen et al. 2010) mediante la expresión del producto recíproco de la traslocación, es decir, de AF4-MLL, causando enfermedad independientemente de la expresión de MLL-AF4. Este modelo, aún siendo el que más se aproxima a lo visto en los pacientes, es murino y presenta una latencia demasiado prolongada. Por tanto, no ha sido posible hasta la fecha establecer un modelo *in vivo* de enfermedad de LLA pro-B MLL-AF4+, posiblemente porque en los mismos se ha obviado el efecto de componentes esenciales que en humano son necesarios para el desarrollo de la enfermedad. Es posible que la célula diana utilizada en estos modelos para originar el clon pre-leucémico no se encuentre en un estadio jerárquico u ontogénico adecuado o que el oncogén de fusión MLL-AF4 trasforme preferentemente células humanas y no de ratón. También se desconoce la existencia de eventos secundarios que podrían cooperar en la trasformación mediada por MLL-AF4. En consecuencia, la ausencia de un modelo que reproduzca la enfermedad ha dificultado el avance del conocimiento sobre el origen celular, patogenia y progresión de esta LLA infantil de fatal pronóstico.

1.5. BIOLOGÍA DEL GEN MLL-LOCUS 11q23.

1.5.1. ESTRUCTURA Y FUNCIÓN DE MLL.

El gen *MLL*, un homólogo del gen *trithorax* de *Drosophila,* también es conocido como *MLL1*, *HRX* (*human trithorax*) y *ALL-1* (*acute lymphocytic leukemia-1*). Está involucrado en el mantenimiento de la memoria epigenético-traslacional de distintos genes involucrados en desarrollo, entre los que destacan los genes *homeobox* (*Hox*). El gen *MLL* está localizado en el cromosoma 11 región q23, y consta de 36 exones. Codifica una proteína de 3.969 aminoácidos con un tamaño de 430 kDa (Hess 2004).

La proteína MLL una vez trascrita es catalizada por una proteasa aspártica llamada Taspasa 1, dando lugar a dos fragmentos (Yokoyama et al. 2002): un fragmento N-terminal de 320 kDa (subunidad MLL^N) y otro C-terminal de 180kDa (subunidad MLL^N) (ver figura 9). Ambos fragmentos forman parte del complejo

MLL. Debido a su gran tamaño no ha podido realizarse un análisis estructural de la proteína completa, pero sí que se conocen algunos de sus dominios bien individuales o bien formando complejos con ligandos relevantes (Cosgrove and Patel 2010).

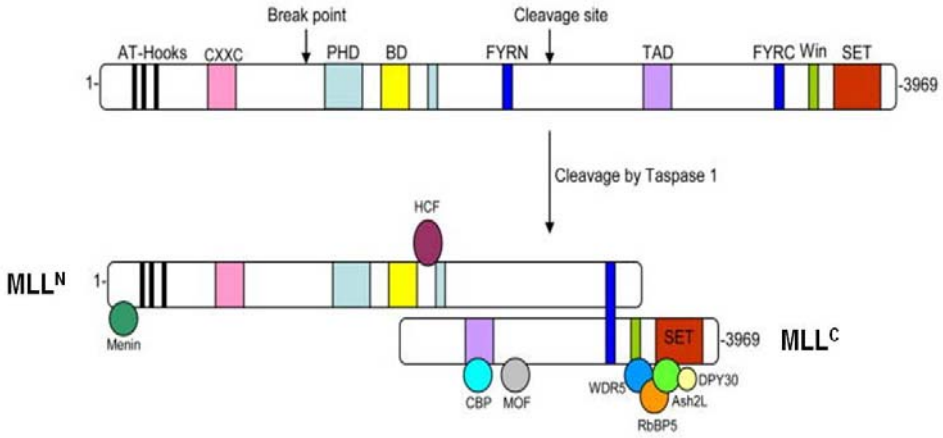

Figura 9. Representación esquemática de los dominios de la proteína MLL. Una vez sintetizada, la proteína es rápidamente procesada por la enzima Taspasa 1 en dos fragmentos, MLL^N y MLL^C, que se reasocian a través de los dominios FYRN y FYRC para formar el complejo estable. La proteína madura se asocia con numerosas proteínas para formar el complejo MLL en la célula. Modificado de (Cosgrove and Patel 2010).

La subunidad MLL^C prepara a la cromatina convirtiéndola en una conformación relajada necesaria para la trascripción. En esta subunidad se encuentran los siguientes dominios; un dominio de trasactivación (*TAD*), motivo de interacción con receptor nuclear (*NR box*), a un motivo *Win* (*WDR5 interaction*) y un dominio *SET* C-terminal, responsable de la actividad histona metiltransferasa de MLL. El dominio *SET* con actividad metiltrasferasa metila la lisina 4 de la histona 3 (H3K4), modificación asociada con regiones trascripcionalmente activas de la cromatina. Entre las proteínas que se asocian a esta subunidad está la proteína *MOF*, una acetiltrasferasa que trasfiere un grupo acetilo a la lisina 16 de la histona 4 (H4K16) y relaja la cromatina al neutralizar la carga de la histona (Dou et al. 2005; Zhang et al. 2012). La proteína WDR5 (de unión al motivo *Win*) reconoce la marca de metilación H3K4 y posiblemente sea necesaria para la señalización posterior (Schuetz et al. 2006). Finalmente, las proteínas RBBP5 y ASH2L son necesarias para la actividad metiltrasferasa, estabilizando la conformación del complejo MLL (Slany 2009).

La subunidad MLL^N se encarga de la unión específica del complejo MLL a su diana. En esta subunidad se encuentran varios dominios conservados, entre los que se incluyen un dominio de unión a regiones de DNA ricas en nucleótidos AT, un dominio rico en cisteína (CxxC) con homología a DNA metiltrasferasas, motivos PHD (*Plant Homeodomain finger*) y un bromodominio (BD). El dominio CxxC se une de manera específica a motivos CpG no metilados del DNA. Esta unión específica es esencial para el reconocimiento génico, trasactivación y trasformación asociada a las proteínas de fusión MLL (Ayton et al. 2004), aunque posiblemente no sea el único responsable de la unión específica ya que existen genes con promotores CpG no metilados que no están regulados por MLL (Cosgrove and Patel 2010). Recientemente, se ha

demostrado que el dominio CxxC interactúa con el complejo *PAFc* (*Polymerase Associated Factor complex*) (Muntean et al. 2010). *PAFc* tiene amplio espectro de actuación que incluye trascripción génica, regulación del ciclo celular, metilación de H3K4, y metilación de H3K79 (en colaboración con DOT1L). Se ha demostrado que la unión *PAFc*-MLL es esencial para la trasactivación génica de MLL, así como de las fusiones de *MLL* (Muntean et al. 2010). Esta subunidad se asocia con la proteína supresora de tumores Menin (*multiple endocrine neoplasia*). Menin y MLL se asocian con LEDGF (*lens epithelium derived growth factor)*, y LEDGF contacta con la cromatina mediante un dominio PWWP.

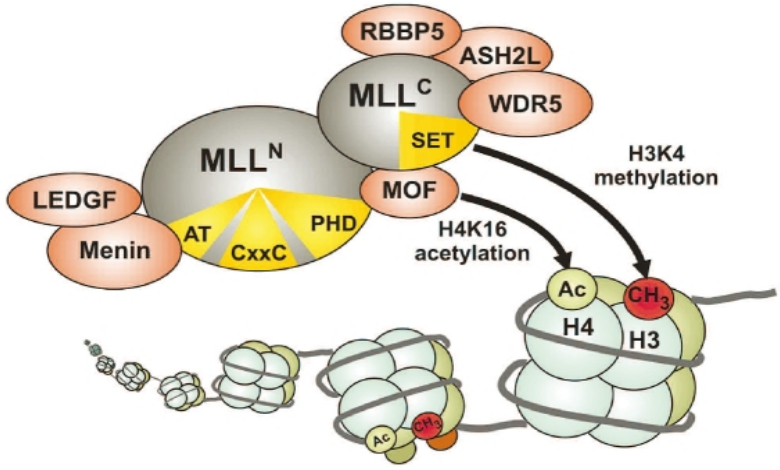

Figura 10. Complejo MLL. Tras el procesamiento proteolítico post-trascripcional, la unidad amino y carboxy terminal se incorporan a un complejo macromolecular con funciones histona metiltrasferasa e histona acetiltrasferasa. Los dominios funcionales del complejo se indican en amarillo. En la subunidad MLL^C, el dominio *SET* ejerce la actividad histona metiltrasferasa H3K4, mientras que la proteína *MOF*, que también se une a esta subunidad del complejo MLL, realiza la función histona acetiltrasferasa H4K16. A ambas subunidades, MLL^C y MLL^N, se unen otra serie de proteínas que colaboran en la estabilización del complejo, el reconocimiento y unión al DNA, así como preparación de la cromatina para las actividades metil- y acetiltrasferasa (Slany 2009).

Por tanto, el complejo MLL coordina los tres mecanismos principales de modificación de la cromatina: metilación y acetilación de histonas, y remodelación de nucleosoma, y, probablemente, los factores de trascripción reclutan al complejo MLL para iniciar el proceso de síntesis de RNA, como es el caso de p53, y se asocian al complejo MLL durante su proceso de activación trascripcional (Dou et al. 2005). Una de sus dianas más estudiadas (por estudios de expresión génica y *ChIP-sequencing*) son los genes *Hox* (*homeodomain genes*), cuya expresión es alta en células troncales y precursores tempranos, y deben ser silenciados para la maduración y diferenciación de los mismos. Los genes *Hox,* dependen más que otros de la función del complejo MLL y se ven más afectados por la pérdida de función de MLL (Slany 2009) (Eguchi et al. 2003) (Eguchi et al. 2005).

1.5.2. REORDENAMIENTOS DE MLL.

Los reordenamientos del gen *MLL* suceden en un grupo heterogéneo de leucemias agudas (linfoides, mieloides y de linaje mixto), y comprenden principalmente fusiones o traslocaciones y duplicaciones parciales en tándem (MLL-PTD) (Zhang et al. 2012). También se han descrito otras alteraciones que se

presentan con menor frecuencia como son amplificación génica de *MLL* (Hess 2004), inversiones y deleciones. *MLL* se reordena en leucemias humanas con cerca de 100 genes conocidos (Meyer et al. 2009). De hecho, existe un consorcio internacional conocido como "Recombinoma de MLL" liderado por los profesores Marschalek y Meyer (Meyer et al. 2006; Meyer et al. 2009)(Meyer et al. sometido).

Tanto las fusiones de *MLL* como MLL-PTD son reguladores trascripcionales de dianas génicas que normalmente son controladas por *MLL*. Varios mecanismos reguladores epigenéticos están implicados en procesos leucémicos mediada por las fusiones de *MLL*, entre ellos metilación de DNA, acetilación de histonas y metilación de histonas. Recientemente, la histona metiltrasferasa DOT1L, el miembro de la familia BET (*bromodomain and extra-terminal*) BRD4, y la proteína Menin se han definido como elementos de unión a *MLL* fusionado con implicaciones en la trasformación oncogénica. En algunas proteínas de fusión como MLL-AF4, MLL-AF5q31 y MLL-ENL, la unión a DOT1L se produce a través del factor de elongación positiva b (pTEFb) (Benedikt et al. 2011; Zhang et al. 2012). La metiltrasferasa DOT1L promueve la marca de metilación H3K79. DOT1L es reclutado por las fusiones de *MLL* y provoca modificaciones importantes en la cromatina. La metilación aberrante H3K79 parece jugar un papel fundamental en la expresión génica asociada a las fusiones de *MLL*. De hecho, se ha comprobado que los pacientes con LLA con *MLL* fusionado presentan un perfil de expresión génica característico (Armstrong et al. 2002), posiblemente debido a la metilación aberrante asociada a las traslocaciones de *MLL*. Sin embargo, no hay que perder de vista el papel que juega el gen al que se fusiona *MLL* en las traslocaciones. En este sentido, Stam *y cols.* mostraron en muestras de pacientes con LLA infantil, que las traslocaciones t(4;11), t(11;19) y t(9;11) presentan una marca de expresión génica específica, que identifica de manera independiente tres grupos de pacientes (Stam et al. 2010).

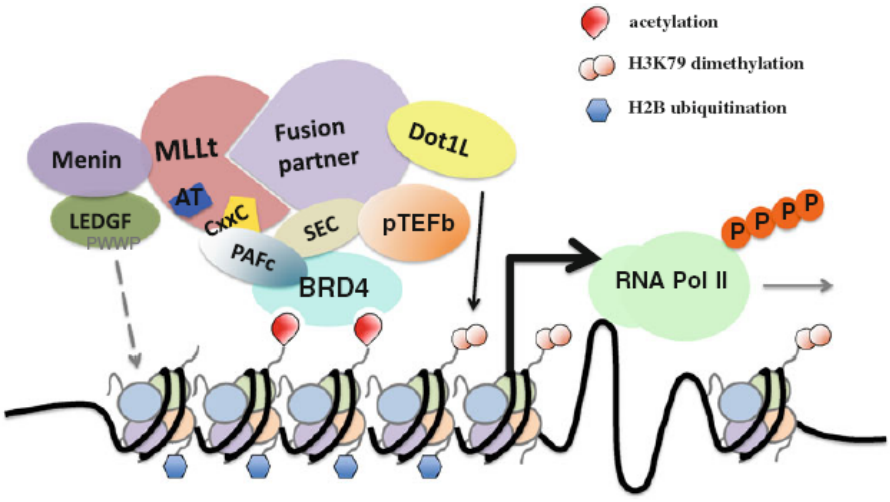

Figura 11. Complejo proteico formado tras la traslocación de *MLL*. La proteína de fusión MLL retiene su interacción con Menin, LEDGF y el complejo PAFc, pero pierde los motivos PHD y el dominio SET, contenidos en la subunidad MLL^C, por lo que pierde la actividad metiltrasferasa H3K4. Sin embargo, la proteína de fusión MLL recluta, directamente o a través de pTEFb, a la metiltrasferasa DOT1L que cataliza la metilación H3K79. La mayoría de los compañeros de fusión de *MLL* interactúan directamente con el supercomplejo de elongación (SEC), junto con PAFc. SEC se une a la proteína BRD4 que interacciona con las histonas acetiladas (Zhang et al. 2012).

El dominio MLL^N se encarga de la selección de la diana génica del complejo MLL, y este complejo se mantiene en las fusiones de *MLL*, por lo que mantendrían, al menos, dichas dianas génicas. Esto se ha confirmado para los genes *Hox*, que se expresan también en las fusiones de *MLL*. De hecho, se ha identificado expresión aberrante de los genes *Hox* en LLA pediátricas en comparación con muestras sanas (Starkova et al. 2010). Como se ha mencionado anteriormente, los genes *Hox* se expresan en hematopoyesis en los precursores inmaduros y esta expresión se inhibe para favorecer la maduración de los mismos hacia las células hematopoyéticas funcionales. Por tanto, una expresión ectópica de los genes *Hox* mediada por la fusión de *MLL*, podría bloquear el proceso de diferenciación y propiciaría la aparición de un precursor pre-leucémico (Slany 2009).

La mayoría de los reordenamientos corresponden con traslocaciones balanceadas en las que la porción N-terminal de MLL se fusiona con la porción C-terminal de otro gen. Como la fusión de estos genes ocurre en el marco de lectura génica, los genes quimera resultantes son trascritos y trasducidos dando lugar a proteínas de fusión altamente oncogénicas. Hasta la fecha se conocen más de 100 genes que se fusionan a *MLL* en este tipo de traslocaciones, habiéndose clonado y caracterizado a nivel molecular más de 70 (Meyer et al. 2009). Las traslocaciones más frecuentes son MLL-AF4, MLL-AF9, MLL-AF10, MLL-ENL y MLL-ELL, suponiendo el 85% de todas las fusiones de *MLL* (Wang et al. 2011). La mayoría de las fusiones de *MLL* tienen lugar entre los exones 8 y 12, en una región de 8.3kb denominada *bcr* (*breakpoint cluster region*) (Thirman et al. 1993). Sin embargo, estudios muy recientes han comprobado que la mayoría de las rupturas de *MLL* en LLA infantiles ocurren en el exón 11, y que este grupo de pacientes presentan además peor pronóstico, lo que indica que el punto de ruptura *MLL* tiene importancia clínica y podría considerarse como un predictor de la evolución de la enfermedad en los pacientes con *MLL* traslocado (Emerenciano et al. 2013).

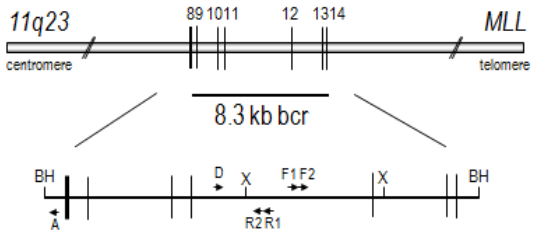

Figura 12. Región génica de *MLL*, donde se producen habitualmente las rupturas (*bcr-breakpoint cluster region*) (Bueno et al. 2009).

En cuanto al origen de las traslocaciones de *MLL*, varias evidencias han señalado a la recombinación no homóloga de DNA de cadena doble como la causa más probable de las traslocaciones de 11q23 (Slany 2009). Una de estas evidencias es el tratamiento con etopósido como responsable de la aparición de reordenamiento de *MLL* en pacientes con leucemias secundarias a tratamiento (Blanco et al. 2004) (Felix 2001; Felix et al. 2006). El etopósido inhibe la acción de la topoisomerasa II y como consecuencia se priva a la célula de los mecanismos de reparación necesarios. Además, el locus 11q23 parece

particularmente susceptible a la acción de etopósido (Bueno et al. 2009), aunque la causa de por qué MLL es un "*hot spot*"/diana génica desconocido.

Las duplicaciones parciales en tándem (PTDs) tienen lugar en los exones de la secuencia N-terminal de MLL (Hess 2004). Suceden en el 10% de los casos de LMA y se asocian con peor pronóstico que las leucemias del mismo tipo que no presentan reordenamiento de *MLL* (Schichman et al. 1994). El mecanismo de trasformación de MLL-PTD parece relacionado con la actividad de dimerización de MLL, ya que la duplicación de las regiones de DNA ricas en nucleótidos AT y del dominio con homología a DNA metiltrasferasas (CxxC) presentes en la porción N-terminal de MLL, incrementa la afinidad por su sitio diana de unión. Además, los exones duplicados distanciarían los dominios autoregulatorios de sus sitios de acción en el complejo MLL. Por otro lado, existen evidencias de que los mecanismos de trasformación oncogénica de MLL-PTD podrían ser diferentes a los de *MLL* traslocado, ya que se ha comprobado que los pacientes con MLL-PTD no muestran sobreexpresión de genes *Hox* ni de otros genes como Meis1, que constituyen una marca específica en la mayoría de las traslocaciones de *MLL*. Así, MLL-PTD no recluta DOT1L, como lo hacen muchas fusiones de *MLL*.

Otro tipo de reordenamiento de *MLL* descrito, aunque minoritario es la amplificación de *MLL* resultando en copias adicionales del gen aparentemente inalterado (Poppe et al. 2004). Aparece en algunos casos de síndromes mielodisplásico (SMD) y LMA. La amplificación de *MLL* también se asocia con la sobreexpresión de algunos genes consistentemente expresados en leucemias con reordenamientos de *MLL*, sugiriendo un mecanismo similar de trasformación. Entre estos genes se encuentran los ya mencionados (*HoxA7*, *HoxA9* y *Meis1*) y otros, que son potencialmente directas o bien indirectas (*PROML1*, *ADAM10*, *NKG2D* y *ITPA*) (Poppe et al. 2004).

1.5.3. REORDENAMIENTO DE MLL-AF4.

Los reordenamientos de MLL-AF4 ocurren como consecuencia de la traslocación t(4;11) (q21;23). En LLA pediátrica, el 50% de las fusiones de *MLL* corresponden con MLL-AF4, siendo la fusión de MLL más frecuente en este tipo de leucemias (Meyer et al. 2009; Pui et al.).

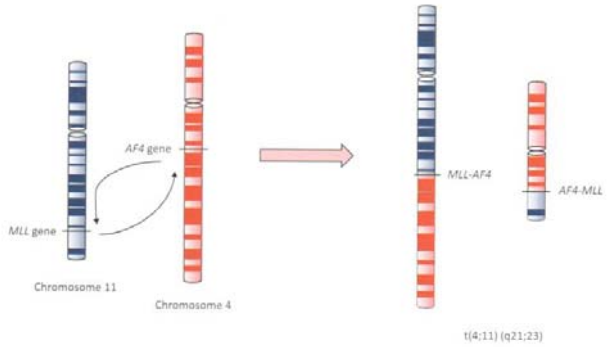

Figura 13. Traslocación cromosómica t(4;11)(q21;23) (Stumpel 2012).

En la LLA pro-B del lactante, la traslocación t(4;11) aparece en la mayoría de los pacientes. Dado el pronóstico fatal de este tipo de leucemias, y su asociación casi exclusiva con MLL-AF4, es probable que esta traslocación induzca un fenotipo leucémico más agresivo. La traslocación MLL-AF4 identifica a pacientes con un perfil de expresión génica característico (Stam et al. 2010). Además, en muestras de LLA infantil MLL-AF4+ se ha podido distinguir dos grupos de pacientes dependiendo de la presencia o ausencia de la expresión de *HoxA9*, *HoxA7*, *HoxA10*, *HoxA5* y *HoxA3*, asociándose la ausencia de expresión de dichos genes con peor pronóstico (Stam et al. 2010).

Como se ha explicado en el apartado 1.4.1.3.1. de esta introducción, no existen hasta la fecha modelos *in vivo* que reproduzcan la enfermedad LLA pro-B MLL-AF4+. Los modelos de enfermedad logrados en ratón no reproducen ni el fenotipo ni la latencia observada en humano. En una de las últimas aproximaciones, Bursen *y cols*. consiguieron desarrollar el proceso leucémico en ratón en presencia de AF4-MLL (producto recíproco de la traslocación (ver figura 8, arriba)), independientemente de la expresión de MLL-AF4, por lo que los autores postulan que es la expresión de AF4-MLL el evento transformante (Bursen et al. 2010). La expresión de MLL-AF4 y AF4-MLL se ha detectado en 80% de pacientes, pero su aportación a la enfermedad en humanos necesita ser elucidada. De hecho, otros autores defienden que la expresión de AF4-MLL es necesaria para la proliferación y supervivencia de leucemias derivadas de pacientes con traslocación t(4;11) pero no para el inicio del proceso leucémico (Kumar et al. 2011).

Tanto el origen prenatal de la traslocación t(4;11) (Greaves 2003), como la corta latencia que presenta la enfermedad LLA pro-B del lactante (aparece antes del primer año de vida), inducen a pensar que el reordenamiento MLL-AF4 podría ser suficiente en humano para desarrollar el proceso leucémico. Esto estaría apoyado por los trabajos de Bardini *y cols.* (Bardini et al. 2010; Bardini et al. 2011), en los se observa ausencia de *CNA* (del inglés *copy number alterations*), es decir, un genoma estable en estos pacientes. Al contrario de lo que ocurre en la gran mayoría de tumores, la LLA pro-B con t(4;11) MLL-AF4+ presenta hipermetilación del genoma lo que se asocia a una estabilidad génica y explicaría, en parte, la ausencia de eventos oncogénicos secundarios (Stumpel et al. 2013). Sin embargo, hay ciertas proteínas que, en un estado de expresión alterada, están presentes de modo más o menos frecuente a la LLA pro-B MLL-AF4+. Es el caso de la proteína K-Ras, que aparece mutada en un 20-40% de leucemias MLL-AF4+ (Liang et al. 2006; Prelle et al. 2012; Driessen et al. 2013), y del receptor tirosina-kinasa FLT3, que también se muestra altamente sobre-expresado (Stam et al. 2007a), y en un 3-20% de los casos se ha descrito mutado, en LLA MLL-AF4+ (Armstrong et al. 2004; Taketani et al. 2004).

1.6. RECEPTOR TIROSÍN-KINASA FLT3.

1.6.1. ESTRUCTURA Y FUNCIÓN DEL RECEPTOR FLT3.

El receptor tirosina kinasa FLT3 (*Fms-like tyrosine kinase 3*), es también conocido como FLK-2 (*fetal liver kinase-2*) y STK-1 (*human stem cell kinase-1*). Pertenece a la familia de receptores tirosina kinasa de clase III (RTKIII) al que también pertenecen FMS, el factor de crecimiento derivado de plaquetas (PDGFR) y KIT. El gen *FLT3* se localiza en el cromosoma 13q12 y consta de 24 exones (Abu-Duhier et al. 2001). Codifica una proteína glucosilada unida a membrana de 993 aminoácidos con un peso molecular de 158-160kDa, así como otra isoforma no glucosilada de 130-143 kDa citoplasmática no asociada a la membrana plasmática (Rosnet et al. 1993). El receptor FLT3 se caracteriza por un N-dominio extracelular glicosilado conformado por cinco subdominios de estructura similar a la inmunoglobulina, por un dominio trasmembrana y dos dominios citoplasmáticos con función tirosina kinasa. Se expresa en células inmaduras del sistema hematopoyético, placenta, gónadas y cerebro.

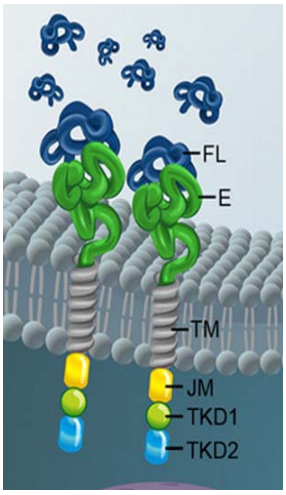

Figura 14. Imagen representativa del receptor tirosina kinasa FLT3 uniéndose a su ligando (FL) por su dominio extracelular (E) y formando homodímeros. También se representan los dominios trasmembrana (TM), y los dominios citoplasmáticos de unión a membrana (JM), y con función tirosina kinasa (TKD1 y TKD2). Modificado de (Fischer 2011).

El ligando de FLT3 (FL) es una proteína trasmembrana tipo 1 que contiene un péptido de señalización N-terminal, cuatro dominios extracelulares helicoidales (Lyman and Jacobsen 1998). El ligando de FLT3 se expresa en la mayoría de los tejidos, incluyendo próstata, ovario, riñón, pulmón, colon, intestino delgado, testículo, corazón, placenta, así como órganos hematopoyéticos (bazo, timo, sangre periférica y MO), correspondiendo su expresión más alta a células mononucleares circulantes en sangre (Stirewalt and Radich 2003).

La unión de FL al receptor FLT3 induce la formación de homodímeros en la membrana plasmática que conduce a la autofosforilación del receptor en los residuos tirosina citoplasmáticos (TKD1 y TKD2). La activación del receptor activa, a su vez, una cascada de señalización mediante la fosforilación y activación de múltiples moléculas efectoras citoplasmáticas involucradas en apoptosis, proliferación y

diferenciación de células hematopoyéticas en MO (Takahashi 2011). El dominio citoplasmático se asocia físicamente con la subunidad p85 de la kinasa fosfoinositol-3 (PI3K), Ras guanosina trifosfatasa (GTP-asa), fofolipasa C-γ 1 (PLCγ), Shc, Grb2 y tirosina kinasas de la familia Src, lo que conlleva a la fosforilación de todas estas proteínas (Dosil et al. 1993). La fosforilación de estas proteínas induce la activación de las vías Akt (*PI3K/protein kinase B*) y MAPK (*mitogen-activated protein kinase*) (Takahashi 2006).

1.6.2. MUTACIONES ACTIVADORAS DE FLT3.

Se han descrito hasta la fecha dos tipos de mutaciones que conducen a la activación constitutiva, es decir, en ausencia de ligando, del receptor FLT3. En ambas mutaciones se pierde la función autoinhibitoria de fosforilación. Una de ellas tiene lugar en el dominio yuxtamembrana del receptor y se conoce como ITD (del inglés *internal tandem duplication*). El otro tipo de mutaciones se conocen como mutaciones puntuales o del bucle de activación, y tienen lugar en los dominios tirosina kinasa citoplasmáticos, siendo las más frecuentes la sustitución del residuo Asp835 (D835 *mutation*) (ver figura 15). En los apartados siguientes se describen con más detalle estas mutaciones activadoras.

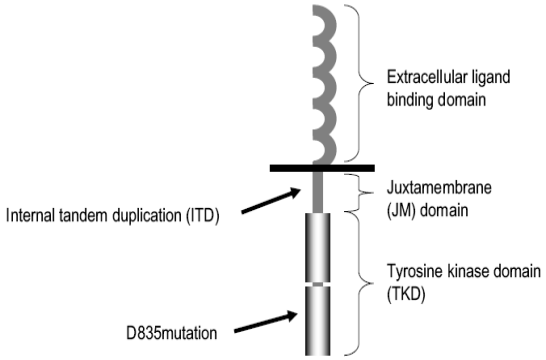

Figura 15. Esquema representativo del receptor FLT3 y de la localización de las mutaciones activadoras (Takahashi 2011). Aunque no representada en el esquema, la mutación I836del (que implica la deleción del aminoácido isoleucina de la posición 836) ocurre en el dominio tirosina-kinasa (TKD) al igual que la mutación D835Y (Yamamoto et al. 2001).

1.6.2.1. MUTACIONES EN EL DOMINIO KINASA.

El bucle de activación es un componente conservado de las tirosina kinasas y presenta dos conformaciones según el estado activo o inactivo de la kinasa (Gilliland and Griffin 2002). Cuando la kinasa está en estado inactivo, el bucle de activación bloquea el acceso de ATP y que éste sirva de sustrato de fosforilación al dominio kinasa. Cuando la kinasa está en estado activo, tras la unión del ligando al receptor FLT3, el bucle adopta la conformación activa permitiendo el acceso de ATP a la kinasa. En el caso del receptor FLT3, en este bucle de activación se han descrito diversas mutaciones puntuales consistentes bien en la sustitución del aminoácido aspártico de la posición 835 (D835), o bien en la eliminación o deleción del aminoácido isoleucina de la posición 836 (I836del). Con respecto a la mutación D835, la mutación más frecuente es la D835Y (aspártico→tirosina), pero también se han

descrito otras, como D835V (aspártico→valina), D835H (aspártico→histidina), D835E (aspártico→glutámico) y D835N (aspártico→asparagina). Estas mutaciones puntuales en el dominio kinasa provocan que el bucle de activación adquiera la conformación "activada" en ausencia de ligando, por lo que el receptor FLT3 se presenta constitucionalmente activo (Till et al. 2001). Esta activación constitutiva conduce a la dimerización del receptor, y a la fosforilación y activación de las vías de señalización Akt, MAPK y STAT5 (*signal transducers and activators of transcription*) (Taketani et al. 2004).

La incidencia de estas mutaciones puntuales es aproximadamente 7% en LMAs en adulto, 3% en síndromes mielodisplásicos y 2.8% en LLAs (Yamamoto et al. 2001). Sin embargo, en el caso de las LLAs infantiles, se ha mostrado hasta un 20% de casos con mutaciones en el dominio kinasa (Armstrong et al. 2004; Taketani et al. 2004). Además, Ono *y cols*. han demostrado que FLT3 puede colaborar con fusiones de *MLL* en procesos leucémicos (Ono et al. 2005).

1.6.2.2. MUTACIONES EN EL DOMINIO YUXTAMEMBRANA.

En el dominio yuxtamembrana del receptor FLT3 se ha descrito una mutación conocida como ITD. Esta mutación tiene lugar en los exones 14 y 15 del gen FLT3 (Abu-Duhier et al. 2001) y consiste en repeticiones seriadas de un fragmento de este dominio que puede variar tanto en la posición y la longitud como en el número de repeticiones, pero siempre manteniendo el marco de lectura de la trascripción. El dominio yuxtamembrana de FLT3 tiene una función inhibitoria reguladora de la autofosforilación de residuos tirosina kinasa, por lo que la mutación ITD en este dominio resulta en una pérdida de la función autoinhibitoria, y por lo tanto dimerización y activación constitutiva del receptor FLT3 (Gilliland and Griffin 2002). Esta activación constitutiva conduce a la activación de las vías de señalización de RAS/MAP kinasas, PI3K/AKT y STAT5 (Hayakawa et al. 2000; Kiyoi et al. 2002; Choudhary et al. 2007).

En 1996, *Nakao y cols.* (Nakao et al. 1996), mostraron por primera vez la presencia de ITDs en LMAs, y en la actualidad se ha descrito que está presente en el 20% de este tipo de leucemias (Meshinchi et al. 2001). También se han descrito en menor medida en síndromes mielodisplásicos (SMDs) (5%), mientras que prácticamente no se han detectado en LLAs (3%) (Yokota et al. 1997; Xu et al. 2000; Meshinchi et al. 2001). Así mismo, se especula que en los pocos casos en que la mutación ITD aparece en LLA, no proporciona la misma ventaja proliferativa, ya que no se asocia con mal pronóstico en este tipo de leucemia (Xu et al. 2000). En cuanto a LMAs, la frecuencia de la mutación ITD es menor en pacientes infantiles que en adultos (Xu et al. 2000). Por otro lado, tanto la longitud como el número de repeticiones de la mutación ITD tiene un impacto clínico, mostrando peor pronóstico cuanto mayor sea la longitud del fragmento que se repite (Kottaridis et al. 2001; Stirewalt et al. 2006; Meshinchi et al. 2008). El trasplante intrafemoral de células de MO trasducidas con la mutación ITD provoca la aparición de un SMD en

ratones, pero no conduce al desarrollo de la leucemia aguda (Kelly et al. 2002), por lo que se piensa que son necesarias otras alteraciones/mutaciones adicionales para el desarrollo de LMA.

1.6.3. SOBREXPRESIÓN DE FLT3 EN AUSENCIA DE MUTACIONES.

La activación constitutiva del receptor FLT3 también se produce por altos niveles de expresión del mismo, por los cuales el receptor dimeriza en ausencia de ligando. La sobreexpresión en ausencia de mutación se ha mostrado en la mayoría de las muestras analizadas correspondientes a pacientes de LLA con traslocación de *MLL* y con hiperdiploidía (Stam et al. 2007a). Guenter *y cols*. (Guenther et al. 2008) han demostrado mediante elegantes experimentos de *ChIP-Sequencing* que *FLT3* es una diana directa de MLL-AF4, lo que puede explicar fehacientemente porque los pacientes MLL-AF4+ expresan altos niveles de *FLT3*.

La expresión de altos niveles de *FLT3* está asociada con las traslocaciones de *MLL* en pacientes con LLA y LMA. En cuanto a la LLA infantil, Armstrong y colaboradores, demostraron que la expresión de *FLT3* es significativamente mayor en pacientes con traslocaciones de *MLL*, que en pacientes con el gen *MLL* intacto (Armstrong et al. 2002). Además, este mismo grupo ha comprobado que las células leucémicas provenientes de pacientes con LLA infantil *MLL* traslocado presentan mayor sensibilidad a inhibidores de FLT3 que pacientes no infantiles con LLA y de LMA con mutación ITD (Stam et al. 2005).

1.6.4. ALTERACIONES EN LA EXPRESIÓN DE FLT3 Y LLA PRO-B DEL LACTANTE.

En el caso de las LLAs pediátricas, la aparición de mutaciones activadoras del receptor FLT3 parece restringida a LLAs con reordenamientos *MLL* o con hiperdiploidía (>50 cromosomas). En estos subtipos de LLA, las mutaciones puntuales TKD de *FLT3* ocurren en un porcentaje variable de 3-21% de los casos (Armstrong et al. 2004; Taketani et al. 2004; Emerenciano et al. 2008; Emerenciano et al. 2013), mientras que son infrecuentes las mutaciones ITD de FLT3 (Xu et al. 2000).

La activación de FLT3 es una característica de las LLAs con reordenamiento de *MLL* (Armstrong et al. 2002). Hay autores que postulan que la activación constitutiva del receptor FLT3 es consecuencia de mutaciones activadoras en un porcentaje muy pequeño (3-20%), y que es la expresión de altos niveles de *FLT3* la causa de dicha activación (Stam et al. 2007a). Por lo tanto, la activación constitutiva de FLT3, bien por mutaciones o por sobreexpresión del receptor, se asocia a las LLA con reordenamiento de *MLL*, y más concretamente con LLA pro-B infantil. Es por esto que la activación constitutiva de FLT3 constituye uno de los eventos candidatos a colaborar con la traslocación t(4;11) en el desarrollo y mantenimiento del proceso leucémico.

HIPÓTESIS DE TRABAJO Y OBJETIVOS

HIPÓTESIS DE TRABAJO

El oncogén de fusión MLL-AF4 se caracteriza por tener un origen pre-natal durante la hematopoyesis embrionaria-fetal. Dicho gen de fusión se asocia con la LLA pro-B MLL-AF4+ del lactante con una latencia muy breve y una gran agresividad con afectación linfoide B-monocítica. Varios estudios han tratado de reproducir esta leucemia neonatal en ratón mediante el empleo de células troncales/progenitoras hematopoyéticas. Sin embargo, estos modelos no han reproducido ni la latencia ni el fenotipo observados en los pacientes. El poco éxito en la consecución de un modelo que reproduzca la enfermedad de manera exacta podría deberse a: (i) la célula diana empleada en los modelos murinos es una célula en un estadio ontogénico o jerárquico equivocado, (ii) MLL-AF4 cuando es expresado bajo los promotores retrovirales LTR acumula niveles mucho más altos que los fisiológicos pudiendo llegar a ser tóxico, (iii) no se ha tenido en cuenta la presencia de alteraciones secundarias o adicionales, o la exposición a factores ambientales con un gran impacto en la etiología de la enfermedad, o (iv) MLL-AF4 sólo es funcional en células humanas, indicando que la patogénesis ha de ser estudiada empleando células troncales/progenitoras humanas en un estadio ontogénico relativamente temprano.

Ante la ausencia de un modelo fiel que reproduzca la LLA pro-B MLL-AF4+ difícilmente lograremos avanzar en el conocimiento sobre el origen celular y patogenia de esta leucemia pediátrica tan devastadora. Hipotetizamos que el empleo de hESCs o hematopoyesis embrionaria y células CD34+ de SCU que representan dos estadios ontogénicos diferentes, prenatal y neonatal, respectivamente, pueden representar la célula diana idónea en la que MLL-AF4 tiene impacto. Así mismo, hipotetizamos que la activación constitutiva (ligando independiente) del receptor tirosina kinasa FLT3 en estas células, bien por mutación bien por sobre-expresión, puede cooperar con MLL-AF4 en el inicio del proceso leucémico.

OBJETIVOS

1. Objetivos relacionados con aspectos técnicos y metodológicos asociados tanto al cultivo y mantenimiento de hESCs y CD34+ de SCU como a la utilización de la cepa de ratón inmunodeficiente más apropiada.
    - 1a. Optimización de las condiciones de mantenimiento y expansión de HSPCs derivadas de SCU.
    - 1b. Optimización de las condiciones de mantenimiento indiferenciado de hESCs.
    - 1c. Selección de la cepa de ratón inmunodeficiente que permita una mejor reconstitución hematopoyética, así como de las condiciones metodológicas más apropiadas
2. Objetivo dirigido a profundizar en la asociación genotipo-fenotipo entre los reordenamientos *MLL* y la expresión del marcador NG2.
3. Objetivos relacionados con el estudio del impacto funcional y oncogénico del gen de fusión MLL-AF4 en células troncales progenitoras neonatales y embrionarias.
    - 3a. Estudiar el impacto de MLL-AF4 en células HSPCs derivadas de SCU.

3b. Estudiar el efecto de MLL-AF4 sobre la especificación hematopoyética a partir de hESCs.

4. Objetivos relacionados con el estudio de la activación constitutiva del receptor FLT3 y su posible papel en el proceso de iniciación del proceso leucémico en colaboración con MLL-AF4.
    4a. Estudiar el estado mutacional y de expresión del receptor FLT3 en muestras de pacientes con leucemias agudas con y sin reordenamiento MLL-AF4.
    4b. Estudiar el efecto de la activación constitutiva de FLT3 en hESCs que expresan MLL-AF4.
    4c. Estudiar la activación constitutiva de FLT3 en HSPCs CD34+ de SCU que expresan MLL-AF4.

MATERIAL, MÉTODOS Y RESULTADOS

La descripción de los materiales y métodos empleados, así como de los resultados obtenidos, están reflejados en los artículos originales publicados en relación con cada uno de los objetivos planteados y que han sido incluidos en esta sección. Cada uno de ellos va precedido por un breve resumen que pretende facilitar una revisión rápida de la información más relevante contenida en los mismos. Los artículos incluidos en este apartado son:

- En relación a los objetivos 1a, 1b y 1c, relacionados con aspectos técnicos y metodológicos asociados tanto al cultivo y mantenimiento de hESCs y CD34+ de SCU como a la utilización de la cepa de ratón inmunodeficiente más apropiada:

1.- Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-β and IGF-II.
Montes R., Ligero G., Sanchez L., Catalina P., de la Cueva T., Nieto A., Melen G.J., Rubio R., García-Castro J., Bueno C., Menendez P.
Cell Res. 2009;19(6):698-709.

2.- The ROCK inhibitor Y-27632 negatively affects the expansion/survival of both fresh and cryopreserved cord blood-derived CD34+ hematopoietic progenitor cells.
Bueno C., **Montes R.**, Menendez P.
Stem Cell Rev and Rep. 2010; 6(2):215-223.

3.- Intra-bone marrow transplantation of human CD34(+) cells into NOD/LtSz-scid IL-2rgamma(null) mice permits multilineage engraftment without previous irradiation.
Bueno C., **Montes R.**, de la Cueva T., Gutierrez-Aranda I., Menendez P.
Cytotherapy. 2010; 12(1):45-49.

- En relación al objetivo 2, dirigido a profundizar en la asociación genotipo-fenotipo entre los reordenamientos *MLL* y la expresión del marcador NG2.

4.- NG2 antigen is expressed in CD34+ HPCs and plasmacytoid dendritic cell precursor: is NG2 expression in leukemia dependent on the target cell where leukemogenesis is triggered?
Bueno C., **Montes R.**, Martín L., Prat I., Hernandez M.C., Orfao A., Menendez P.
Leukemia. 2008; 22: 1475-1478.

- En relación a los objetivos 3a y 3b, relacionados con el estudio del impacto funcional y/o oncogénico del gen de fusión MLL-AF4 en células troncales progenitoras neonatales y embrionarias:

5.- Enforced expression of MLL-AF4 fusion in cord blood CD34+ cells enhances the hematopoietic repopulating cell function and clonogenic potential but is not sufficient to initiate leukemia.

Montes R., Ayllón V., Gutierrez-Aranda I., Prat I., Hernández-Lamas C., Ponce L., Bresolin S., te Kronnie G., Greaves M., Bueno C., Menendez P.
Blood. 2011; 117(18):4746-4758.

6.- A human ESC model for MLL-AF4 reveals an impaired early hemato-endothelial specification.
Bueno C., **Montes R.**, Melen G.J., Ramos-Mejia V., Real P.J., Ayllón V., Sánchez L., Ligero G., Gutiérrez-Aranda I., Fernandez A., Fraga M.F., Moreno-Gimeno I., Burks D., Plaza M.C., Rodriguez-Manzaneque J.C., Menéndez P.
Cell Res. 2012; 22(6):986-1002.

- En relación con los objetivos 4a, 4b y 4c, relacionados con el estudio de la activación ligando-independiente del receptor FLT3 en combinación con la expresión del gen de fusión MLL-AF4:

7.- Prognostic significance of FLT3 mutational status and expression levels in MLL-AF4+ and MLL-germline acute lymphoblastic leukemia.
Chillón M.C., Gómez-Casares M.T., López-Jorge C.E., Rodriguez-Medina C., Molines A., Sarasquete M.E., Alcoceba M., Miguel J.D., Bueno C., **Montes R.**, Ramos F., Rodríguez J.N., Giraldo P., Ramírez M., García-Delgado R., Fuster J.L., González-Díaz M., Menendez P.
Leukemia. 2012; 26(11):2360-2366.

8.- FLT3 activation cooperates with MLL-AF4 fusion protein to abrogate the hematopoietic specification of hESCs.
Bueno C., Ayllón V., **Montes R.**, Romero-Moya D., Navarro-Montero D., Ramos-Mejia V., Real P.J., Arauzo M., Menendez P.
Blood 2013, in press. doi: 10.1182/blood-2012-11-470146.

9.- Ligand-independent FLT3 activation does not cooperate with MLL-AF4 to immortalize/transform cord blood CD34+ cells.
Montes R., Bursen A., Prelle C., Marschalek R., Chillón M.C., Romero-Moya D., Prieto C., Navarro-Montero O., Bueno C., Menendez P.
(Sometido, Leukemia 2013)

1.- Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-β and IGF-II.

Montes R., Ligero G., Sanchez L., Catalina P., de la Cueva T., Nieto A., Melen G.J., Rubio R., García-Castro J., Bueno C., Menendez P.

Cell Res. 2009;19(6):698-709.

INTRODUCCIÓN. El crecimiento de hESCs en MEF-CM es dependiente de una regulación paracrina en las que las hESCs se diferencian de forma espontánea a células fibroblastoides que mantienen la homeostasis del cultivo mediante la producción de TGF-β e IGF-II en respuesta a la adición al medio de bFGF. La importancia de la familia TGF-β en el mantenimiento de la pluripotencia de hESCs ya ha sido ampliamente establecida, pero, sin embargo, poco se sabe sobre el papel de IGF-II.

OBJETIVO. Con el fin de reducir el uso de componentes xenogénicos, en este trabajo proponemos estudiar si las hESCs se mantienen estables y pluripotentes usando para su cultivo HFF-CM y hMSC-CM. Además, quisimos analizar si la cooperación de bFGF con TGF-β e IGF-II descrita en el mantenimiento de hESCs cultivadas en MEF-CM, se produce cuando estas células se cultivan en HFF-CM y hMSC-CM.

MATERIAL Y MÉTODOS. La expresión de los receptores de bFGF en los HFFs y en las hMSCs fue confirmada por RT-PCR. Los niveles de TGF-β en los medios de cultivo se determinaron por ELISA. Las hESCs se mantuvieron en cultivo en HFF-CM y hMSC-CM, valorándose la morfología del cultivo, la estabilidad cromosómica mediante estudios de bandeo G, la expresión de los marcadores de pluripotencia por citometría, la expresión de los factores de transcripción asociados a hESCs indiferenciadas por RT-PCR, y la capacidad de diferenciación tanto *in vitro* como *in vivo* mediante la formación de cuerpos embrionarios y teratomas en ratón, respectivamente.

RESULTADOS. Tanto los HFFs como las hMSCs expresan los cuatro receptores para bFGF y producen específicamente TGF- β en respuesta a bFGF. Sin embargo, en respuesta a bFGF, las hMSCs no producen IGF-II, mientras que los HFFs sí que lo producen. Respecto a la ausencia de IGF-II en el MSC-CM, las hESCs crecidas con este medio mantienen la morfología típica, euploidia, la expresión de marcadores típicos de pluripotencia, así como su potencial de diferenciación a las tres capas germinales *in vivo* e *in vitro*. Por otro lado, el bloqueo específico de IGF-II en HFF-CM y hMSC-CM no tenía ningún efecto en el mantenimiento de la homeostasis de las hESCs.

CONCLUSIÓN. Nuestros resultados indican que las hESCs pueden mantenerse en hMSC-CM en ausencia de IGF-II, y que el modelo previamente propuesto de regulación paracrina por el que bFGF coopera con TGF-β e IGF-II en el mantenimiento de la homeostasis y pluripotencia de hESCs en MEF-CM, no parece extrapolable a hESCs mantenidas en hMSC-CM.

Cell Research (2009) 19:698-709.

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ORIGINAL ARTICLE

Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-β and IGF-II

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A paracrine regulation was recently proposed in human embryonic stem cells (hESCs) grown in mouse embryonic fibroblast (MEF)-conditioned media (MEF-CM), where hESCs spontaneously differentiate into autologous fibroblast-like cells to maintain culture homeostasis by producing TGF-β and insulin-like growth factor-II (IGF-II) in response to basic fibroblast growth factor (bFGF). Although the importance of TGF-β family members in the maintenance of pluripotency of hESCs is widely established, very little is known about the role of IGF-II. In order to ease hESC culture conditions and to reduce xenogenic components, we sought (i) to determine whether hESCs can be maintained stable and pluripotent using CM from human foreskin fibroblasts (HFFs) and human mesenchymal stem cells (hMSCs) rather than MEF-CM, and (ii) to analyze whether the cooperation of bFGF with TGF-β and IGF-II to maintain hESCs in MEF-CM may be extrapolated to hESCs maintained in allogeneic mesenchymal stem cell (MSC)-CM and HFF-CM. We found that MSCs and HFFs express all FGF receptors (FGFR1-4) and specifically produce TGF-β in response to bFGF. However, HFFs but not MSCs secrete IGF-II. Despite the absence of IGF-II in MSC-CM, hESC pluripotency and culture homeostasis were successfully maintained in MSC-CM for over 37 passages. Human ESCs derived on MSCs and hESCs maintained in MSC-CM retained hESC morphology, euploidy, expression of surface markers and transcription factors linked to pluripotency and displayed in vitro and in vivo multilineage developmental potential, suggesting that IGF-II may be dispensable for hESC pluripotency. In fact, IGF-II blocking had no effect on the homeostasis of hESC cultures maintained either on HFF-CM or on MSC-CM. These data indicate that hESCs are successfully maintained feeder-free with IGF-II-lacking MSC-CM, and that the previously proposed paracrine mechanism by which bFGF cooperates with TGF-β and IGF-II in the maintenance of hESCs in MEF-CM may not be fully extrapolated to hESCs maintained in CM from human MSCs.

Keywords: TGF-β, IGF-II, bFGF, human ESCs, mesenchymal stem cells, conditioned media, feeder-free
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Introduction

Human embryonic stem cells (hESCs) are pluripotent stem cells derived from human blastocyst-stage embryos [1]. Human ESCs are defined by both robust self-renewal capacity and pluripotent developmental potential *in vitro* and *in vivo* [1-4]. These distinctive properties of hESCs are not autonomously achieved, and recent evidence points to a level of external control from the microenvironment, through cell-cell interactions and soluble extrinsic factors [5, 6]. Human ESCs are cultured in either feeder-based or feeder-free systems. Despite the fact that mouse embryonic fibroblasts (MEFs) are the most commonly used feeders for hESC co-culture [1], human feeders including human foreskin fibroblasts (HFFs) [7, 8] and human mesenchymal cells [9] have also been successfully explored in order to reduce xeno-components.

Among the feeder-free systems, MEF-conditioned

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media (MEF-CM) are the most widely employed [10, 11]. Moreover, more defined culture conditions such as serum replacement media containing high concentrations of basic fibroblast growth factor (bFGF) [12] or other exogenous factors such as TGF-β, Activin A, Noggin, insulin-like growth factor-II (IGF-II) or the Wnt agonist BIO among others have also been reported to be essential for undifferentiated growth of hESCs [reviewed in 13]. Despite the fact that hESCs have been successfully maintained on human fibroblasts [7, 8] and mesenchymal cells [9], only in recent work hESCs have been maintained in human fibroblast-CM [14]. However, to date no study has attempted to maintain hESCs in mesenchymal stem cell (MSC)-CM.

Recently, several members of the TGF-β family of signalling molecules (Activin, Nodal and TGF-β) and IGF-II have been shown to be necessary for the maintenance of pluripotency in hESCs [5, 15-18]. Importantly, despite the evolution of the hESC culture conditions and the finding of these several key factors, the supplementation with bFGF to sustain hESC potential is still required. In fact, both TGF-β and IGF-II have been proposed to cooperate with the FGF pathway to maintain feeder-free hESC cultures in xenogenic MEF-CM by establishing a regulatory stem cell niche [5, 18]. Thus, a model of paracrine regulation was recently proposed [5] within feeder-free hESC cultures maintained in MEF-CM, where hESC colonies spontaneously differentiate into hESC-derived fibroblast-like cells (hdFs) to maintain culture homeostasis. These autologous hdFs support hESC culture homeostasis by releasing, in response to bFGF, the hESC supportive factors TGF-β and IGF-II, which have a direct role in the survival and self-renewal of hESCs [5].

In order to facilitate hESC culture conditions and to reduce xenogenic components, we sought to determine whether different hESC lines can be maintained stable and sustain pluripotency in a feeder-free system using CM from human feeders such as MSCs and HFFs rather than xenogeneic MEF-CM. Additionally, we aimed at establishing whether the previously proposed cooperation of exogenous bFGF with TGF-β and IGF-II to maintain hESCs in MEF-CM may be extrapolated to hESC cultures maintained in CM from allogeneic human feeders.

We found that MSCs and HFFs express the four putative FGF receptors (FGFR1-4) and specifically produce TGF-β in response to bFGF. However, HFFs but not MSCs secrete IGF-II. Despite the absence of IGF-II in MSC-CM, hESC pluripotency and culture homeostasis were successfully maintained in MSC-CM beyond 23 passages. Similar to HFF-CM, hESCs derived on MSCs and those maintained in MSC-CM retained typical hESC morphology, euploidy, expression of pluripotency-associated surface markers and transcription factors and displayed *in vitro* and *in vivo* differentiation potential into tissues representing mesoderm, ectoderm and endoderm. These data suggest that hESCs can be successfully maintained feeder-free in IGF-II-lacking MSC-CM, and that the previously proposed paracrine mechanism by which bFGF cooperates with TGF-β and IGF-II in the maintenance of hESCs in MEF-CM may not be fully extrapolated to hESCs maintained in CM from human feeders.

Results

TGF-β is secreted by MSCs and HFFs, and its production increases in response to bFGF

Many efforts have been made to develop hESC culture systems based on the use of human feeders [8, 9, 19-24]. However, among the feeder-free culture systems, the most widely established is still based on the use of xenogenic MEF-CM. Only in very recent work have hESCs been maintained feeder-free using human fibroblast-CM [14]. To date no study has analyzed whether hESCs may be successfully maintained in MSC-CM. We therefore sought to determine whether hESCs can be maintained pluripotent and feeder-free in MSC-CM and HFF-CM.

We initially analyzed whether allogeneic human mesenchymal stem cells (hMSCs) and HFFs used to produce CM of human origin release TGF-β and IGF-II in response to bFGF as recently reported for autologous hdFs [5]. Accordingly, the expression of all known putative FGF receptors was analyzed in both proliferating hMSCs (Figure 1A) and HFFs (Figure 1C). RT-PCR analysis revealed that both hMSCs (Figure 1B) and HFFs (Figure 1D) expressed the four FGF receptors (FGFR1-4). Next, we determined whether TGF-β, a well-established hESC-supportive factor that cooperates with bFGF, is produced by these human feeders in response to exogenous bFGF [5, 15, 18]. Human TGF-β was readily detectable at low levels in MSC-CM (Figure 1E) and HFF-CM (Figure 1F). Importantly, its concentration significantly increased in response to exogenous bFGF: 3.6-fold increase in MSC-CM (Figure 1E) and 5.8-fold increase in HFF-CM (Figure 1F). To further confirm TGF-β production in response to bFGF, MSC-CM and HFF-CM were independently harvested on day 2 (1st harvest) and day 5 (2nd harvest) after cell seeding and a reproducible trend was observed for both hMSCs and HFFs, which clearly produced additional TGF-β upon bFGF exposure regardless of the time point of CM harvesting. Chemical inhibition of FGFR1 prior to supplementation of bFGF reduced the release of TGF-β by 36% in HFFs and 62% in MSCs (Figure 1G), demonstrating that this functional cooperation between bFGF and TGF-β seems specific. Together,

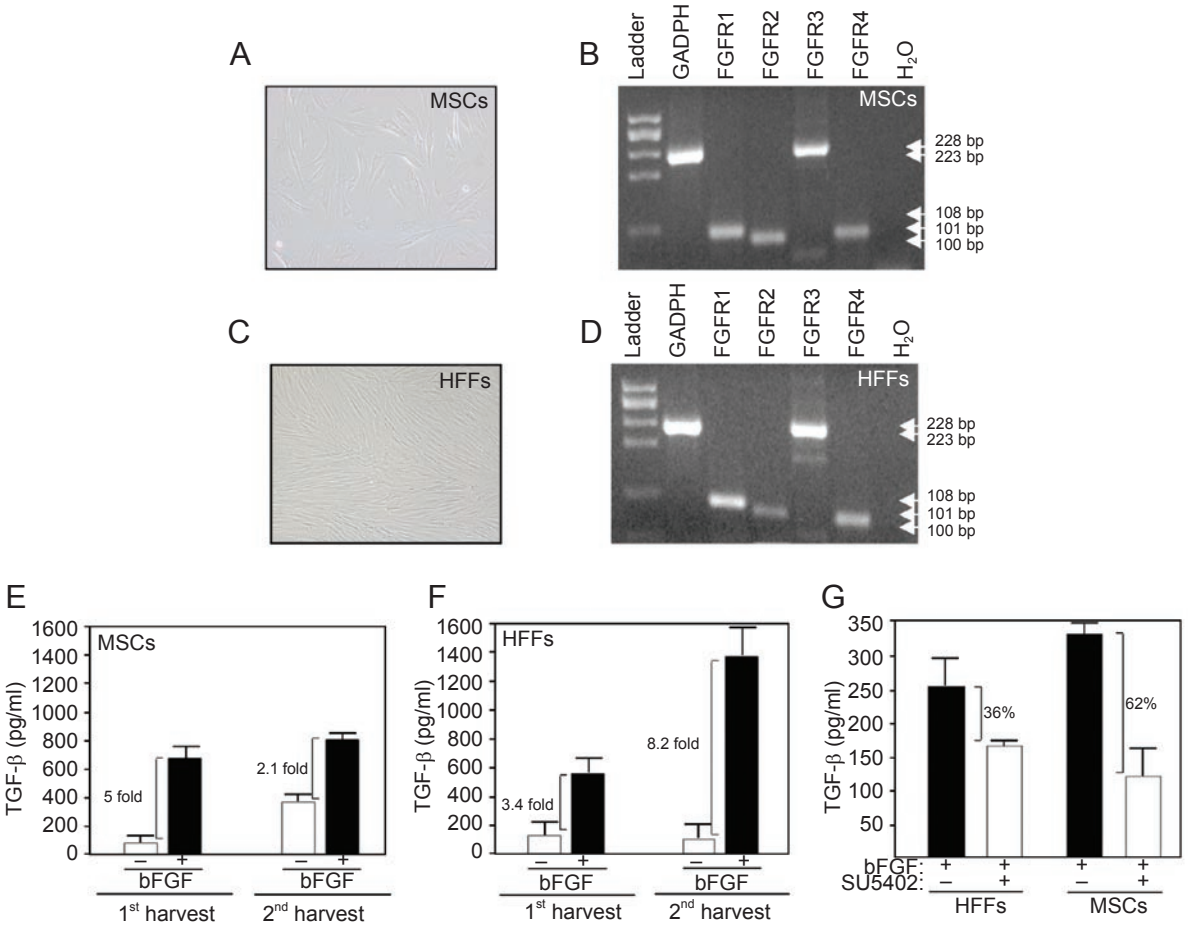


Figure 1 TGF-β is produced in response to bFGF by MSCs and HFFs. Phase-contrast morphology of MSCs **(A)** and HFFs **(C)**. RT-PCR showing mRNA expression of FGFR1-4 in MSCs **(B)** and HFFs **(D)**. Amount of TGF-β produced by MSCs **(E)** and HFFs **(F)** in response to bFGF. **(G)** Specific blockage of TGF-β production by the FGFR chemical inhibitor SU5402.

these results indicate that similar to autologous hdFs, allogeneic hMSCs and HFFs produce TGF-β in response to exogenous bFGF.

HFFs but not MSCs secrete IGF-II, and the production of IGF-II is not augmented in response to bFGF in either human-derived feeders

Still very little is known about the role of IGF-II in hESC pluripotency. IGF-II has been implicated in clonal outgrowth of hematopoietic stem cells [25] and IGF-II and IGFR are expressed in both mouse and human blastocysts [26]. Moreover, a paracrine mechanism by which bFGF cooperates not only with TGF-β but also with IGF-II to maintain feeder-free hESCs in MEF-CM was recently reported for the first time [5]. We therefore sought to determine whether this functional cooperation between bFGF and IGF-II to maintain hESCs in MEF-CM may be extrapolated to hESC cultures maintained in MSC-CM/HFF-CM. In line with previous data [5], we first confirmed that IGF-II is undetectable in MEF-CM and basal hESC media but that it is detectable in media conditioned by autologous hdFs differentiated from hESCs (data not shown). Interestingly, although HFFs produce significant basal levels of IGF-II (2800±260 pg/ml), IGF-II concentration does not increase in response to exogenous bFGF (Figure 2A). Human MSCs, however, do not secrete IGF-II prior to or after exposure to bFGF (Figure 2A). To confirm the ELISA data, we determined by real-time RT-PCR the mRNA expression level of IGF-II in HFFs and hMSCs in the presence or absence of bFGF. In line with the ELISA results, HFFs showed high expression of IGF-II mRNA (Figure 2B). The IGF-II mRNA expression was even higher than that observed in the MCF7 cell line used as positive control (*C*t values: 20 *vs* 26, respectively). As expected, exogenous bFGF did not change the IGF-II mRNA expression (Figure 2B;

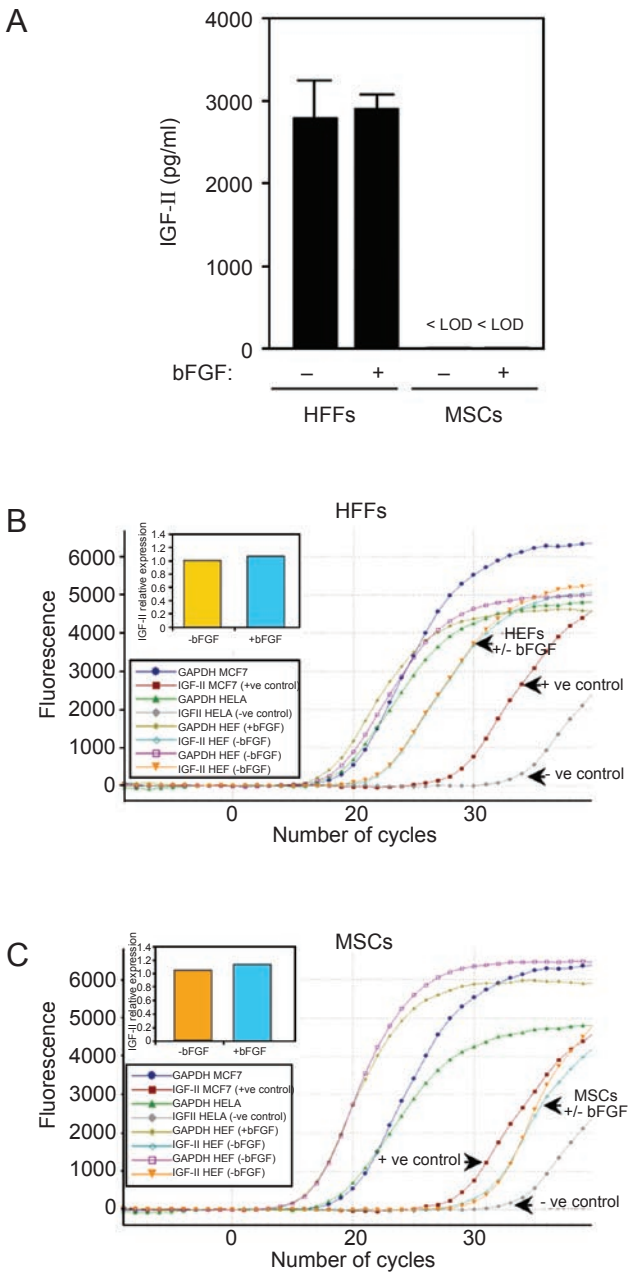


Figure 2 IGF-II production is not altered in response to bFGF by either MSCs or HFFs. **(A)** Levels of soluble IGF-II produced by HFFs and MSCs in the presence or absence of bFGF. In HFFs soluble IGF-II is readily detectable but its concentration does not increase in response to bFGF. In contrast, MSCs do not produce IGF-II. LOD=limit of detection. **(B, C)** Representative real-time RT-PCR confirming the absence of IGF-II in MSC-CM observed in the ELISA. **(B)** HFFs expressed higher levels of IFG-II mRNA (C_t=20) than the positive control (MCF-7 cell line). However, no differences were observed in the mRNA levels in the presence or absence of bFGF (inset). **(C)** According to the ELISA data, MSCs express low levels of mRNA for IGF-II (C_t=32). Similar to HFFs, the relative amounts of mRNA IGF-II was identical in the absence or presence of bFGF, together indicating that at least in these human feeders, bFGF cooperates with TGF-β but not with IGF-II.

inset panel). The real-time RT-PCR also confirmed the lack of IGF-II production by MSCs measured by ELISA (Figure 2C). As expected, hMSCs showed very low expression of IGF-II mRNA (Figure 2C) and the IGF-II mRNA expression remained low regardless of the presence or absence of bFGF (Figure 2C; inset panel). These data are consistent with previous reports suggesting that the distinct ability of mouse and human feeders in promoting undifferentiated growth of hESCs is attributable to their hESC-supportive growth factor production [20]: both HFFs and hMSCs produce TGF-β, whereas MSCs, in contrast to HFFs, are unable to release IGF-II.

Human ESCs retain in vitro and in vivo pluripotency in IGF-II-lacking MSC-CM

The analysis performed on MSC-CM and HFF-CM unravelled significant differences regarding the TGF-β and IGF-II composition. HFF-CM contains both TGF-β and IGF-II, while MSC-CM contains TGF-β but lacks IGF-II. We then wanted to determine whether hESCs can be maintained stable and pluripotent using allogeneic MSC-CM and HFF-CM rather than MEF-CM, taking into account the differences in IGF-II production between the two human feeders.

Two hESC lines, HS181 and SHEF1, were cultured for 23-37 passages feeder-free either in MSC-CM or in HFF-CM. Despite the absence of IGF-II in MSC-CM, both hESC lines retained pluripotency and undifferentiated growth in MSC-CM as they did in HFF-CM, which contains both factors, TGF-β and IGF-II. No differences in *in vitro* and *in vivo* pluripotency and culture homeostasis were observed among hESC lines maintained in MSC-CM versus HFF-CM. Identical to hESCs cultured in HFF-CM, those maintained in MSC-CM retained typical hESC morphology and culture homeostasis (Figure 3; left panels) and expression (analyzed by immunocytochemistry and flow cytometry) of the pluripotency-associated surface markers SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81 (Figure 3). Similarly, both hESC lines expressed the transcription factors Oct3/4, Nanog, Rex-1 and Sox-2 (Figure 4A) and remained karyotypically stable (Figure 4B). Functionally, both hESC lines successfully differentiated *in vitro* through EB formation (Figure 5A; left panel) into tissues representing the three germ layers: endoderm (α-fetoprotein+ cells; Figure 5A), mesoderm (Actin+ cells; Figure 5A) and ectoderm (β-III-Tubulin+ cells; Figure 5A). The gold-standard pluripotency assay relies on the ability to form *in vivo* teratomas upon injection into immune-deficient mice. HS181 and SHEF1 hESC lines maintained either in MSC-CM or in HFF-CM formed teratomas 8-10 weeks after inoculation. These complex and disorganized tumours contained

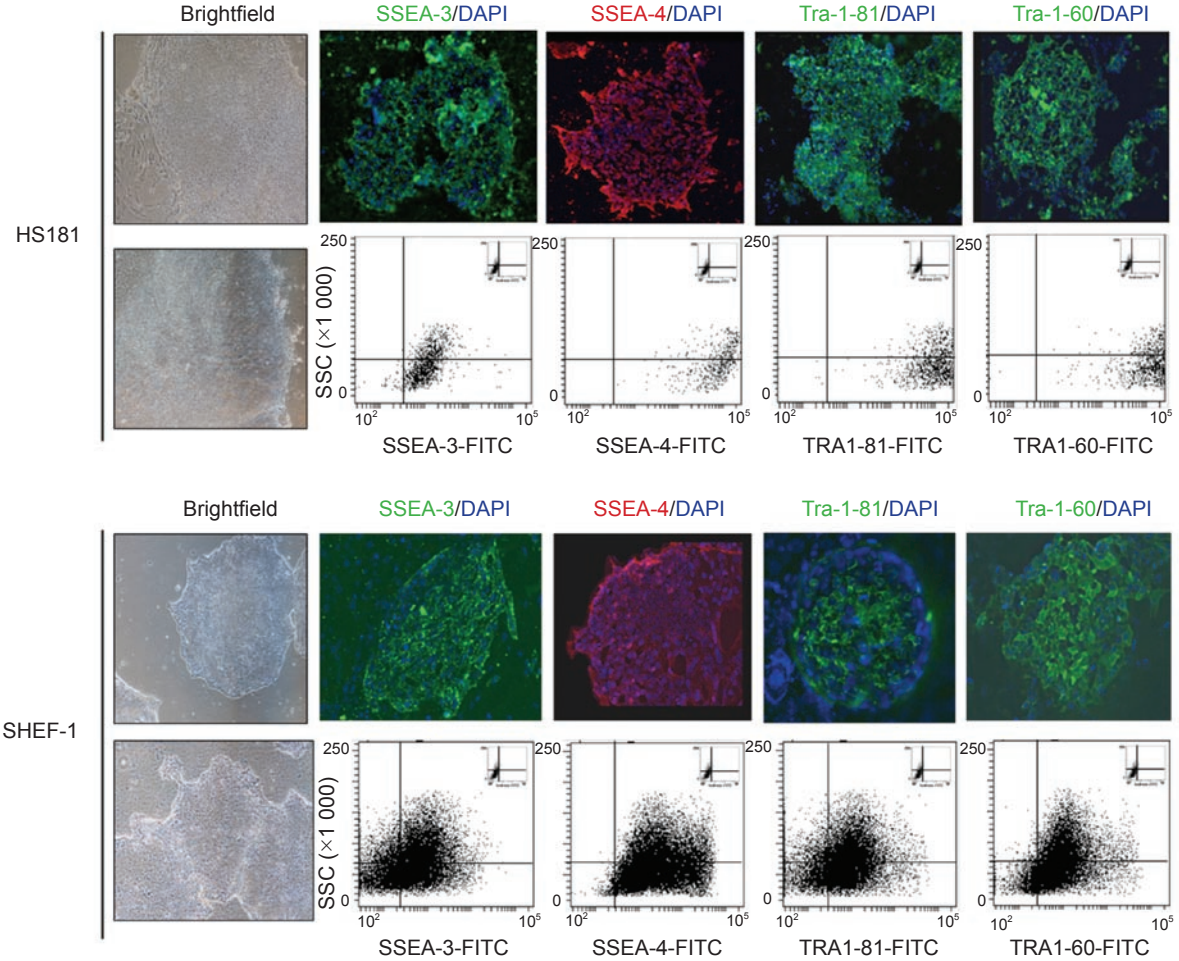


Figure 3 Representative immunophenotypic characterization of HS181 and SHFF1 hESC lines maintained feeder-free with MSC-CM or HFF-CM. Phase-contrast photomicrographs showing typical hESCs morphology (left panels). Expression of the pluripotency-associated surface markers SSEA-3, SSEA-4, Tra-1-60 and Tra-81 was detected both by immunocytochemistry (top row) and by flow cytometry (bottom row; insets represent isotype controls). For immunocytochemistry staining, colours are indicated. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, blue).

a variety of tissues representing the three germ layers (Figure 5B and 5C), thus demonstrating the pluripotent features of hESC lines, regardless of their growth in IGF-II-lacking MSC-CM or IGF-II-containing HFF-CM.

Human ESC cultures maintained in MSC-CM displayed growth kinetics and doubling time similar to those maintained in HFF-CM. A slight proliferative disadvantage could be observed just during the first 2-3 passages for those hESCs grown in MSC-CM (Figure 6A). However, no differences in cellular death/apoptosis were observed between hESCs grown in either condition (Figure 6B), suggesting a slower culture adaptation for those hESCs grown in MSC-CM. These data indicate that hESCs are successfully maintained feeder-free in MSC-CM lacking IGF-II, suggesting that IGF-II may be dispensable for hESC pluripotency. Additionally, the cooperation of bFGF with TGF-β and IGF-II in the maintenance of hESCs in MEF-CM might not be fully extrapolated to hESCs maintained in allogeneic MSC-CM lacking IGF-II.

Distinct requirements of IGF-II for hESC maintenance in MEF-CM versus allogeneic MSC-CM or HFF-CM

IGF-II was recently reported to be a candidate hESC-supportive factor [5], but its supplementation is not required in the absence of feeders to maintain hESCs in MEF-CM, suggesting endogenous IGF-II production in hESC cultures maintained in MEF-CM [5]. Accordingly, a paracrine regulation seems to occur in feeder-free hESC cultures grown in MEF-CM, by which hESCs spontaneously differentiate into hdFs to maintain culture homeostasis by producing IGF-II in response to bFGF.

Here, we first confirmed that IGF-II is undetectable in MEF-CM and basal hESC media, but that its levels

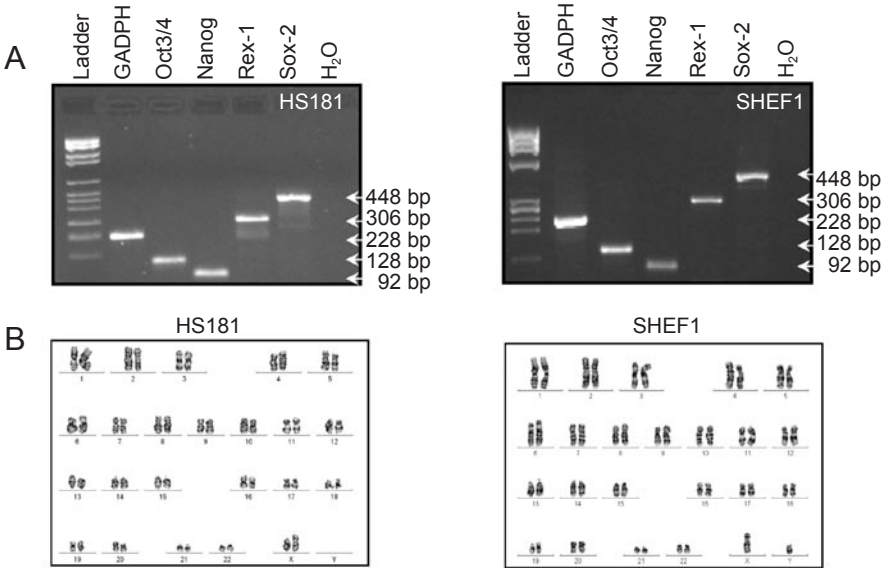


Figure 4 Molecular and cytogenetic characterization of HS181 and SHEF1 hESC lines maintained either with MSC-CM or with HFF-CM. **(A)** RT-PCR analysis displaying mRNA expression of the undifferentiated markers Oct3/4, Nanog, Rex-1 and Sox-2. GAPDH was used as a housekeeping gene. **(B)** Conventional G-banding confirming karyotypic stability of hESCs maintained for 30 passages feeder-free in MSC-CM or HFF-CM.

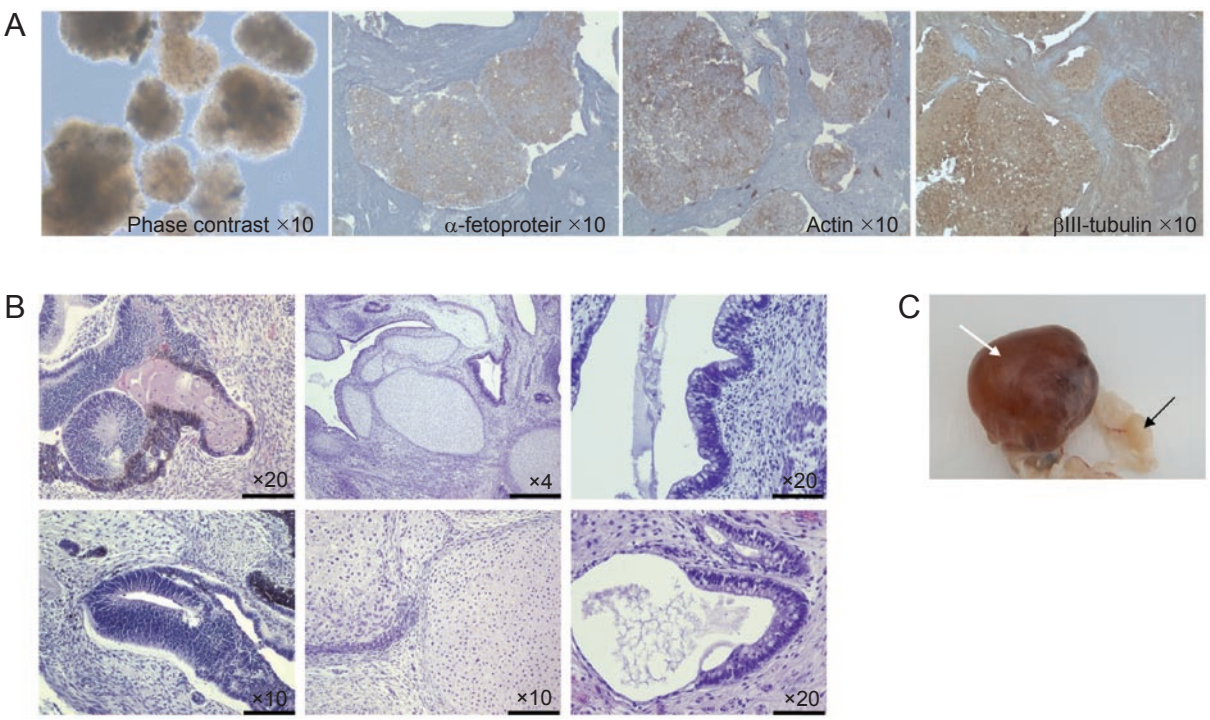
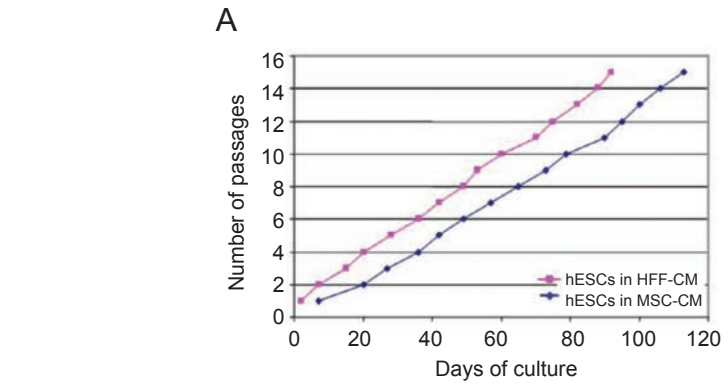
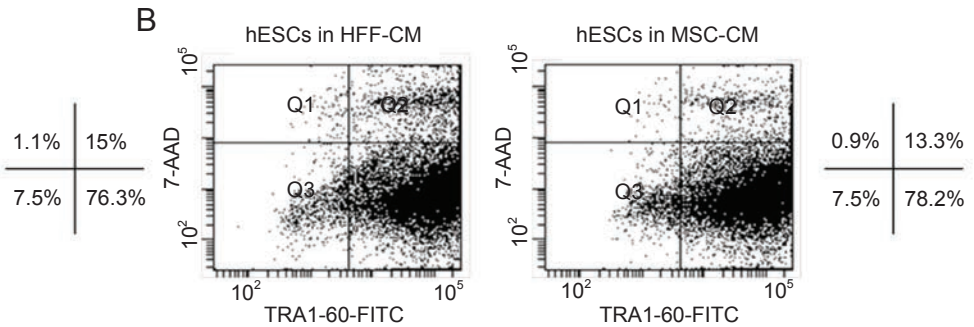


Figure 5 Representative images of *in vitro* and *in vivo* multi-lineage differentiation capacities of HS181 and SHEF1 hESC lines maintained feeder-free in MSC-CM or HFF-CM. **(A)** Phase-contrast (left panel) and immunohistochemistry images showing the ability of hESC lines to differentiate into tissues representing the three germ layers: endoderm (α-fetoprotein), mesoderm (actin) and ectoderm (βIII-tubulin). **(B)** Human ESCs cultured either in MSC-CM or in HFF-CM form teratomas containing tissues representing the three germ layers. A representative hematoxylin-eosin histology of HS181 (top panels) and SHEF1 (bottom panels) shows primitive neural and epidermal epithelia (ectoderm-origin; left panels), cartilage (mesoderm-origin; middle panels) and cylindrical epithelial and goblet cells (endoderm-origin; right panels). **(C)** Representative macroscopic image of 8-weeks teratoma after injection of hESCs maintained in MSC-CM (white arrow). Note the size increase as compared to PBS-injected testis (black arrow).





C

Human IGF-II (pg/mL)

HFF-CM	HFF-CM hESC conditioned	MSC-CM	MSC-CM hESC conditioned	hESC media conditioned by hESC derived and co-cultured over hMSCs
6300±70	3700±213	< LOD	< LOD	< LOD

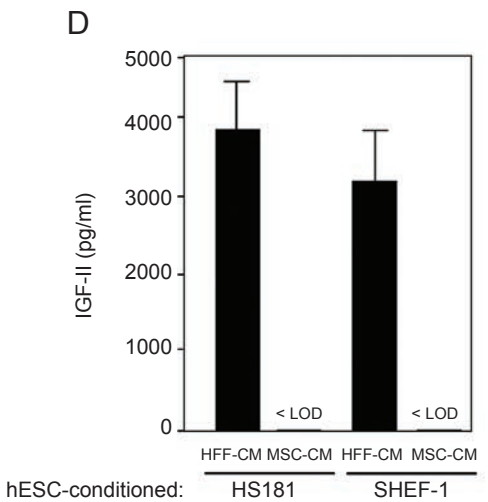


Figure 6 Growth and survival of hESCs maintained in MSC-CM versus HFF-CM and IGF-II concentration in hESC-conditioned MSC-CM or HFF-CM. **(A)** Cell growth curves of hESCs in MSC-CM (blue line) and in HFF-CM (red line). **(B)** Frequency of hESCs undergoing cell death/apoptosis in MSC-CM (right panel) and in HFF-CM (left panel) analyzed by flow cytometry after 7-AAD staining and/or Annexin-V staining. **(C)** Concentration of IGF-II in the indicated media. LOD: limit of detection. **(D)** Concentration of IGF-II detailed for HS181- and SHEF-1-conditioned MSC-CM or HFF-CM. LOD: limit of detection.

increase, which are readily detectable in media exposed to autologous hdFs [5 and data not shown]. On the other hand, HFFs readily produce basal levels of IGF-II, with an average concentration in HFF-CM of 6300±70 pg/ml (Figure 6C). However, in contrast to autologous hdFs, IGF-II concentration did not increase in response to bFGF measured by ELISA and Q-RT-PCR (Figure 2A and 2B). Human MSCs do not secrete IGF-II either before or after exposure to bFGF (Figure 2A and 6C). Similar to HFFs, the very low basal expression of IGF-II transcript in hMSCs was not increased in response to bFGF (Figure 2C). These data indicate that while autologous hdFs (either FACS-isolated or *in vitro* differentiated from hESCs) produce IGF-II in response to bFGF, human feeders such as HFFs and MSCs fail to do so. Moreover, despite clear differences in IGF-II content between HFF-CM and MSC-CM, hESC lines are equally maintained pluripotent in IGF-II-containing HFF-CM or IGF-II-lacking MSC-CM, suggesting that (i) autologous hdFs spontaneously differentiating from hESCs cultured in MSC-CM or HFF-CM, similar to what we have shown for hESCs maintained in MEF-CM, produce IGF-II, compensating its absence in the MSC-CM, or (ii) IGF-II is dispensable for maintenance of hESC pluripotency in hMSC-CM.

In order to test these two possibilities, the concentration of IGF-II was measured in IGF-II-containing HFF-CM and IGF-II-lacking MSC-CM before and after exposure to hESC cultures (Figure 6C and 6D). Detectable amounts of IGF-II were found in hESC-conditioned HFF-CM. However, no IGF-II was detectable in MSC-CM either prior to or after exposure to hESC cultures (Figure 6C and 6D). Furthermore, the undetectable levels of IGF-II in MSC-CM were reproducible after exposure to distinct hESC lines (HS181 and SHEF1) (Figure 6C and 6D). These data indicate that it is unlikely that hdFs derived from hESCs cultured in MSC-CM produced IGF-II, suggesting that MSC-CM might contain unknown factors interfering with the IGF-II production by hdFs. Alternatively, it remains plausible that allogeneic hMSCs produce other uncharacterized hESC-supportive factors, which make the presence of IGF-II irrelevant for hESC maintenance [20]. Overall, our data support that IGF-II may be dispensable for maintenance of hESC pluripotency in MSC-CM.

To gain further insights into the role of IGF-II in hESC derivation and maintenance, we measured the presence of IGF-II during the co-culture of a fully characterized hESC line recently derived in our laboratory on hMSCs (Cortes *et al*., submitted). Up until now, this hESC line (termed AND-1) has been successfully maintained on hMSCs with hESC basal media supplemented with 8 ng/ml of bFGF beyond 30 passages, while displaying typical hESC morphology, euploidy, expression of pluripotency-associated surface markers and transcription factors, and *in vitro* and *in vivo* multi-lineage developmental potential (Cortes *et al*., submitted). In line with the undetectable IGF-II levels measured by ELISA in MSC-CM and the very low IGF-II transcript expression in MSCs, no IGF-II could be detected in hESC media conditioned by this hESC line derived on and maintained in a MSC co-culture (Figure 6C). The successful derivation, maintenance of pluripotency and culture homeostasis of this hESC line derived and grown on hMSCs, together with the absence of IGF-II in the media conditioned by this hESC line, further demonstrate that IGF-II is dispensable for the maintenance of hESCs feeder-free in MSC-CM or on MSCs feeder layer.

To further confirm that IGF-II is dispensible for the maintenance of hESCs in HFF-CM and MSC-CM and to exclude the possibility of IGF-II secretion from hESCs themselves, IGF-II was specifically blocked with an IGF-II-neutralizing antibody. After 16 days, IGF-II blocking had no effects on the overall homeostasis of hESC cultures maintained either on HFF-CM (Figure 7A-7C) or on MSC-CM (Figure 7D-7F). Treatment with an IGF-II-neutralizing antibody did not induce morphological changes (Figure 7A and 7D) and had little to no effect on cell proliferation, apoptosis, expression of the ESC-associated markers Tra-1-81 and SSEA-3 (Figure 7B and 7E) and expression of the ESC-specific transcription factors Nanog, Oct3/4, Rex1 and Sox2 (Figure 7C and 7F).

Discussion

Many efforts have been made to develop hESC culture systems based on human feeders [8, 9, 19, 20, 22-24]. However, among the feeder-free culture systems, the most widely established is still based on the use of MEF-CM. Only in very recent work have hESCs been maintained feeder-free using human fibroblast-CM [14]. No study has analyzed so far whether hESCs may be successfully maintained in MSC-CM. The rationale behind maintaining hESCs in MSC-CM or even deriving them on MSCs is two-fold: (i) to reduce the presence of xeno-components, and (ii) because hESC maintained with MSC-CM or derived on MSCs might be more prone to differentiation toward mesoderm lineages.

We have recently shown that hESCs maintained in MEF-CM differentiate into autologous hdFs, which maintain culture homeostasis by producing TGF-β and IGF-II in response to bFGF [5]. Very little is known about the role of IGF-II in the maintenance of hESC pluripotency. Here, we aimed at determining whether

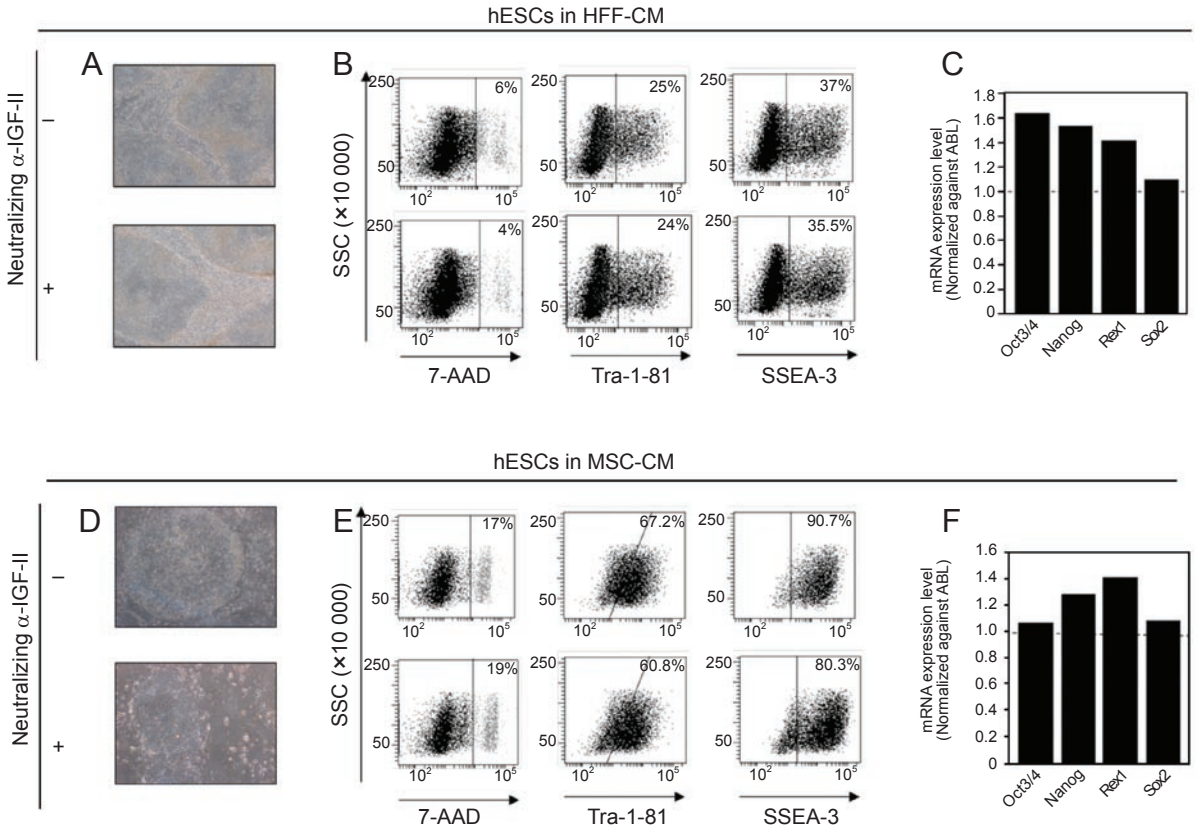


Figure 7 IGF-II blocking does not affect the homeostasis of hESCs maintained in HFF-CM or MSC-CM. Phase-contrast images of hESC cultures maintained in HFF-CM **(A)** or MSC-CM **(D)** with or without the IGF-II neutralizing antibody. 7-AAD staining and expression of Tra-1-81 and SSEA-3 in hESC cultures maintained in HFF-CM **(B)** or MSC-CM **(E)** with or without the neutralizing α-IGF-II. Oct3/4, Nanog, Rex1 and Sox2 transcript expression in hESCs maintained in HFF-CM **(C)** or MSC-CM **(F)** in the presence of the neutralizing α-IGF-II. The mRNA expression is relative to that from hESCs maintained in the absence of α-IGF-II and normalized against the indicated housekeeping gene.

hESCs can be maintained stable and pluripotent using allogeneic MSC-CM and HFF-CM rather than xenogenic MEF-CM, and to analyze whether the cooperation of bFGF with TGF-β and IGF-II in MEF-CM may be extrapolated to hESCs maintained in allogeneic MSC-CM and HFF-CM.

Similar to feeder-free culture in MEF-CM, both MSCs and HFFs specifically produce TGF-β, a well-established hESC-supportive factor, in response to exogenous bFGF. As previously reported [5], IGF-II was undetectable in MEF-CM and basal hESC media but was detectable in media conditioned by autologous hdFs differentiated from hESCs. Interestingly, HFFs readily produce IGF-II whereas MSCs do not secrete IGF-II at all, and the production of IGF-II is not induced in response to bFGF in either human feeder. Our data reveal differences in the composition of TGF-β and IGF-II factors between HFF-CM and MSC-CM and confirm that the distinct ability of mouse and human feeders to promote undifferentiated hESC growth may be attributable to their hESC-supportive growth factor production [20].

Despite the significant differences regarding the composition of IGF-II between HFF-CM and MSC-CM, several hESC lines can be equally maintained stable and pluripotent for over 37 passages using allogeneic MSC-CM and HFF-CM. Human ESCs derived on MSCs and those maintained in MSC-CM retained typical morphology, euploidy, expression of surface markers and transcription factors associated with pluripotency and displayed *in vitro* and *in vivo* multilineage developmental potential. Human ESCs maintained in MSC-CM displayed during the first 2-3 passages slower growth as compared to hESCs grown in HFF-CM. Due to the lack of differences in doubling time and apoptosis between hESCs grown in MSC-CM and those grown in HFF-CM, it may be plausible that rather than a proliferative disadvantage, a delayed culture adaptation of hESCs grown in MSC-CM occurred. Thus, hESCs are successfully main-

tained feeder-free in MSC-CM lacking IGF-II, suggesting that IGF-II may be dispensable for hESC pluripotency. The functional cooperation of bFGF with TGF-β and IGF-II reported for hESCs in MEF-CM might not be extrapolated to hESCs maintained in allogeneic IGF-II-lacking MSC-CM.

While autologous hdFs produce IGF-II in response to bFGF, allogeneic human feeders fail to do so. Moreover, despite clear differences in the IGF-II content between HFF-CM and MSC-CM, hESC lines are equally maintained pluripotent in IGF-II-containing HFF-CM or IGF-II-lacking MSC-CM, suggesting that (i) autologous hdFs derived from hESCs cultured in MSC-CM or HFF-CM produce IGF-II, thus compensating its absence in the MSC-CM or (ii) IGF-II is dispensable for maintenance of hESC pluripotency in human feeders-derived CM. To distinguish between these two possibilities, the concentrations of IGF-II in HFF-CM and MSC-CM before and after exposure to hESC cultures were measured. hdFs derived from hESCs cultured in MEF-CM produced IGF-II [5], whereas those derived from hESCs cultured in allogeneic HFF-CM or MSC-CM failed to produce IGF-II, suggesting that allogeneic human-derived CM might contain unknown factors interfering with IGF-II production by hdFs. Alternatively, it remains plausible that human feeders produce alternative uncharacterized hESC-supportive factors, which make the presence of IGF-II irrelevant for hESC maintenance [20]. Two-dimensional protein fractionation experiments are being considered in our laboratory in order to facilitate a comprehensive mapping of soluble protein profiles in distinct mouse- and human-derived CM. Overall, our data support that IGF-II may be dispensable for maintenance of hESCs in human feeder-derived CM, rather than the hypothesis that autologous hdFs differentiated from hESCs cultured in MSC-CM produce IGF-II, which compensates for its absence in the MSC-CM.

Materials and Methods

hESC maintenance

Human ESC lines HS181 and SHEF1 (kindly provided by Prof. Outi Hovatta, Karolinska Institute, Sweden and Prof. Harry Moore and Peter Andrews, University of Sheffield, UK, respectively) were maintained in a feeder-free culture over matrigel-coated T25 flasks (BD Biosciences) in MSC-CM or HFF-CM. The basal media used to prepare the CM consisted of 80% KO-DMEM supplemented with 20% KO Serum Replacement, 1% non-essential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol and 8 ng/ml of bFGF (all from Invitrogen, CA). MSC-CM and HFF-CM were prepared and collected as described in detail for MEF-CM [11, 12]. HFFs were purchased from ATCC (SCD-1112SK). Human MSCs were obtained from post-natal adipose tissue from healthy donors upon informed consent as described previously [27, 28]. During routine maintenance, HFFs and MSCs were grown in IMDM and advanced-DMEM, respectively, plus 10% FCS and 2 mM L-glutamine and split in the ratio 1:2 when they reached 80-90% confluence. MSCs were fully characterized and showed typical fibroblast-like morphology, immunophenotype ($CD44^+CD90^+CD73^+CD105^+CD45^-CD34^-CD14^-HLA\text{-}DR^-$) and *in vitro* differentiation capacity into osteoblasts, adipocytes and chondrocytes [7, 27-29]. Both hESC lines were maintained feeder-free for 23-37 passages and were fed with MSC-CM or HFF-CM. The media was changed daily and the hESC cultures were split (1:2) weekly using collagenase IV [10, 12].

Immunocytochemistry and flow-cytometric characterization of hESCs

Human ESC lines were phenotypically characterized by immunocytochemistry and flow cytometry for SSEA-3, SSEA-4 (both from DSHB, Iowa), and Tra-1-81 and TRA-1-60 (both from Chemicon, CA). For immunocytochemistry, hESC colonies were fixed in 4% of paraformaldehyde for 20 min, followed by 30-min incubation in 10% normal goat serum in PBS. Colonies were incubated with primary antibodies (1:100 dilution) for 1 h at RT. Conjugated secondary antibodies (1:100) were used for 30 min at RT as follows: goat anti-rat IgG AMCA-conjugated for SSEA-4 and goat anti-mouse IgM-FITC conjugated for SSEA-3, TRA-1-60 and Tra-1-81. The slides were mounted in Vectashield containing DAPI. An irrelevant monoclonal antibody of the same isotype was used as a negative control.

For flow cytometry, collagenase IV-dissociated hESCs were suspended in PBS+3%FBS at a concentration of $2\text{-}5\times10^4$ cells per 100 µl and incubated with the specific primary antibody for 30 min at 4 ºC. After being washed, cells were incubated with 2.5 µl of FITC-conjugated goat anti-mouse IgG antibody (Immunotech, Marseille, France). After 30 min at 4 ºC, stained cells were washed and stained with 7-aminoactinomycin D (7-AAD) viability dye (Immunotech) for 15 min at RT. Live cells identified by 7-AAD exclusion were analyzed for expression of SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81 using a FACSCanto-II flow cytometer equipped with the FACSDiva software (BDB) [30].

RNA extraction, cDNA synthesis and RT-PCR detection of hESC markers and FGFRs

Total RNA from hESCs, HFFs and MSCs was obtained using Trizol Reagent (Invitrogen). The first-strand cDNA was synthesized with 10 µl of total RNA, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The samples were incubated at 25 ºC for 10 min, followed by 2-h incubation at 37 ºC. The reaction was stopped at 85 ºC for 5 s.

Expression of the pluripotency-associated transcription factors Oct3/4, Nanog, Sox-2 and Rex-1, as well as FGFR1-4, was assessed by end-point RT-PCR. GAPDH or ABL was used as the housekeeping gene. The primers used for the PCR were as follows: Oct3/4: 5′-TCT GCA GAA AGA ACT CGA GCA A-3′ and 5′-AGA TGG TCG TTT GGC TGA ACA C-3′; Nanog: 5′-TGC AGT TCC AGC CAA ATT CTC-3′ and 5′-CCT AGT GGT CTG CTG TAT TAC ATT AAG G-3′; Sox-2: 5′-CCC CCG GCG GCA ATA GCA-3′ and 5′-TCG GCG CCG GGG AGA TAC AT-3′; Rex-1: 5′-CAG ATC CTA AAC AGC TCG CAG AAT-3′ and 5′-GCG TAC GCA AAT TAA AGT CCA GA-3′; FGFR1: 5′-GGA CTC TCC CAT CAC TCT GCA T-3′ and 5′- CCC CTG TGC AAT AGA

TGA TGA TC-3′; FGFR2: 5′-ACG TGG AAA AGA ACG GCA GTA-3′ and 5′- AGC CAG CAC TTC TGC ATT GG-3′; FGFR3: 5′-AGG ATG CCT GCA TAC ACA CTG C-3′ and 5′- ACA CCC TAC GTT ACC GTG CTC AAG-3′; FGFR4: 5′-GAA CCG CAT TGG AGG CAT T-3′ and 5′-TTC TCT ACC AGG CAG GTG TAT GTG-3′; and GAPDH: 5′-GAA GGT GAA GGT CGG AGT C-3′ and 5′-GAA GAT GGT GAT GGG ATT TC-3′. For Oct3/4, Nanog, Sox-2 and Rex-1, the conditions used for end-point PCR and Q-PCR were as follows: end-point PCR [5 min at 94 ºC, 35 cycles of 30 s at 94 ºC followed by 50 s at 60 ºC and 50 s at 72 ºC and a final extension of 10 min at 72 ºC] and Q-PCR [10 min at 95 ºC, 40 cycles of 30 s at 95 ºC followed by 60 s at 55 ºC and 60 s at 72 ºC]. For the FGFRs the following PCR conditions were used: 2 min at 50 ºC, 10 min at 95 ºC, 40 cycles of 15 s at 95 ºC followed by 60 s at 60 ºC.

Conventional karyotyping

G-banding studies were performed as described previously [31-34]. Briefly, hESC lines were cultured in hESC medium supplemented with 0.1 mg/ml colcemid (Biological Industries) for up to 3-4 h. The cells were then washed in Versene solution (Gibco) and subsequently trypsinized and spun down. The pellet was resuspended carefully in a KCl hypotonic solution (0.075 mol/L), rinsed to remove the cytoplasm, and then fixed in methanol/acetic acid 3:1. The fixing procedure was repeated three times. Finally, the pellet was resuspended in a final volume of 1 ml of fixative, and the cells were dropped onto glass slides. Chromosomes were visualized by using a modified Wright's staining. Twenty metaphases were analyzed for each cell line using a conventional microscope and the IKAROS-software (Metasystems, GmbH, Altlussheim, Germany).

Embryoid body (EB) formation and in vitro differentiation

Human ESCs were harvested, transferred to low-attachment plates and allowed to differentiate spontaneously through EB formation in KO-DMEM supplemented with 20% FCS (Invitrogen), 1% L-glutamine, 0.1 mM non-essential amino acids and 0.1 mM β-mercaptoethanol, but without bFGF, with media changes every 4 days. After 21 days of differentiation, EBs were spun down, fixed with 4 % paraformaldehyde for 10 min and embedded in paraffin [31]. For each staining, three sections per specimen were used. The EB cells were incubated (1 h at RT) with primary antibodies for anti-α-fetoprotein, anti-βIII-tubulin and anti-smooth muscle actin (Chemicon, 1:100). Subsequently, the sections were incubated with a biotinylated secondary antibody (30 min at RT) and a streptavidin peroxidase complex (30 min at RT) (Vector Laboratories Inc). The immunostaining was visualized using diaminobenzidine and counterstained with hematoxylin. The washing steps were done in PBS. Negative controls were prepared by replacing the primary antibody by PBS.

In vivo teratoma formation

Animal protocols were approved by the Local University Hospital Council On Animal Care and Experimentation. In vivo pluripotency was tested as described previously [31, 33, 34]. In brief, HS181 and SHEF1 hESCs were harvested with Collagenase IV. Human ESCs were implanted beneath the testicular capsule of 6-8-week-old NOD/SCID IL2Rγ$^{-/-}$ male mice (n = 17) (The Jackson Lab, Bar Harbor, MA). Teratoma growth was determined by weekly palpation every week, and the mice were killed 7-10 weeks after implantation. Teratomas were fixed, embedded in paraffin, and sections were stained with hematoxylin and eosin.

TGF-β and IGF-II detection and FGFR1 and IGF-II blocking experiments

To determine whether bFGF cooperates with TGF-β and IGF-II, MSCs and HFFs were plated and treated with or without 8 ng/ml bFGF. The concentration of soluble TGF-β released into the media was measured by ELISA (Human TGF-β ELISA Set BD OptEIA™; BD Biosciences). For soluble IGF-II detection, the human IGF-II ELISA Kit (Diagnostic System Laboratories, Inc., Webster, TX) was used as described previously [5]. The production of IGF-II in response to bFGF was also assessed at the RNA level by real-time Q-PCR. Q-PCR was performed with Quantitect™ SYBR® Green PCR Master Mix (Qiagen, Valencia, CA) and analyzed with the Mx3005P real-time PCR system (Stratagene, La Jolla, CA). IGF-II primer sequences used were 5′-TTG CTC TAC CCA CCC AAG AC-3′ and 5′-GAT GGA ACC TGA TGG AAA CG-3′. Real-time PCR conditions were as follows: 10 min at 95 ºC, 40 cycles of 30 s at 95 ºC followed by 60 s at 60 ºC and 30 s at 72 ºC. IGF-II mRNA expression was normalized against GAPDH. Inhibition of bFGF signals has been previously demonstrated with the FGFR1-specific chemical inhibitor SU5402 [5]. Before adding bFGF to MSCs or HFFs cultures, FGFR1 was inhibited for 60 min with 20 μM SU5402 (Calbiochem) or with an equivalent volume of DMSO as control. The media was harvested 24 h later and the levels of soluble TGF-β were further quantified by ELISA. Interference with IGF-II signalling in hESC cultures was accomplished by specific ligand neutralization through addition of 2 μg/ml of the antibody α-hIGF-II (R&D Systems; AF-292-NA), as described previously [5]. As an appropriate control, an immunoglobulin isotype match was used. After 16-day treatment with the IGF-II neutralizing antibody, cell proliferation, cell death, hESC colony morphology, expression of hESC-associated surface markers and transcription factors were assessed.

Acknowledgments

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2.- The ROCK inhibitor Y-27632 negatively affects the expansion/survival of both fresh and cryopreserved cord blood-derived CD34+ hematopoietic progenitor cells.

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INTRODUCCIÓN. La SCU constituye una fuente de fácil acceso de HSPCs de gran utilidad clínica. Las HSPCs CD34+ derivadas de SCU han sido utilizadas de manera exitosa en niños consiguiendo una reconstitución hematopoyética relativamente rápida tras el trasplante. Sin embargo, los métodos actuales para la expansión *ex vivo* de HSPCs conducen a pérdida del potencial de diferenciación multilinaje. Por otro lado, los protocolos utilizados para la congelación-descongelación conllevan muerte celular así como a la pérdida de fenotipo CD34+. La causa principal de la pérdida de viabilidad es la apoptosis directamente inducida por el daño asociado a la criopreservación. Trabajos recientes han mostrado que Y-27632, una molécula inhibidora de la kinasa ROCK, es un potente inhibidor de la apoptosis y potencia de manera eficiente la supervivencia post-descongelación y la recuperación de embriones humanos congelados, hESCs, iPSCs y hMSCs.

OBJETIVO. En el presente estudio se pretende valorar el efecto del inhibidor de ROCK Y-27632 en la expansión y supervivencia celular de HSPCs CD34+ derivadas de SCU, tanto recién aisladas como criopreservadas.

MATERIAL Y MÉTODOS. Las HSPCs CD34+ de SCU fueron aisladas mediante separación magnética y cultivadas en presencia o ausencia de 10μM de Y-27632. El efecto de Y-27632 en estas células, bien recién aisladas o bien congeladas, se determinó mediante estudios de proliferación, ciclo celular, apoptosis, así como en ensayos de clonogenicidad.

RESULTADOS. Nuestros resultados indican que Y-27632 no sólo inhibe de manera dramática la expansión celular de HSPCs CD34+, frescas o criopreservadas, sino que también inhibe la supervivencia/recuperación de CD34+ tras la descongelación tanto si se añade al medio de criopreservación como al de expansión, de manera independiente a la presencia de citocinas hematopoyéticas en el medio.

CONCLUSIÓN. Este estudio muestra por primera vez el efecto negativo de Y-27632 en la expansión y supervivencia de HSPCs CD34+ de SCU, sugiriendo que este inhibidor podría tener un impacto diferente dependiendo de la especificidad linaje/tisular, dado el beneficio mostrado en la supervivencia de otras células como son las hESCs. Por tanto, desaconsejamos el uso de Y-27632 en el desarrollo de protocolos de criopreservación y expansión *ex vivo* de HPCs de SCU.

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The ROCK Inhibitor Y-27632 Negatively Affects the Expansion/Survival of Both Fresh and Cryopreserved Cord Blood-Derived CD34+ Hematopoietic Progenitor Cells

Y-27632 negatively affects the expansion/survival of CD34 + HSPCs

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Abstract Cord blood (CB) is an unlimited source of hematopoietic stem and progenitor cells (HSPC). The use of cryopreserved CB-derived CD34+ HSPCs is successful in children and usually leads to rapid hematopoietic recovery upon transplantation. However, current methods for ex vivo expansion of HSPCs still result in a loss of multilineage differentiation potential and current freeze-thawing protocols result in significant cell death and loss of CD34+ HSPCs. The major cause for the loss of viability after slow freezing is apoptosis induced directly by cryoinjury. Very recent reports have demonstrated that Y-27632, a selective and robust ROCK inhibitor is a potent inhibitor of the apoptosis and is efficient in enhancing the post-thaw survival and recovery of different human stem cells including human embryos, hESCs, induced pluripotent stem cells and mesenchymal stem cells. Here, we analyzed the effect of such an inhibitor in CB-derived CD34+ HSPCs. CB-derived CD34+ HSPCs were MACS-isolated and treated with or without 10 μM of Y-27632. The effect of Y-27632 on culture homeostasis was determined in both fresh and cryopreserved CB-derived CD34+ HSPCs. Our results indicate that the Y-27632 not only dramatically inhibits cell expansion of both fresh and cryopreserved CD34+ HSPCs but also impairs survival/recovery of CD34+ HSPCs upon thawing regardless whether Y-27632 is added to both the cryopreservation and the expansion media and or just to the expansion culture medium with or without hematopoietic cytokines. This study identifies for the first time a detrimental effect of Y-27632 on the expansion and survival of both fresh and cryopreserved CB-derived CD34+ HSPCs, suggesting that Y-27632 may have a differential impact on distinct lineage/tissue-specific stem cells. Our data suggest different functions of Y-27632 on human stem cells growing in suspension versus those growing attached to either treated tissue culture plastic or extracellular matrix. We discourage any clinical application of Y-27632 in potential technical developments aimed at improving cryopreservation procedures of CB-derived cells and/or in vitro expansion of HSPCs without spontaneous differentiation.

Keywords Human CB-CD34+ HSPCs · Rock inhibitor Y-27632 · Cryopreservation · Survival · Expansion

Introduction

Hematopoietic stem cell transplantation (HSCT) may be curative in a large variety of diseases. Cord blood is an unlimited source of hematopoietic stem and progenitor cells (HSPC) for allogeneic HSCT. Cord blood transplantation (CBT) has extended the availability of allogeneic HSCT to patients who would not otherwise be eligible for this curative approach [1]. CB units are usually banked for future unrelated or related CBT. There are currently over 400.000 banked CB units registered worldwide [1]. The use of cryopreserved CB-derived CD34+ HSC is successful in

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children and usually leads to rapid hematopoietic recovery [2–5]. However, there is clearly room for improvements as current methods for ex vivo expansion of HSPC still result in a loss of multilineage differentiation potential and current freeze-thawing protocols result in significant cell death and loss of CD34+ HSPC populations essential for engraftment [6–11].

The major cause for the loss of viability after slow freezing is apoptosis induced directly by cryoinjury [12]. Very recent reports have demonstrated that Y-27632, a selective and robust inhibitor of p160-Rho-associated coiled-coil kinase (ROCK), is a potent inhibitor of the apoptosis and is efficient in enhancing the post-thaw survival and recovery of frozen human embryos [13], cryopreserved human embryonic stem cells (hESCs) [14–20], induced pluripotent stem cells (iPS cells) [21] and human mesenchymal stem cells (hMSCs) [22].

In the present study, therefore, we attempted to assess the effect of the ROCK inhibitor Y-27632 on the expansion and cellular survival of both fresh and cryopreserved CD34+ HSPCs which, in contrast, to the aforementioned human stem cells (blastocysts, hESCs, iPS cells, MSCs) grow in suspension rather than attached or as colony aggregates.

Material and Methods

Cord Blood-Derived $CD34^+$ HSPC Isolation and Culture

Different fresh Cord Blood (CB) units from healthy newborns were obtained from local hospitals upon approval by our local Ethics and Biozahard Board Committee. CB samples were pooled to reduce variability between individual freshly-isolated CB units (n=5 CB pools). Mononuclear cells were isolated using Ficoll-Hypaque (GE Healthcare, Stockholm, Sweden). After lysing the red blood cells (Lysis solution, StemCell Technologies, Vancouver, Canada), $CD34^+$ cells were purified by magnetic bead separation using the human CD34 MicroBead kit (Miltenyi, Munich, Germany) and the AutoMACS Pro separator (Miltenyi) as per manufacturer's instructions. After washing in phosphate-buffered saline (PBS), equal numbers of $CD34^+$ cells were plated in liquid culture: Stem Spam (Stem Cell Technologies) supplemented with SCF (100 ng/mL), FLT3L (100 ng/mL) and IL-3 (10 ng/mL) (Peprotech, London, UK) in the presence or absence of 10 μM of the ROCK inhibitor Y-27632 (Calbiochem, San Diego, CA, USA). In some experiments, the liquid culture was not supplemented with hematopoietic cytokines to address the potential effect of the ROCK inhibitor in the absence of such cytokine stimulation. Y-27632 was added to the culture medium daily.

As aforementioned, identical experiments (n=4) were performed using MACS-isolated CB-derived $CD34^+$ cells from cryopreserved CB units. CB-derived cells were frozen using current protocols (culture medium supplemented with 10% *v/v* dimethyl sulfoxide (DMSO) using a slow linear cooling curve). Ten μM of Y-27632 was added to both the cryopreservation medium and the expansion medium or just to the expansion medium daily. Cells were kept frozen in liquid nitrogen for several weeks. Then, cells were thawed at 37°C, washed in PBS and equal numbers of $CD34^+$ cells were cultured for up to 13 days in the presence or absence of 10 μM of Y-27632. Analysis of CD34+ cells in the culture was determined by flow cytometry at days 0, 4, 9 and 13.

Cell Cycle Analysis

CB-derived $CD34^+$ cells were harvested after 4, 9 and 13 days of culture and pelleted by centrifugation. Cell pellets were washed with PBS, fixed in 70% ice-cold ethanol, and stored for up to 2 weeks at −20°C. Cells were washed with PBS followed by incubation in 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A (Sigma) for 30 min. Stained nuclei were analyzed on a FACS Canto-II using the FACS Diva software (Becton Dickinson, San Jose, CA, USA). Modfit software (Becton Dickinson) was used to discriminate among apoptotic cells (Sub-G_0/G_1), quiescent cells (G_0/G_1) and cycling cells ($S/G_2/M$). Cell death was also analysed by 7-actinomycin D (7-AAD) staining [23].

Caspase 3 and PI Staining

CB-derived $CD34^+$ cultured cells were harvested after 4, 9 and 13 days and pelleted by centrifugation. Unfixed cells were assayed for active caspase 3 immediately after harvesting using CaspGLOW Fluorescein Active Caspase-3 staining Kit according to the manufacturer´s instructions (MBL Corp., Woburn, MA). Subsequently, they were resuspended in PBS containing 50 μg/ml of PI, 100 μg/ml of RNAse A and 0.1% (*v/v*) of Triton X-100. After 30 min incubation, the cell suspension was analyzed by flow cytometry for active caspase-3 and cell cycle simultaneously on a FACS Canto-II (Becton Dickinson).

Colony Forming Unit (CFU) Assay

On day 0, 4, 9, 13 of culture, CB-derived $CD34^+$-enriched fraction was plated ($2\times10^3/cm^2$) in methycellulose assays supplemented with SCF (50 ng/mL), GM-CSF (10 ng/mL), IL-3 (10 ng/mL) and Erytropoietin (3U/mL) (Methocult GF H4434; StemCell Technologies) in the presence or absence of Y-27632 (10 μM). After 12–14 days in culture, colonies were counted and scored [3, 24–26].

Statistical Analysis

All data are expressed as mean ± standard errors of the mean (SEM). Statistical comparisons were performed with a paired Student's *t* test. Statistical significance was defined as a *P* value <0.05 [4, 5, 27, 28].

Results

Y-27632 Dramatically Inhibits Proliferation But Do Not Impair Differentiation of Freshly-Isolated CD34+ HSPCs

CD34+ HPSCs were MACS-isolated from fresh CB units. Purity was consistently higher than 91% (Fig. 1a). Equal numbers of CD34+ HSPCs were cultured in liquid culture supplemented with hematopoietic cytokines for up to 13 days in the presence or absence of Y-27632. Proliferation was measured on days 4, 9 and 13 relative to the number of CD34+ HSPCs initially plated. As shown in Fig. 1b, overall cell expansion was reduced up to 8-fold ($p<0.001$) when Y-27632 was added into the culture medium. As it is well-established, CD34+ HSPCs gradually differentiated in vitro losing CD34 expression, indicating that Y-27632 does not impair differentiation/maturation of CD34+ HSPCs (Fig. 1c, d). Accordingly, the absolute numbers of both CD34+ and CD34- cell subsets throughout the 13-day period in vitro were measured in the presence or absence of Y-27632. As shown in Fig. 1e and f, Y-27632 significantly ($p<0.03$) inhibited the proliferation of CD34+ HSPCs and, at least extend, CD34- mature cells (Fig. 1e, f). Interestingly, Y-27632 inhibited proliferation of CD34+ HPSCs (Fig. 1e) but did not impair the differentiation/maturation of CD34+ HSPCs in liquid culture (Fig. 1c, d) or in colony-forming units (CFU) assays (Fig. 1g). Taken together, this data indicates that Y-27632 dramatically inhibits the proliferation of freshly-isolated CD34+ HSPCs without impairing CD34+ HSPCs differentiation/maturation.

Y-27632 Induces Cell Death Coupled to Cell Cycling of CD34+ HSPCs

Because of the robust loss of cellularity in CB cell cultures treated with Y-27632, we next analysed cell death and cell cycle distribution in Y-27632-treated and untreated CB cultures. Cell death was determined as the Sub G_0/G_1 fraction (Fig. 2a) or 7-ADD+ fraction. Both assays provided identical results (data not shown). As shown in Fig. 2b, cell death was significantly higher (between 2–3 fold higher) in those cultures treated with Y-27631: 14% vs 5%, 41% vs 18% and 37% vs 18% on day 4, 9 and 13, respectively. As for cell cycle distribution, no differences were observed at day 4 after Y-27632 treatment (Fig. 2c, d). From day 9 onwards, a significantly higher (1.45 fold higher) number of cycling cells ($S/G_2/M$) were detected in those CB-cultures treated with Y-27632: 44% vs 30%, and 32% vs 22% on day 9 and 13, respectively. CB-derived cells were stained with an antibody anti active Caspase 3 and PI in order to determine at which phase of the cell cycle apoptosis is mainly occurring. As depicted in Fig. 2e, over 80% of the caspase-3+ apoptotic cells were at G_0/G_1 cell cycle phase in Y-27632-treated cultures. These data suggest that Y-27632 dramatically inhibits CD34+ HSPCs expansion through apoptosis/cell death induction which contributes more than cell cycling (2–3 fold versus 1.45 fold) to Y-27632-induced inhibition of proliferation.

We next wondered whether the Y-27632 effect may be linked, to some extend, to the cytokine cocktail (SCF, FLT3, IL3) used to pre-stimulate CB-derived CD34+ HSPCs. Accordingly, the same cell cycle/apoptosis assay was performed under cytokine-free conditions (Fig. 2f). In hematopoietic cytokines-free cultures, Y-27632 similarly inhibited cell expansion by inducing cell death (Sub G_0/G_1: 71% vs 58%). Taken together, regardless the use of hematopoietic growth factors, Y-27632 robustly inhibits cell proliferation of freshly-isolated CD34+ HSPCs by inducing apoptosis/cell death.

Y-27632 Improves Neither Expansion Nor Survival of Cryopreserved CD34+ HSPCs

In an autologous transplantation setting, previously harvested and frozen CD34+ HSPCs need to be thawed before the infusion into the patient. The major cause for the loss of viability after slow freezing is apoptosis induced directly by cryoinjury [22]. Very recent reports have demonstrated that Y-27632, is efficient in enhancing the post-thaw survival and recovery of different cryopreserved human stem cells when added either to the cryopreservation medium or expansion medium or to both [13, 15–20]. We thus wanted to determine the effect of Y-27632 on cryopreserved CB-derived CD34+ HSPCs. Y-27632 was added to both the cryopreservation and the expansion media or just to the expansion medium.

An initial set of experiments was performed in which the effect of Y-27632 addition was analysed only in the expansion culture medium used to grow and maintain thawed CD34+ HSPCs. Identical with the effect of Y-27632 on freshly-isolated CD34+ HSPCs, overall cell expansion of frozen-thawed CB-derived cells was profoundly reduced (10-fold; $p<0.0001$) from day 4 onwards when Y-27632 was added into the medium (Fig. 3a). As reported for freshly-isolated CD34+ HSPCs, frozen-thawed CD34+ cells also differentiated normally in vitro, losing CD34 expression within 13 days (Fig. 3b, c). Accordingly, regarding absolute numbers, Y-27632 significantly inhibited the expansion of CD34+ HSPCs ($p<0.01$)

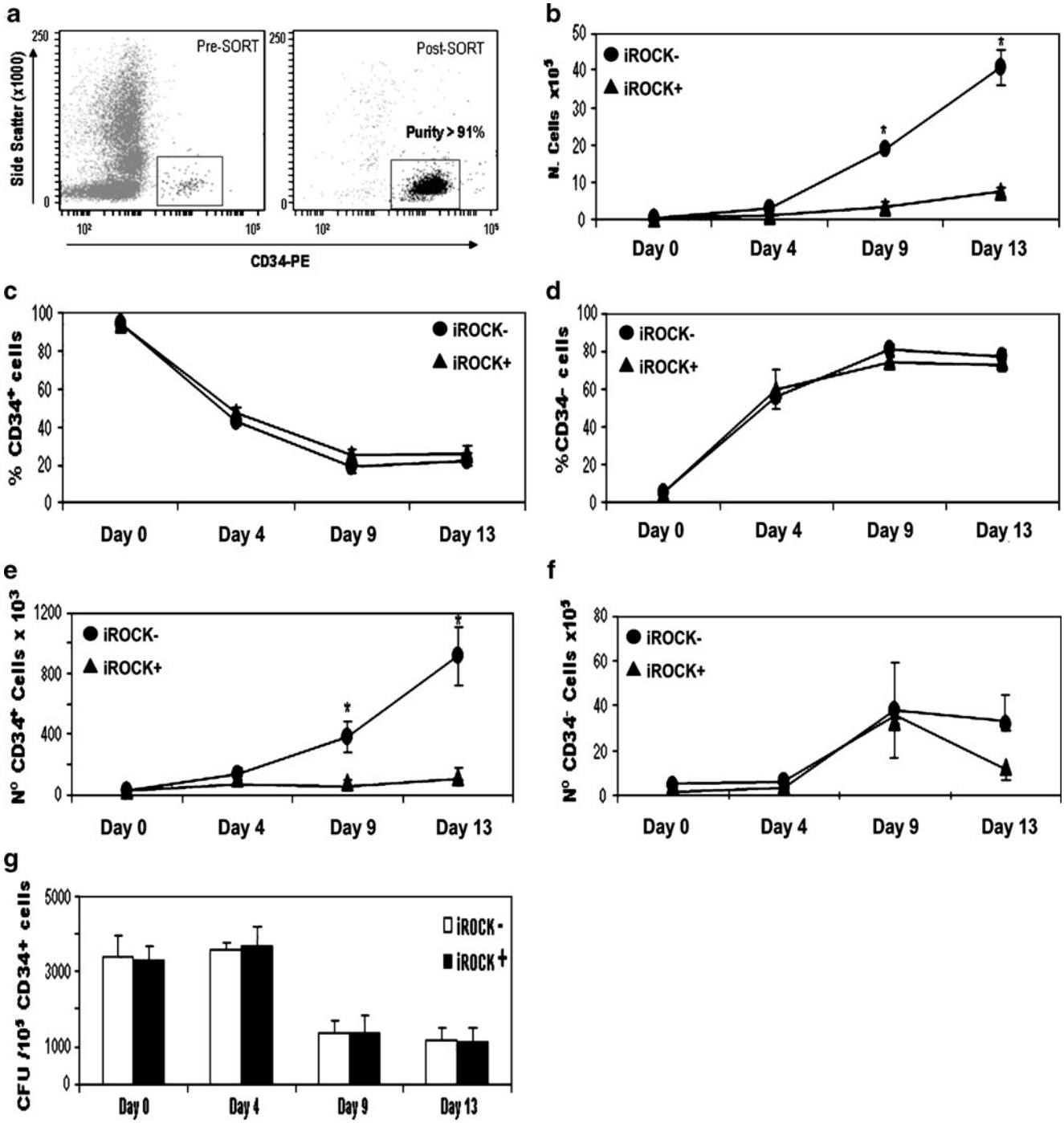

Fig. 1 Y-27632 inhibits cell expansion but not differentiation of freshly isolated CD34+ HSPCs. **a** Representative flow cytometry dot plots displaying purity upon CD34+ FACS sorting. **b** Growth curves (cell expansion) in the presence or absence of Y-27632 measured for up to 13 days. Percentage of CD34+ (**c**) and CD34- cells (**d**) in the culture at different time points in the presence or absence of Y-27632. Absolute numbers of CD34+ (**e**) and CD34- cells (**f**) at different time points in the presence or absence of Y-27632. **g** Total number of hematopoietic colonies per 10^5 CD34+ cells plated in methylcellulose assays in the presence or absence of Y-27632. Asterisks represent statistical significant differences ($p<0.05$)

(Fig. 3d) and CD34- mature cells ($p<0.001$) (Fig. 3e) throughout the 13-day period. Figure 3f shows a representative CD34 versus 7-ADD staining displaying the higher apoptotic rate (7-ADD+ cells) in both CD34+ and CD34- cell subsets.

We finally wondered whether the supplementation of the standard cryopreservation medium containing 10% *v/v* DMSO (slow linear cooling curve) with Y-27632 would facilitate the survival/recovery of cryopreserved CD34+ HSPCs upon thawing. Addition of Y-27632 to both the

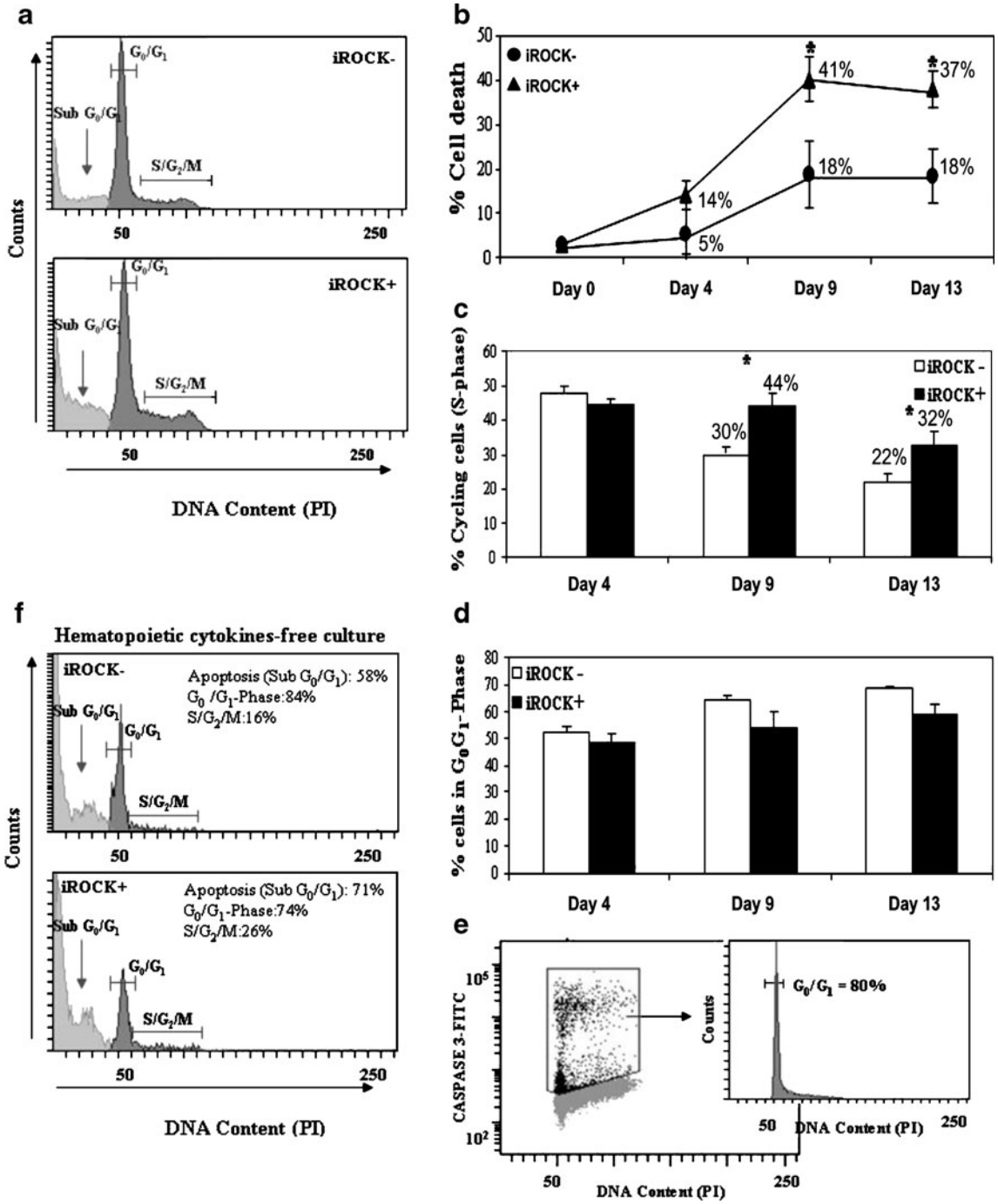

Fig. 2 Y-27632 induces cell death but also cell cycling of CD34+ HSPCs. **a** Representative flow cytometry cell cycle distribution in the presence or absence of the Rock inhibitor Y-27632. **b** Cell death induced by Y-27632 on CD34+ HSPCs measured as Sub G_0/G_1 cell fraction. **c** Proportion of cycling CD34+ cells ($S/G_2/M$) in in vitro cultures with or without Y-27632. **d** Proportion of dormant CD34+ cells (G_0/G_1 phase) in in vitro cultures with or without Y-27632. **e** Representative flow cytometry analysis of active caspase-3 and PI. **f** Representative flow cytometry cell cycle distribution in the presence or absence of the Rock inhibitor Y-27632 under hematopoietic cytokines-free culture conditions. Asterisks represent statistical significant differences ($p<0.05$)

cryopreservation and expansion media also resulted in a strong reduction (7 to 11-fold) of overall cell expansion of frozen-thawed CB-derived cells (Fig. 4a). CD34+ cells cryopreserved in the presence of Y-27632 differentiated normally in vitro, losing CD34 expression within 13 days (Fig. 4b, c). Regarding absolute numbers, Y-27632-supplemented cryopreservation medium and expansion media dramatically inhibited the expansion of CD34+

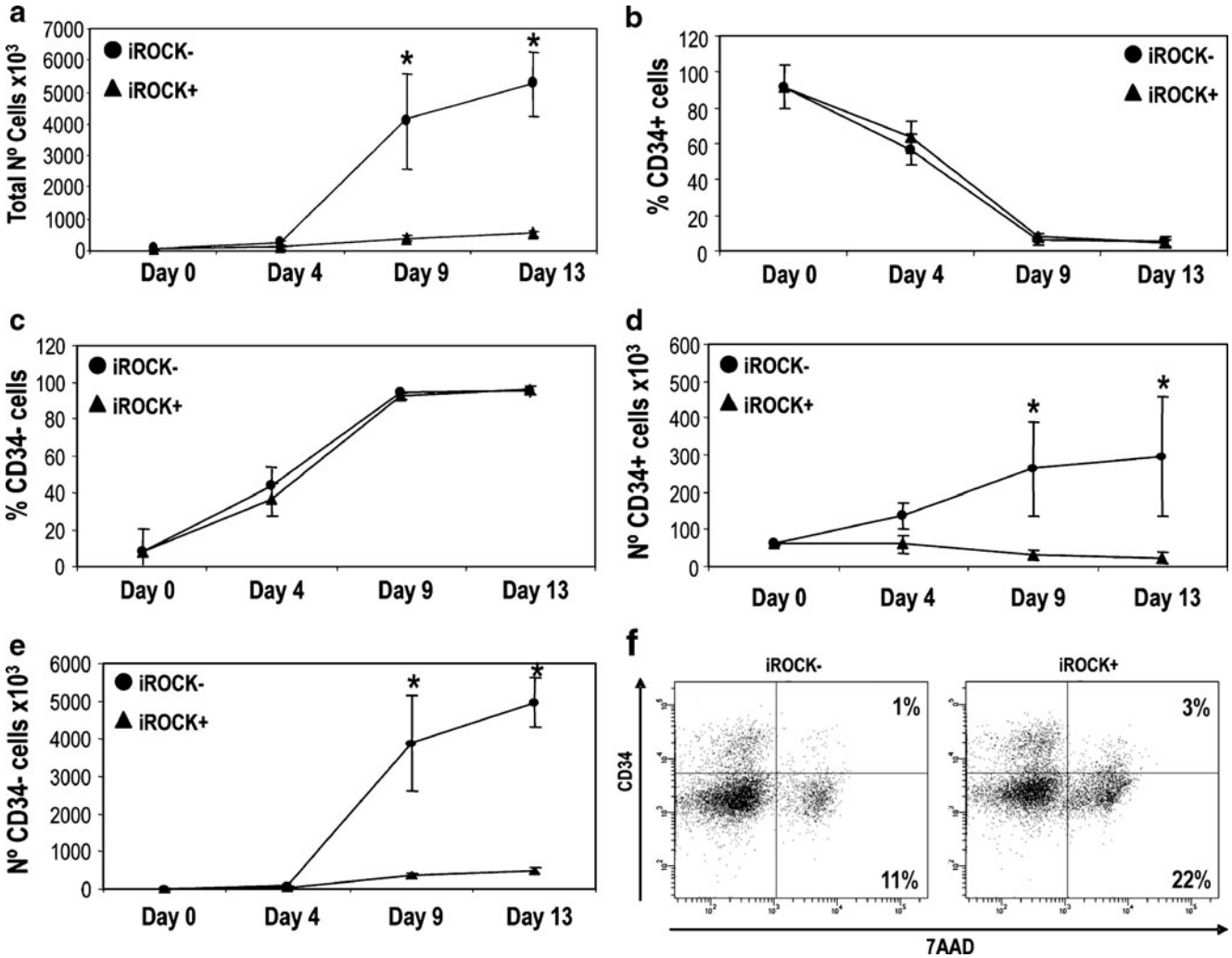

Fig. 3 Y-27632 reduces survival/recovery of cryopreserved CB CD34+ and CD34- cells. **a** Growth curves (cell expansion) of cryopreserved CB-derived CD34+ cells in the presence or absence of Y-27632 measured for up to 13 days. Post-thawing percentage of CD34+ (**b**) and CD34- cells (**c**) at different time points in the presence or absence of Y-27632. Post-thawing absolute numbers of CD34+ (**d**) and CD34- cells (**e**) at different time points in the presence or absence of Y-27632. **f** Representative flow cytometry panels displaying cell death of CD34+ and CD34- cell subsets analysed by 7-AAD staining. Asteriskis represent statistical significant differences ($p<0.05$)

HSPCs (Fig. 4d) and CD34- mature cells (Fig. 4e) throughout the 13-day period. In sum, our results indicate that the ROCK inhibitor Y-27632 not only profoundly inhibits cell expansion of both fresh and cryopreserved CD34+ HSPCs but also improves neither survival nor recovery of CD34+ HSPCs upon thawing regardless Y-27632 being added to both the cryopreservation and the expansion media or just to the expansion medium.

Discussion

The role of HSCT in the treatment of hematologic and non-hematologic malignancies is rapidly expanding. In certain situations fresh HSPCs can be employed in the setting of allogeneic transplantation. However, the current therapeutic strategies demand that the HSPCs are cryopreserved for virtually all autologous and many allogeneic transplants. This strategy has been proven to be safe and not associated with significant adverse outcomes regarding engraftment failure or graft versus host disease [6, 29]. The cryopreservation process is of importance for all types of stem cell collection, but is perhaps particularly critical for CB. The actual transplant is here harvested at the time of birth and used at a later point in time. Despite existing slight variations among transplant centers, the most widely used crypreservation method involves addition of 10% *v/v* DMSO and a constant cooling rate of 1°C per minute. Current methods for ex vivo expansion of HSPCs still result in a loss of multilineage differentiation potential and current freeze-thawing protocols result in significant cell death and loss

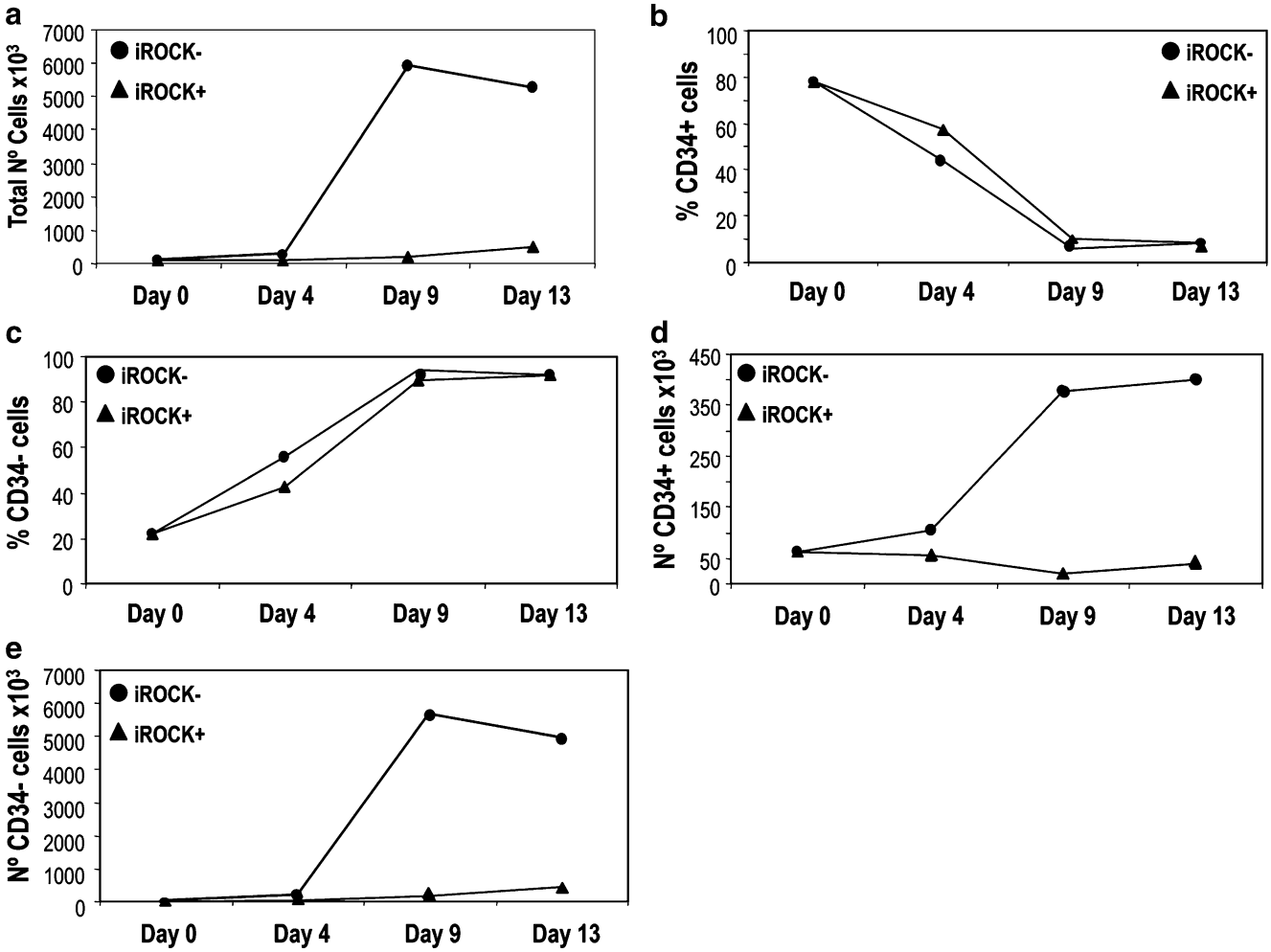

Fig. 4 Supplementation of the cryopreservation medium with Y-27632 does not improve the survival and recovery of thawed CB-derived cells. **a** Y-27632-supplemented cryopreservation medium does not facilitate survival/recovery of thawed CB-derived cells. Post-thawing percentage of CD34+ (**b**) and CD34- cells (**c**) at different time points in the presence or absence of Y-27632 in the expansion media. Post-thawing absolute numbers of CD34+ (**d**) and CD34- cells (**e**) at different time points in the presence or absence of Y-27632 in the expansion media. All the data displayed in Fig. 4 have been generated from CD34+ HSPCs cryopreserved in the presence of Y-27632

of CD34+ HSPCs essential for engraftment, being the apoptosis induced directly by cryoinjury the major cause for the loss of viability after slow freezing.

Very recent reports have demonstrated that Y-27632, a selective and robust inhibitor of p160-Rho-associated coiled-coil kinase (ROCK), is a potent inhibitor of the apoptosis and is efficient in enhancing the post-thaw survival and recovery of frozen human blastocysts [13], hESCs [14–20], iPS cells [21] and hMSCs [22]. Of note, all these human stem cells have in common that they grow attached to the treated tissue culture plastic or extracellular matrix and in many cases they also form physiological cell aggregates. Interestingly, however, the potential effect of Y-27632 on human CB-derived CD34+ HSPCs has not been explored yet. Furthermore, it is worth mentioning that, in contrast to the aforementioned human stem cells (blastocysts, hESCs, iPS cells, MSCs), CD34+ HSPCs grow in suspension rather than attached or as colony aggregates. In the present study, the effect of Y-27632 on culture homeostasis was determined in both fresh and cryopreserved CB-derived CD34+ HSPCs.

Our results indicate that the Y-27632 dramatically inhibits cell expansion of both fresh and cryopreserved CD34+ HSPCs but also improves neither survival nor recovery of CD34+ HSPCs upon thawing regardless whether Y-27632 is added to both the cryopreservation and the expansion media and or just to the expansion medium. These data discourage any clinical application of Y-27632 in potential technical developments aimed at improving cryopreservation procedures of CB-derived cells and/or in vitro expansion of HSPCs without spontaneous differentiation. Our data could be reproduced in the presence and absence of hematopoietic cytokines, suggesting that Y-27632 negatively affects the culture homeostasis

of both fresh and cryopreserved CB-derived CD34+ HSPCs in a cytokine-independent manner.

From a mechanistic standpoint, several recent studies have suggested that hESCs, iPS cells and hMSCs treated with Y-27632 are able to escape apoptosis when dissociated into single cells or detaching them from the treated tissue culture plastic, reinforcing the concept of Y-27632 as a potent inhibitor of apoptosis based upon Rho´s role in accepting signals from G protein-coupled receptors and extracellular matrix (ECM) [30, 31]. In contrast, however, our data on CB-derived CD34+ HSPCs does not support an anti-apoptotic role of the Y-27632. Our data is in line with previous observations indicating that if cell interactions are prevented, Y-27632-treated cells do not survive [15]. Rho kinase and ROCK are ubiquitous proteins which have been found to play a role in regulating multiple cellular processes including cell proliferation, apoptosis, cell-cycle progression, migration, actin cytoskeleton, cell polarity and cell-cell interaction [15, 30]. In contrast to previous reports [12, 13, 16–21], this study identifies for the first time a detrimental effect of Y-27632 on the expansion and survival of both fresh and cryopreserved CB-derived CD34+ HSPCs. Therefore, in agreement with Rancourt´s Lab [15] our data supports that the effect of iROCK on cell-cell interaction and cell adherence may be crucial. We speculate that Y-27632 may have a differential impact not only on distinct lineage/tissue-specific stem cells but may also function differentially on human stem cells growing in suspension versus those growing attached to either treated tissue culture plastic or extracellular matrix. Future work needs to be done to gain further insights into the cellular and molecular mechanisms underlying the apparently differential role of Y-27632 among distinct types of human stem cells.

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Authorship C.B: designed and performed experiments, analysed the data and wrote the manuscript. R.M: performed experiments and analysed the data. P.M: conceived and supervised the study and wrote the paper. We thank Dr. René Rodriguez for helpful discussions and Dr Isidro Prat and M.C Rodríguez Lamas for provision of CB Units.

Conflict of Interest Disclosures The authors reported no potential conflicts of interest.

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3.- Intra-bone marrow transplantation of human CD34(+) cells into NOD/LtSz-scid IL-2rgamma(null) mice permits multilineage engraftment without previous irradiation.

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INTRODUCCIÓN. La utilización de ratones inmunodeficientes no irradiados como receptores de xenotrasplante hematopoyético proporciona una herramienta de gran valor para el estudio de la función de la HSCs. Entre las cepas más comúnmente utilizadas hasta la fecha están NOD/SCID, NOD/SCID $\beta 2m^{-/-}$ y NOD/SCID IL-$2R\gamma^{-/-}$. Por otro lado, la irradiación previa al trasplante se viene utilizando para debilitar inmunológicamente aún más al ratón y para aplasiar su MO y dejar espacio a las células trasplantadas. Sin embargo, se cree que un receptor no irradiado supone la aproximación más fisiológica para estudiar la función hematopoyética, ya que el nicho de la MO tiene un papel importante en hematopoyesis.

OBJETIVO. Comparar la utilización de las cepas NOD/SCID, NOD/SCID $\beta 2m^{-/-}$ y NOD/SCID IL-$2R\gamma^{-/-}$ como receptoras de xenotrasplante. Valorar la función de repoblación hematopoyética de las células progenitoras CD34+ derivadas de SCU, sin irradiación previa en dichas cepas.

MATERIAL Y MÉTODOS. Las HSPCs CD34+ se aislaron de SCU mediante separación magnética con una pureza superior al 91%. Estas células fueron inyectadas a ratones pertenecientes a cada una de las tres cepas mediante trasplante intratibia y tras 9-10 semanas fueron sacrificados para su análisis. Se aislaron células provenientes de la MO de la tibia inyectada, de la MO contraria (no inyectada), de bazo y de sangre periférica, y se analizó mediante citometría tanto la repoblación hematopoyética por parte de células humanas, como la diferenciación multilinaje de las mismas. Por otro lado, se confirmó la presencia de quimerismo humano mediante PCR.

RESULTADOS. En ausencia de irradiación previa al trasplante, en las cepas NOD/SCID y NOD/SCID $\beta 2m^{-/-}$ no se obtuvo quimerismo humano, mientras que en la cepa NOD/SCID IL-$2R\gamma^{-/-}$ se consiguió repoblación hematopoyética humana.

CONCLUSIÓN. La combinación de la cepa NOD/SCID IL-$2R\gamma^{-/-}$ y del trasplante intratibia facilita la repoblación hematopoyética multilínea y la migración de células CD34+, incluso sin necesidad de irradiación previa.

NOTA: La relevancia de este artículo mereció, según el editor, el comentario de un experto en el tema, publicado en el misma edición. Este comentario se añade tras el artículo.

Intra-bone route of administration offers new perspectives for safer transplantation of hematopoietic stem cells.

Frassoni F.

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Intra-bone marrow transplantation of human CD34$^+$ cells into NOD/LtSz-*scid* IL-2rγ^{null} mice permits multilineage engraftment without previous irradiation

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Abstract

Background aims. Non-irradiated immunodeficient recipients provide the best physiologic setting for revealing hematopoietic stem cell (HSC) functions after xenotransplantion. An approach that efficiently permits the detection of human hematopoietic repopulating cells in non-irradiated recipients is therefore highly desired. *Methods.* We compared side-by-side the ability to reconstitute hematopoiesis via intra-bone marrow transplantation (IBMT) in three commonly used mouse strains avoiding previous irradiation. *Results.* Non-irradiated NOD/SCID and NOD/SCID β2m–/– mouse strains prevent engraftment even after IBMT. In contrast, combining the robustness of the NOD/SCID IL-2Rγ–/– recipient with the sensitivity of IBMT facilitates the detection, without previous host irradiation, of human SCID-repopulating cells 10 weeks after transplantation. The level of chimerism averaged 14% and multilineage engraftment (lymphoid dominant) was observed consistently in all mice. Analysis of injected and non-injected bones, spleen and peripheral blood demonstrated that engrafting cells were capable of in vivo migration and expansion. *Conclusions.* Combining the robustness of the NOD/SCID IL-2Rγ–/– mouse strain with the sensitivity of IBMT strongly facilitates long-term multilineage engraftment and migration for human CD34$^+$ cells without the need for previous irradiation.

Key Words: *CD34$^+$ cells, engraftment, NOD/SCID mouse strains, NOD/LtSz-scid IL-2Rγ^{null}, irradiation*

Introduction

Multiple areas of biomedical research, including hematopoietic transplantation, cell therapy and cancer research, require the use of xenotransplant systems (1). The 'gold standard' functional measure of a long-term renewable hematopoietic stem cell (HSC) is the capacity to engraft myeloablative recipients, repopulate the hematopoietic system and sustain long-term multilineage hematopoiesis *in vivo* (2). Repopulation assays based on xenotransplant models require the use of immunodeficient mice.

Upon discovery of the *scid* (severe-combined immunodeficiency) mutation (3), the immunodeficient non-obese diabetic NOD/SCID mouse was developed and rapidly became the standard model within the stem cell research community, still being widely employed (3). This strain displays a low level of innate immunity and natural killer (NK) function, allowing the engraftment of human HSC. A major drawback of the NOD/SCID strain is the relatively short lifespan because of thymic lymphoma development (4,5). A few years afterwards, the NOD/SCID β2m$^{-/-}$ strain was generated (4,6). This strain lacks β2 microglobulin, leading to lack of major histocompatibility complex (MHC) class I expression (4,6). As a consequence, the NK function is much lower than in the conventional NOD/SCID mice, permitting an increased hematopoietic engraftment. Unfortunately, however, this NOD/SCID β2m$^{-/-}$ strain has an even shorter lifespan than the NOD/SCID strain because of very rapid lymphoma development. Recently, a new generation of the NOD/SCID mouse has been developed: the NOD/SCID IL-2Rγ$^{-/-}$ mouse. This strain carries an interleukin-2 receptor-γ (IL-2Rγ)-chain deficiency that blocks signaling through multiple cytokine receptors, leading to many innate immune defects (4,5,7). The NOD/SCID IL-2Rγ$^{-/-}$ mouse allows higher levels of engraftment and does not develop thymic lymphoma, therefore displaying a much longer lifespan. In fact, Ishikawa *et al*. (7) recently reported a more than two-fold (73% versus 31%) higher hematopoietic chimerism in sublethally

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irradiated NOD/SCID IL-2Rγ$^{-/-}$ mice compared with NOD/SCID β2m$^{-/-}$ mice.

Despite the development of these new generations of humanized immunodeficient mice, limitations of these models remain that still need to be overcome. The histocompatibility barriers and lack of space in the bone marrow (BM) niches represent the major limitations for HSC xenotransplantation (8). Despite the belief that the BM niche needs to be opened by myeloablative irradiation, otherwise the donor HSC cannot engraft, non-irradiated recipients provide the best physiologic setting for revealing HSC functions after HSC xenotransplantation (9). Thus there is increasing evidence of the impact of biologic rhythms on the traffic of HSC and their proliferation, differentiation and engraftment capacities. Recent evidence supports the role of the sympathetic nervous system (SNS) in the regulation of HSC behavior, both directly and through supporting stromal cells. In addition, the SNS transduces circadian information to the BM microenvironment, directing circadian oscillations in hematopoiesis and HSC migration (10).

HSC xenotransplantation into non-irradiated hosts would also eliminate the requirement to co-transplant accessory cells along with HSC (8). And it is worth mentioning that for many biomedical research centers and hospitals world-wide (i.e. many developing countries) that do not have simultaneous access to an animal barrier and an irradiator, the use of a non-irradiated immunodeficient recipient that permits engraftment upon xenotransplantation of human HSC will open up new avenues of research.

Intra-bone marrow transplantation (IBMT) has recently emerged as an alternative delivery method for intravenous transplantation for experimentally assessing the human hematopoietic reconstituting ability in NOD/SCID mice (10–13). Delivering cells directly into the femur or tibia bypasses the requirement for homing to the BM niche and has revealed that human CD34$^+$ cells display an enhanced (up to 12-fold higher) (14) multilineage reconstituting hematopoietic potential than previously predicted based on intravenous transplantation in NOD/SCID mice.

We hypothesized that combining the robustness of the NOD/SCID IL-2Rγ$^{-/-}$ recipient with the sensitivity of IBMT delivery would facilitate human hematopoietic repopulating cell function in non-irradiated recipients. Therefore, we compared side-by-side the ability to reconstitute hematopoiesis via IBMT in three commonly used mouse strains avoiding previous irradiation. We report that combining the NOD/SCID IL-2Rγ$^{-/-}$ strain with the sensitivity of IBMT greatly facilitates long-term multilineage engraftment and migration of human CD34$^+$ cells without the need for previous irradiation.

Methods

Cord blood-derived CD34$^+$ isolation

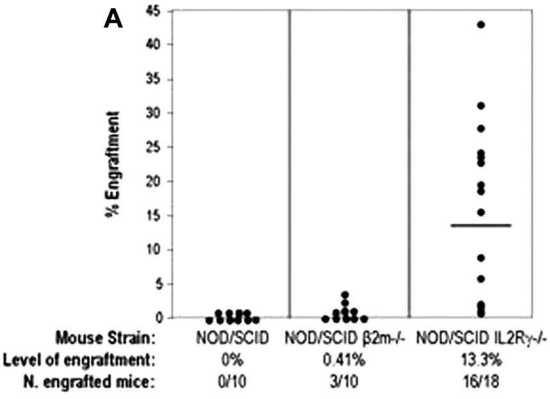
Fresh cord blood (CB) samples from healthy newborns were obtained from local hospitals upon approval by our local ethics and biohazard board committee (University of Granada, Granada, Spain). CB samples were pooled to reduce variability between individual CB units. Mononuclear cells were isolated using Ficoll–Hypaque (GE Healthcare, stockholm, Sweden). After lysing the red blood cells (lysis solution; StemCell Technologies, Vancouver Canada), CD34$^+$ cells were purified by magnetic bead separation using a human CD34 MicroBead Kit (Miltenyi, Munich Germany) and an AutoMACS Pro separator (Miltenyi) as per the manufacturer's instructions. Purity was consistently higher than 91% (median 93%, range 91–97%).

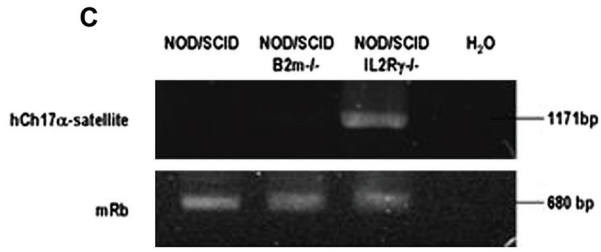
Mice transplantation

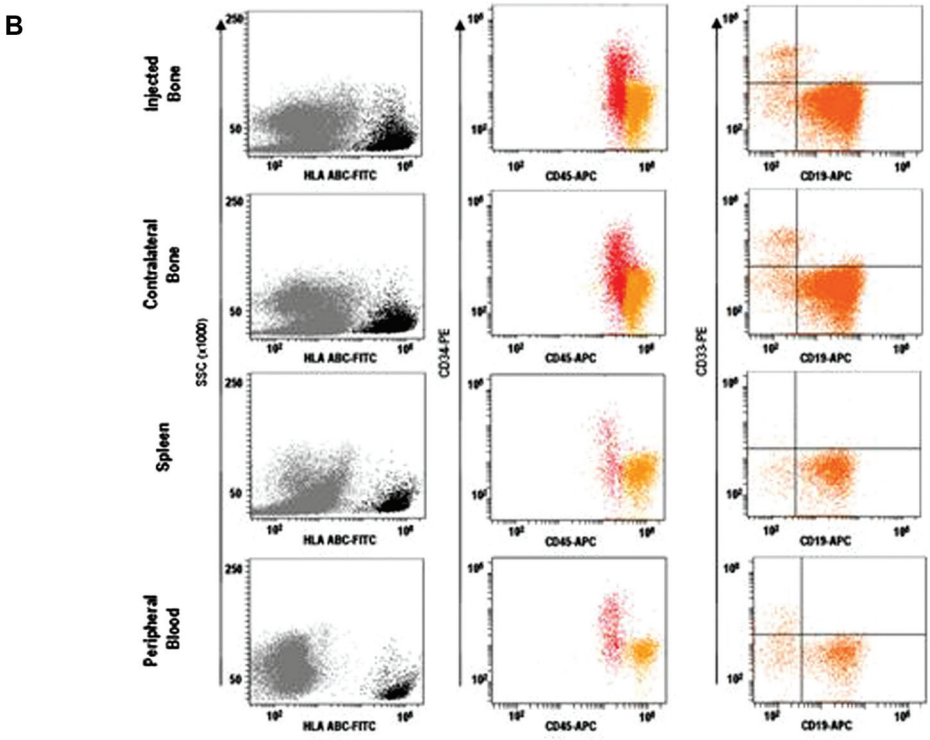
NOD/SCID (n = 10), NOD/SCID β2m$^{-/-}$ (n = 10) and NOD/SCID IL-2Rγ$^{-/-}$ (n = 18) mice (all originally from Jackson Laboratory, Bar Harbor, ME, USA) were housed under sterile conditions in a barrier facility at Vivotecnia (Madrid, Spain). The animal care committee of the University of Granada approved all mouse protocols. The mice did not receive irradiation in any form. For IBMT, they were anesthetized with an intraperitoneal injection of 0.014–0.018 mL/g body weight of a 2.5% solution of tribromoethanol. The knee joint was flexed and a hole was drilled into the femur with a short 27-gauge needle attached to a 3-mL syringe filled with phosphate-buffered saline (PBS), then the first needle was removed and replaced with a 28-gauge needle/0.3-mL insulin syringe containing 1.5×10^5 CD34$^+$ cells in a total volume of 30 μL for delivery. For pain relief, 1.0 mg buprenorphine in 300 mL PBS was administered subcutaneously immediately after transplantation and the day after.

Analysis of transplanted mice by flow cytometry

Mice were killed 9–10 weeks post-transplant and BM was flushed into the following pools: injected femur only and a pool from the contralateral femur, tibiae and iliac crests. Peripheral blood (PB) and spleen were also collected and analyzed for human chimerism. Cells from BM, PB and spleen were stained with anti-HLA-ABC–fluorescein isothiocyanate (FITC), anti-CD45–Allophycocyanine and 7-Actinomycin D to analyze human chimerism by flow cytometry. All engrafted mice were assessed for multilineage analysis using anti-CD33–FITC (Becton-Dickinson) for myeloid cells, anti-CD19–phycoerythrin (PE) (Becton-Dickinson, san Jose, CA, USA) for B-lymphoid cells and anti-CD34–FITC (Becton-Dickinson) for







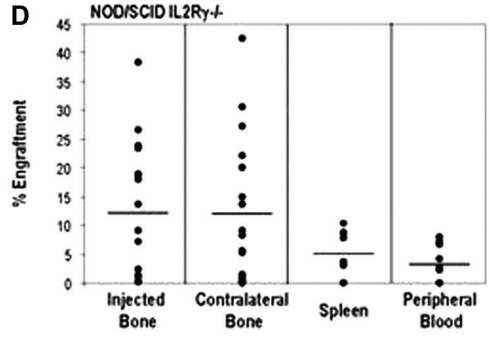


Figure 1. *(Legend Continued)*.

primitive cells. Appropriate isotype-matched control antibodies were used for all flow cytometric analyzes. Cells were analyzed on a FACSCanto and data were analyzed using CellQuest software (Becton-Dickinson).

Polymerase chain reaction for human chromosome 17-specific α-satellite marker

The presence or absence of human chimerism as detected by flow cytometry was confirmed by polymerase chain reaction (PCR) in some mice, as described previously (10). Genomic DNA was isolated from the BM using standard phenol-chloroform extraction. PCR for the human chromosome 17-specific α-satellite marker was done using 100 ng genomic DNA and the following primers: F 5′-ACACTCTTTTTGCAGGATCTA-3′ and R 5′-AGCAATGTGAAACTCTGGGA-3′. PCR conditions were 96°C for 2 min, 30 cycles of 94°C for 45 s, 65°C for 1 min and 72°C for 2 min and a final extension at 72°C for 7 min. The 1171-base pair product was resolved on a 1% agarose gel, stained with ethidium bromide and visualized.

Results and discussion

The immunodeficient mouse strains NOD/SCID, NOD/SCID $\beta2m^{-/-}$ and NOD/SCID IL-2R$\gamma^{-/-}$ were compared as recipients of hematopoietic engraftment without receiving prior irradiation; 1.5×10^5 CB-derived $CD34^+$ cells were transplanted by IBMT injection and human chimerism was assessed 10 weeks post-transplant. As shown in Figure 1A, $CD34^+$ cells had generated a human graft in 90% (16 out 18) of the non-irradiated NOD/SCID IL-2R$\gamma^{-/-}$ mice 10 weeks after transplant. The average level of engraftment was 13.5 ± 12.9% (range 0.5–44%). In contrast, engraftment was barely detected in non-irradiated NOD/SCID $\beta2m^{-/-}$ (0.4 ± 0.1%) and totally absent in non-irradiated conventional NOD/SCID (Figure 1A) mice. Human chimerism was detected by flow cytometry analyzing the presence of human $CD45^+$/HLA-ABC^+ cells in the BM of recipient mice 10 weeks after transplantation (Figure 1B). The presence or absence of chimerism was confirmed by PCR for the human chromosome 17-specific α-satellite marker (Figure 1C).

We next characterized the engraftment composition of the human graft. Multilineage engraftment was consistently observed in non-irradiated NOD/SCID IL-2R$\gamma^{-/-}$ mice. Similar to grafts in conditioned recipients, the graft in non-irradiated recipients was composed predominantly of B-lymphoid cells ($CD45^+$ $CD19^+$), followed by myeloid cells ($CD45^+$ $CD33^+$) and immature cells ($CD45^+$ $CD34^+$) (Figure 1B).

One advantage of IBMT over intravenous transplantation is that direct injection of cells into one femur provides the opportunity to assess migration of transplanted $CD34^+$ cells *in vivo*. Accordingly, *in vivo* migration of transplanted $CD34^+$ cells from the injected bone site of NOD/SCID IL-2R$\gamma^{-/-}$ recipients was assessed by analyzing the level of human chimerism not only in the injected femur but also in non-injected BM, spleen and PB. Human cells were never observed exclusively in a non-injected bone site, which would indicate migration of a transplanted SCID-Repopulating Cell and complete retention of the progeny to an alternative bone site. Multilineage engraftment in all bone sites, spleen and PB was observed routinely even in the absence of myeloablative irradiation (Figure 1B, D), suggesting that the lack of space in the BM niches does not represent a major limitation for HSC xenotransplantation (8). These data demonstrate that, following localized intrafemoral delivery, transplanted $CD34^+$ cells are capable of migration to other bone sites, spleen and PB in non-irradiated NOD/SCID IL-2R$\gamma^{-/-}$ recipients.

Our study shows that combining the robustness of the NOD/SCID IL-2R$\gamma^{-/-}$ mouse strain with the sensitivity of IBMT greatly facilitates long-term multilineage engraftment and migration of human $CD34^+$ cells without the need for prior irradiation. Although myeloablative irradiation is typically used in animal models to enhance host engraftment, a frequently overlooked concern is that this severely damages the BM architecture and may therefore mask defects in

Figure 1. Long-term multilineage hematopoietic reconstitution of non-irradiated NOD/SCID IL-2Rγ^{null} mice after IBMT IL-2Rγ null means the same that IL-Rγ-/-. Please standardise. Should they be standardized? (A) Engraftment was compared between three non-irradiated immunodeficient mouse strains: NOD/SCID, NOD/SCID $\beta2m^{-/-}$ and NOD/SCID IL-2R$\gamma^{-/-}$. The percentage of human CD45+ HLA-ABC+ was measured in BM cells of recipient mice analyzed 10 weeks after transplantation with 1.5×10^5 CD34+ cells. Each dot represents an individual mouse and the bar indicates the mean of each experimental group. Below the graph the frequency of engrafted mice and level of human chimerism are shown. (B) Representative flow cytometry analysis showing multilineage engraftment. All human cells co-express HLA-ABC and the pan-hematopoietic marker CD45. The CD45+ human hematopoietic fraction comprises immature cells (CD34+), myeloid cells (CD33+) and B-lymphoid cells (CD19+). (C) Representative genomic PCR for the human-specific chromosome 17 α-satellite marker. In line with the flow cytometry data, engraftment was confirmed by PCR in NOD/SCID IL-2R$\gamma^{-/-}$ mice but not in NOD/SCID or NOD/SCID $\beta2m^{-/-}$ strains. (D) Human chimerism in non-injected BM, spleen and PB indicates migration of human cells from the injected femur.

HSC engraftment, survival, homing and trafficking (9). Thus this assay opens up new avenues for studying the phenotype and *in vivo* behavior of normal SRC and leukemia-initiating cells in a mouse strain very sensitive to hematopoietic engraftment with no need for BM-damaging myeloablative conditioning and with a lifespan long enough to undertake leukemogenesis studies (15).

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Authorship and disclosures: CB designed and performed experiments, analyzed the data and wrote the manuscript. RM performed experiments and analyzed the data. TC and IG-A performed *in vivo* transplants and supervised animals. PM conceived and supervised the study and wrote the paper. The authors report no potential conflicts of interest.

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COMMENTARY

Intra-bone route of administration offers new perspectives for safer transplantation of hematopoietic stem cells

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In this issue of *Cytotherapy*, Bueno et al. (1) describe an experimental setting in which hematopoietic stem cells (HSC) can be transplanted without conditioning the recipient mouse. *Cytotherapy* readers will immediately understand the formidable potential of this approach. If we could transplant healthy cells into patients without the need for a toxic conditioning regimen, the hematopoietic transplantation field could take a big leap forward. Overcoming the need for a conditioning regimen represents a long-held dream in the field of transplantation (2).

The study of Bueno et al. (1) opens up exciting new possibilities but several considerations need to be addressed first. For many years, two barriers have been considered the major obstacles to the full success of hematopoietic transplantation: (i) creating *space*, to allow the incoming HSC to seed in the marrow; and (ii) abrogation of *immune resistance* because of histocompatibility differences between donor and recipient, to overcome rejection and graft-versus-host disease (GvHD). The problem of space is discussed extensively in this paper but it is not a novel concept (3,4 and others). The possibility of transplanting HSC without conditioning challenges the assumption that empty 'niches' need to be created for marrow to permit engraftment of incoming cells. In considering previous studies showing that HSC can engraft with minimal or no conditioning, two features have to be borne in mind. Firstly, Stewart et al. (4), for example, used stem cell doses of 40×10^6 cells/mouse, which represent a very high equivalent dose in the clinical setting. Secondly, the syngeneic transplants described do not predict what might be achieved in an allogeneic HSC setting. It should also be emphasized that HSC are most frequently used to eliminate hematopoietic malignancies where elimination of malignant cells is a priority irrespective of whether or not empty niches exist.

The problem of seeding efficiency must also be considered, namely how many cells from those injected into the vein reach the hematopoietic sites. Seeding efficiency calculated in mice is around 10% or less after intravenous injection. This suggests that 90% of the cells that we transplant may be lost. Intra-bone transplantation, the alternative route of administration (5), is already showing promise in clinical transplantation for improving the seeding efficiency by allowing direct homing of stem cells to bone marrow (6). However, it is not known how many cells injected into the bone remain at the site of injection and how many leave the site of injection to colonize distant hematopoietic sites. When we first found that intra-bone injection was associated with better engraftment, we proposed the idea of an 'engraftment store', where HSC would learn how to colonize other remote hematopoietic sites following *physiologic* cross-talking with the micro-environment. This could explain efficient seeding in distant sites (5). Whether this is a major mechanism is not yet clear.

In their discussion, Bueno et al. (1) state that 'immunodeficient mice [the NOD/SCID IL-2R$\gamma^{-/-}$] provide the most physiologic setting'; however, it must be pointed out that NOD/SCID IL-2R$\gamma^{-/-}$ mice are experimentally engineered animals that do not represent either physiologic or pathophysiologic conditions. The mechanisms by which intra-bone transplant of human CD34$^+$ cells in NOD/SCID IL02R$\gamma^{-/-}$ mice allows the engraftment and spreading of HSC are largely characterized by this study. Nevertheless, the work offers the prospect of utilizing the intra-bone technique to identify further

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mechanisms contributing to abrogation of rejection or induction of tolerance. Although it may be coincidental, the clinical experience utilizing intra-bone transplantation of cord blood cells (6) shows better engraftment rates and a lower incidence and severity of acute GvHD. We are only just beginning to understand all the consequences and possibilities of alternative routes of HSC administration.

What Bueno et al. (1) have not addressed so far is whether local radiation of a limb could facilitate local intra-bone HSC transplantation. These experiments could determine whether HSC can colonize the rest of the hematopoietic system from the local area of injection. If this was possible, HSC transplantation for thalassemia might be achieved by irradiating only a single limb and transplanting HSC locally intra-bone, thus avoiding the toxicity of total body irradiation or other conditioning regimens. Although it could be argued that the defect in thalassemia manifests itself at a later stage of differentiation and maturation and normal HSC may not have a proliferative advantage, even achieving stable mixed chimerism might be sufficient to avoid the need for transfusion. Possibly the most important immediate implication of Bueno et al.'s (1) study (considering the short post-engraftment follow-up) is the opportunity of using the NOD/SCID IL-2R$\gamma^{-/-}$ mice to study leukemic cell engraftment. This is important because, so far, transplantation of leukemic cells (notably in chronic myeloid leukemia) into NOD/SCID mice has yielded contradictory and inconsistent results.

In conclusion, the work of Bueno et al. (1), although in a very special experimental setting, is a significant contribution to the intra-bone HSC transplantation approach and encourages the further exploration of this new and rapidly expanding avenue of transplantation research.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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4.- NG2 antigen is expressed in CD34+ HPCs and plasmacytoid dendritic cell precursor: is NG2 expression in leukemia dependent on the target cell where leukemogenesis is triggered?

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Leukemia. 2008; 22: 1475-1478.

INTRODUCCIÓN. La expresión del antígeno neuro-glial 2 (NG2) se ha venido asociando de manera específica a los reordenamientos de *MLL*. NG2 es reconocido por el anticuerpo monoclonal 7.1., por lo que este anticuerpo forma parte desde hace años en los paneles diagnósticos para el inmunofenotipaje de pacientes leucémicos, dado el potencial papel predictivo de NG2 en LMAs adultas y pediátricas con reordenamiento de *MLL*. Incluso, se ha venido asociando la expresión de NG2 de manera específica con las traslocaciones de *MLL* t(4;11) y t(9;11). Sin embargo, existe controversia en los datos publicados, ya que existen pacientes con *MLL* reordenado que no expresan NG2, a la vez que se han descrito leucemias agudas y leucemias de células dendríticas plasmocitoides (pDC), que expresan NG2 sin tener reordenado *MLL*.

OBJETIVO. Profundizar en la asociación entre la expresión de NG2 y los reordenamientos de *MLL*, determinando si dicha expresión depende del gen con el que *MLL* esté reordenado. Así mismo, comprobar si la expresión de NG2 en leucemias sin reordenamiento de *MLL* se pudiera deber al inicio del proceso leucémico en una subpoblación HSPCs que co-expresan CD34+ y NG2+.

MATERIAL Y MÉTODOS. Se utilizaron las siguientes líneas celulares: líneas con *MLL germline,* KG1a, REH y 293T; y líneas con *MLL* reordenado, MV4;11 (MLL-AF4+ LLA pro-B), RS4;11 (MLL-AF4+ LLA pro-B) y THP-1 (MLL-AF9+ LMA). También se utilizaron hESCs (línea HS181), HSPCs CD34+ de SCU, sangre periférica movilizada y MO provenientes de donantes sanos. En todas ellas se comprobó por citometría de flujo la expresión de NG2. Así mismo, se buscó la expresión de NG2 en alguna subpoblación de CD34+ en muestras de donantes sanos.

RESULTADOS. Con respecto a las líneas celulares sin *MLL* reordenado, KG1a no mostró expresión de NG2, mientras que REH y 293T mostraban expresión de NG2. Respecto a las líneas celulares con *MLL* reordenado, THP-1 y MV4;11 mostraban positividad para NG2, mientras que RS4;11 fue negativa en esta expresión. En relación a la expresión de NG2 con el origen celular donde la alteración leucemogénica tiene lugar, se observó positividad para NG2 en la subpoblación HPCs CD34+CD38+ de cordón umbilical (2.1±2.4%), MO (0.83±2.2%) y sangre periférica movilizada (1.3%), así como en el 5-6% de las hESCs. Así mismo, los precursores pDC (con inmunofenotipo $CD34^{+}$ $CD45^{+}$ $CD38^{+}$ $CD123^{high}$ $HLADR^{+}$) mostraban co-expresión de NG2 en el 12.2±13% de dichos precursores.

CONCLUSIÓN. La expresión de NG2 no depende del gen traslocado con *MLL*. Por otro lado, el hecho de que los progenitores CD34+, precursores pDC y hESCs expresen NG2, indica que NG2 podría

expresarse únicamente en los pacientes donde el proceso leucémico se inició en una célula NG2+, independientemente del tipo de leucemia.

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EDITORIAL

NG2 antigen is expressed in CD34+ HPCs and plasmacytoid dendritic cell precursors: is NG2 expression in leukemia dependent on the target cell where leukemogenesis is triggered?

Leukemia (2008) **22,** 1475–1478; doi:10.1038/leu.2008.134

The neuron-glial antigen 2 (NG2) molecule and its human homolog was first reported on oligodendrocyte progenitor cells.[1] NG2 is recognized by the 7.1 monoclonal antibody.[2] The physiological role of this molecule remains to be elucidated.[3] The expression pattern of NG2 in leukemia is controversial. NG2 expression was initially claimed to be specifically associated with 11q23/mixed-lineage leukemia (MLL) gene rearrangements.[4] In fact, over the past years, NG2 has been incorporated in diagnostic panels for immunophenotyping of leukemic patients because of its potential predictive value for MLL rearrangements in childhood and adult acute myeloid leukemias.[2,4–9] However, it has been suggested that 7.1 expression could be specifically associated with only two specific subtypes of leukemia harboring either the translocations t(4;11) (q21;q23) or t(9;11) (p13;q23), which encode for the leukemic fusion genes MLL-AF4 and MLL-AF9, respectively, but not to other MLL rearrangements.[10] Moreover, in the clinic, many leukemic patients harboring MLL rearrangements but lacking NG2 expression are commonly seen (Supplementary Table 1). In turn, we and many others have reported the existence of acute leukemias and plasmacytoid dendritic cell (pDC) leukemias (>50%) lacking MLL rearrangements but expressing NG2[11] (Supplementary Table 1).

Over 80 different partners of the human MLL gene have been identified so far, most being associated with a poor clinical outcome.[12–14] Clearly, this renders the characterization of MLL fusion alleles and its correlation with NG2 expression a very demanding task. Intriguingly, it has been suggested that the expression of NG2 antigen may be dependent on the particular gene(s) paired to MLL when it is rearranged, explaining, at least in part, why some patients with 11q23 balanced translocations can be NG2 negative.[5,10]

On the basis of the controversial data about the clinical relevance of NG2 expression and its inclusion in diagnostic immunophenotypic panels as well as the existence of NG2-expressing acute leukemias lacking MLL rearrangements, in particular pDC leukemias,[11] we aimed (i) to gain further insights into the biological association between NG2 expression and MLL rearrangements; (ii) to analyze whether the expression of NG2 may depend on the particular gene(s) paired to MLL when it is rearranged and (iii) to explore the hypothesis that the expression of NG2 in leukemias lacking MLL rearrangements, such as NG2+ pDC-leukemias, may be due to the existence of a minor subset of CD34+ hematopoietic stem/progenitor cells readily coexpressing NG2 where the leukomogenesis process may be initially triggered.

The following cell lines, chosen based on the status of the MLL locus were used in this study: KG1a (stem cell like-AML), REH (TEL-AML1+ pre-B ALL), 293T (kidney embryonic epithelial cell line), MV4;11 (MLL-AF4+ pro-B ALL), RS4;11 (MLL-AF4+ pro-B ALL) and THP-1 (MLL-AF9+ AML). Three out of the six (KG1a, REH and 293T) are cell lines without MLL translocations, whereas the other three cell lines (MV4;11, RS4;11 and THP-1) harbor balanced MLL translocations. Importantly, AF4 is the partner gene paired to rearranged MLL in both MV4;11 and RS4;11 cell lines, whereas AF9 is paired to MLL in THP-1. The cell lines were maintained in standard culture conditions: RPMI or DMEM with 10% fetal bovine serum supplemented with nonessential amino acids, 2 mM L-glutamine and antibiotics (all from Gibco, Invitrogen, Grand Island, NY, USA). Genetically stable (manuscript submitted) and fully characterized pluripotent undifferentiated human embryonic stem cells (hESCs) HS181 (kindly provided by Professor O Hovatta, Karolinska Institute) were maintained on mitotically inactivated human embryonic fibroblasts as described previously.[15] Cord blood samples (CB; $n=5$) from healthy newborns were obtained from local hospitals with Ethics Board approval. Normal bone marrow ($n=6$) and granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood ($n=1$) were obtained from healthy volunteers upon informed consent. The mononuclear cells were isolated using Ficoll-Hypaque (GE Healthcare, Bucks, UK), washed with phosphate-buffered saline + 3% fetal bovine serum and the red blood cells lysed (Red Blood Cell-Lysis Solution, BD Biosciences, Erembodegem, Belgium). Then, the mononuclear cells were stained by direct immunofluorescence (see below) and ready for multicolor high-speed flow cytometry analysis.[16]

The expression of NG2 in CD34+ pDC precursors[16] was assessed by flow cytometry using the following combination of monoclonal antibody in a six-color staining: CD38-FITC/NG2-PE/HLADR-PerCP/CD123-APC/CD34-PECy7/CD45-APC-Cy7. All monoclonal antibody were purchased from Becton Dickinson (San Jose, CA, USA) except the NG2 that is from Immunotech (Marseille, France) and CD123-APC from Miltenyi (Bergisch, Gladbach, Germany). Of note, as many as 2×10^5 CD34+ hematopoietic stem/progenitor cells were gated and analyzed. Plasmacytoid DC precursors were identified as being $CD34^+/CD45^+/CD38^+/CD123^{high}/HLADR^+$, as described recently.[16] From an ontogeny standpoint, the expression of NG2 was assessed in hESCs (prenatal stage of *in utero* development) and CB samples (neonatal stage of hematopoietic development), because a high frequency of primary leukemias with rearranged MLL gene occurs during infancy/childhood.[17] NG2 expression was also analyzed in adult bone marrow samples.

Among the cell lines without MLL rearrangements, the myeloid cell line KG1a lacked NG2 expression, whereas REH was NG2+ (Figure 1a). Interestingly, the nonhematopoietic cell line 293T was also clearly positive for NG2. This data confirm the expression of NG2 in hematopoietic and nonhematopoietic cell lines without MLL rearrangements, further supporting the existence of human leukemias lacking MLL translocations but expressing the NG2 antigen[11] (Figure 1a and Supplementary Table 1).

Among the MLL translocation-harboring cell lines, THP-1 displayed clear expression of NG2. Intriguingly, both the

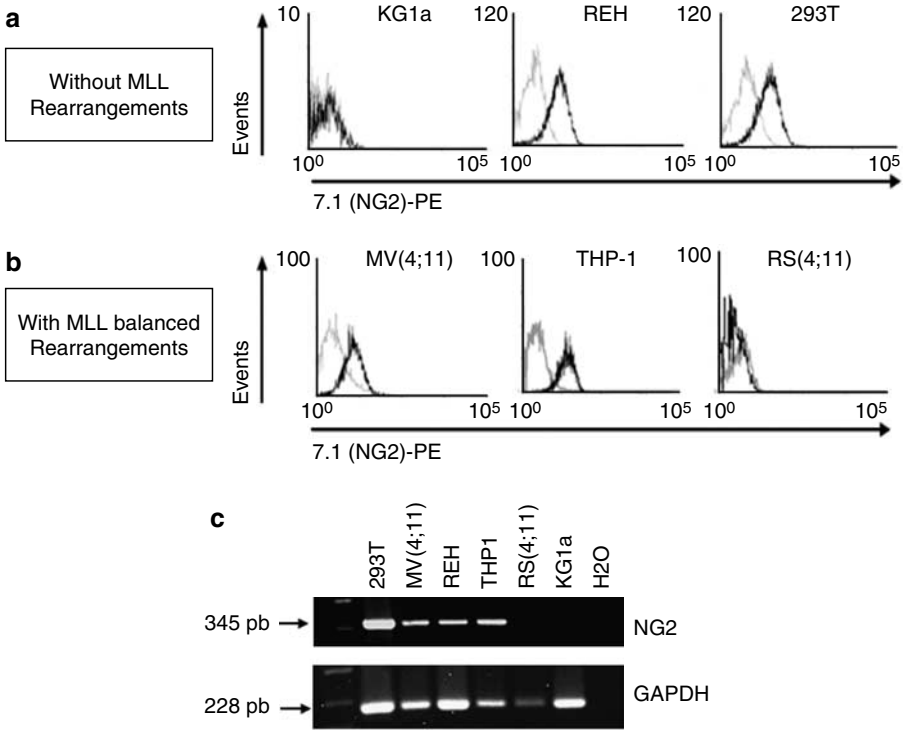

Figure 1 Flow cytometry analysis of NG2 (7.1) antigen in transformed hematopoietic cell lines. (**a**) Expression of NG2 in transformed cell lines lacking MLL gene rearrangements. (**b**) Expression of NG2 in transformed cell lines carrying balanced MLL gene rearrangements. Gray lines represent the irrelevant isotype-matched control. (**c**) reverse transcriptase showing the NG2 mRNA expression in different leukemic cell lines. MLL, mixed-lineage leukemia; NG2, neuron-glial antigen 2.

MV4;11 and RS4;11 cell lines, while sharing MLL gene rearrangements fused to the same partner gene (AF4), displayed a different profile of NG2 expression: MV4;11 cells were NG2-positive, whereas RS4;11 were negative (Figure 1b). To add more mechanistic insight, NG2 expression was also analyzed at the RNA level by reverse transcriptase-PCR. As shown in Figure 1c, NG2 mRNA is present in all the NG2+ leukemic cell lines and absent in all the NG2− leukemic cell lines, indicating a full correlation between NG2 mRNA detection and NG2 transcribed protein.

Our experimental data support the clinical finding of both primary human leukemias with balanced MLL rearrangements coexpressing NG2 and human leukemia cases harboring balanced MLL gene translocations but lacking NG2 expression (Supplementary Table 1). This data not only highlight the lack of association between NG2 antigen and MLL rearrangements but also provide preliminary experimental evidence against the hypothesis that NG2 expression in human leukemia may be dependent on the particular gene(s) paired to MLL when rearranged, as we demonstrate that two pro-B ALL cell lines (MV4;11 and RS4;11) with the MLL gene fused to the same partner, AF4, have a completely different NG2 antigen expression profile (Figure 1).

Several cellular and molecular mechanisms, intrinsic molecular determinants and extrinsic signals may contribute to the controversial correlation between MLL rearrangements and NG2 regulation. The confused association observed in actual leukemic samples makes an explanation based on a potential role for '*in vivo*' (bone marrow, CB and so on) occurring cell–cell interactions unlikely. Similarly, our '*in vitro*' data, rule out the possibility that potential hits/mutations secondary to MLL translocations are required for triggering NG2 expression, as we used fully transformed/immortalized cell lines derived from patients with overt disease, therefore carrying a paramount of cooperating mutations and genetic insults. The possibility that NG2 expression could be associated with nonbalanced MLL rearrangements such as deletions or inversions has previously been ruled out.[4,5] Although unlikely, the possibility that NG2 expression could be linked to MLL internal duplications, which are not prospectively analyzed in human leukemias at diagnosis, should not be excluded.

Here, we hypothesize that NG2 expression may be dependent on the cell of origin where a specific leukemic abnormality initially occurs. For instance, NG2 might only be regulated when the leukemic abnormality arises either in a lineage-specific progenitor (hematopoietic progenitor cell; HPC) or in a more immature, less committed stem cell (hematopoietic stem cell; HSC). When a large number (2×10^5 cells) of CD34+ hematopoietic stem/progenitor cells are gated and analyzed by flow cytometry, coexpression of NG2 is readily observed in a subset of CD34+CD38+ HPCs from CB (2.1 ± 2.4%; Figure 2a), bone marrow (0.83 ± 2.2%) and mobilized peripheral blood (1.3%; Supplementary Figure 1a), suggesting that HPCs rather than HSCs may be the target cell for transformation. To verify that this CD34+CD38+NG2+ cell subset represents truly HPCs, this population was enriched (74% post-sort purity) by flurescence-activated cell sorting from CB and the cells plated in methylcellulose assays. Importantly, multilineage (CFU-G, CFU-M; CFU-Mix, BFU-E) hematopoietic colonies were obtained in *in vitro* colony-forming unit (CFU) assays[18] (Supplementary Figure 1a), suggesting that HPCs rather than HSCs may be the target cell for transformation. Recent studies from Vormoor laboratory[19,20] support our data as they found that NG2 is not expressed in MLL-rearranged HSC populations (CD34+CD38−) but was up-regulated in differentiated MLL-

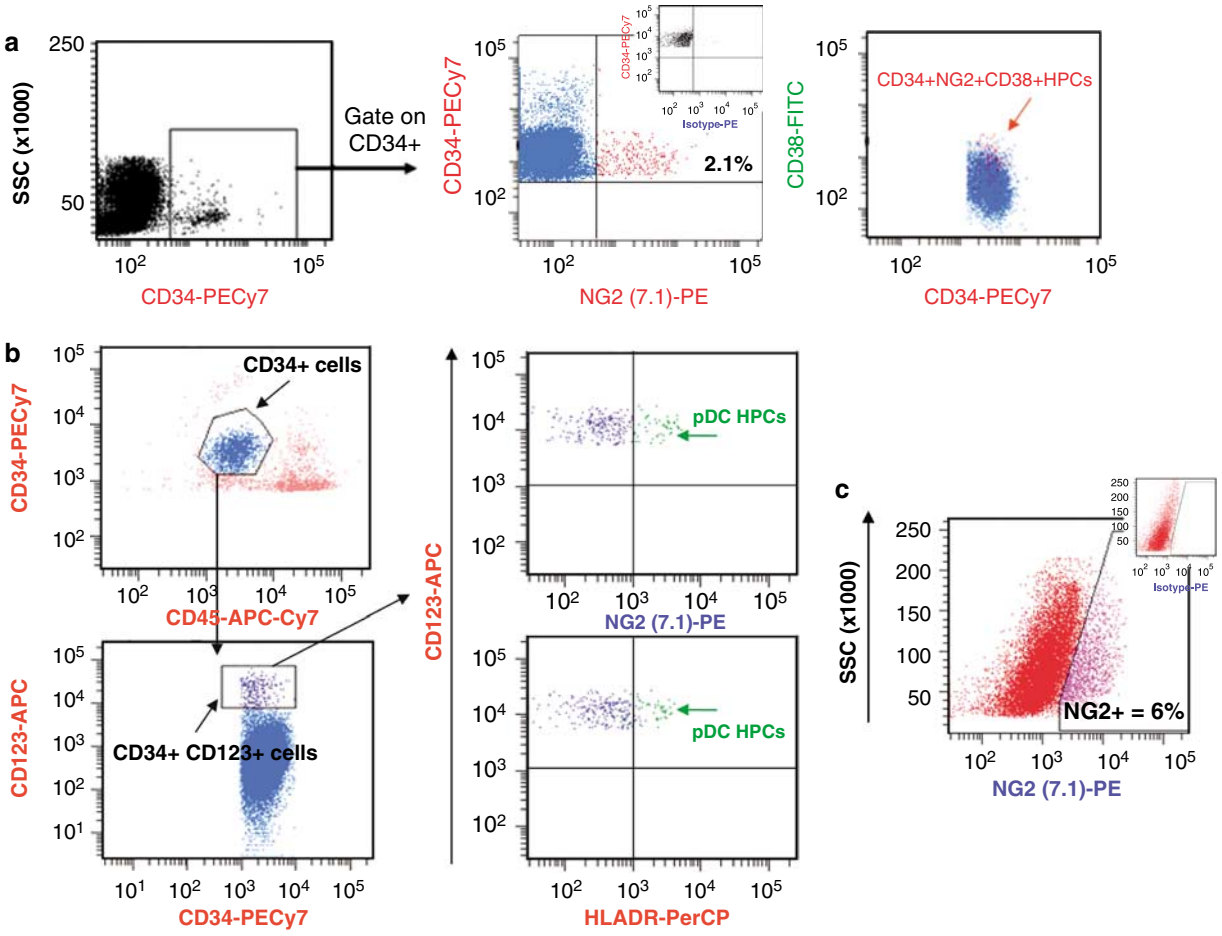

Figure 2 Representative flow cytometry analysis of NG2 (7.1) antigen in normal CD34+ progenitors, CD34+ pDC precursors and hESCs. (**a**) Expression of NG2 in gated CB-derived CD34+ cells. Analyses of as many as 2×10^5 CD34+ cells revealed that 2.1 ± 2.4% of the CD34+ cells coexpress NG2+ cells. All these CD34+NG2+ cells are CD38+, therefore, representing HPCs. An irrelevant isotype-matched antibody was used as a negative control (inset panel). (**b**) Six-color high-speed flow cytometry analysis showing the expression of NG2 (green dots) in a population of pDC precursors (CD45+ CD34+ CD38+ CD123++ and HLADR+).[16] (**c**) Representative NG2 expression in genetically stable hESCs. An irrelevant isotype-matched antibody was used as a negative control (inset panel). CB, cord blood; HPCs, hematopoietic progenitor cells; NG2, neuron-glial antigen 2; pDC, plasmacytoid dendritic cell.

rearranged leukemic blasts (CD19+ B-lineage blasts and CD33+ myeloid blasts) in pediatric MLL-rearranged AMLs and MLL-AF4+ ALLs.

The expression of NG2 in 60% of pDC leukemias lacking MLL rearrangements have recently been reported[11] (our unpublished observations). We, therefore, next addressed whether pDC CD34+ precursors[16] coexpress NG2. Interestingly, we found that 12.2 ± 13% of the pDC precursors ($CD34^+/CD45^+/CD38^+/CD123^{high}/HLADR^+$) readily coexpress NG2 (Figure 2b). This suggests that, regardless of the status of the MLL locus, the NG2 antigen may be expressed in pDC leukemias, if the leukomogenesis process is initially triggered in a pDC CD34+ precursor readily expressing NG2, which might act as a leukemic-initiating cell. In fact, based on the existence of NG2+ cell lines and NG2+ human primary leukemias [11](our unpublished observations) lacking MLL rearrangements our data illustrate that the leukemic abnormality underlying NG2 expression does not necessarily need to be an MLL rearrangement.

Unlike other MLL fusion proteins, MLL-AF4 fusion gene resulting from the t(4;11) is always found in infant pro-B-ALL with a dismal prognosis and arises prenatally.[17] From an ontogeny standpoint, two possible scenarios have been proposed: (i) MLL-AF4 arises and has its preleukemic impact during early human embryonic hematopoiesis or (ii) MLL-AF4 arises during early hematopoiesis but has its preleukemic impact only in later fetal hematopoiesis.[17] We analyzed the expression of NG2 in undifferentiated hESCs, a potential prenatal target cell for MLL-AF4 occurrence. Intriguingly, NG2 expression is also readily found in 5–6% of genetically stable hESCs (Figure 2d).

Despite NG2 and its human homolog was first reported on oligodendrocyte progenitor cells, the possibility that the cell subset CD34+ NG2+ might represent a potential circulating neural progenitors should be ruled out since, as shown by six-color high-speed flow cytometry, the NG2-expressing cells coexpress the panhematopoietic marker CD45 and the HPCs markers CD34 and CD38. More importantly, NG2 expression in CB was confined to the CD45+CD34+ cell subset, indicating that, NG2 is solely expressed in hematopoietic cells, especially in committed HPCs; pDC-progenitors (CD45+ CD34+ CD38+ CD123++ and HLADR+)[16] among others, in line with the occurrence of NG2+ pDC-leukemias lacking MLL.[11] Taken together, we throw more light on the association between NG2 expression and MLL rearrangements and report preliminary data supporting that NG2 expression does not depend on the particular gene(s) paired to MLL. The fact that CD34+ progenitors, pDC precursors and hESCs express NG2 indicates that NG2 in leukemia might uniquely be expressed in those

patients where the leukemogenesis process is triggered in a NG2-expressing cell subset, regardless the type of leukemia. More comprehensive clinical, functional, molecular, cytogenetic and immunophenotypic studies are warranted to resolve the current puzzling and confusing significance of NG2 expression in human leukemias.

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Author contribution:
CB and RM designed and perfomed experiments and analyzed data. LM performed and analyzed experiments. IP and MCH provided CB samples and clinical data. AO contributed key clinical data, analyzed data and revised the manuscript. PM conceived, supported and supervised the work and analyzed the data. CB and PM wrote the paper.

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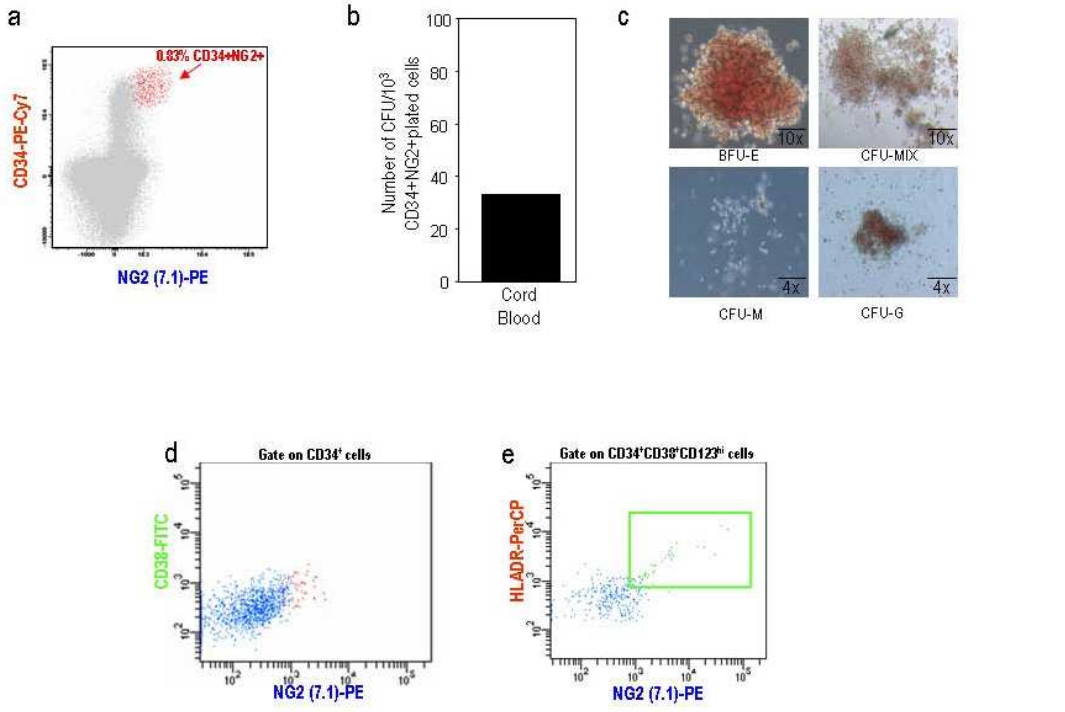
Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Supplementary Figure. Bueno *et al*



Supplementary Table 1: Published clinical data supporting the lack of association between MLL rearrangements and NG2 Expression.

Reference	Diagnosis	MLL rearranged / NG2$^-$ (% cases)	MLL germline / NG2$^+$ (% cases)
Hilden JM, Blood 1997	Infant AML	45%	
Mauvieux L, Br. J. Hematol 1999	Pediatric AML	34%	
	Adult AML	64%	
Wuchter C, Leukemia 2000	Childhood ALL	14%	
	Childhood AML	23%	
	Adult AML	36%	61%
Schwartz, Leukemia 2003	Childhood ALL	16%	5-10%
Neudenberger J, Br. J. Hematol 2006	Pediatric AL	18%	
Bueno C, Haematologica 2004	DC Leukemia		60%
Our Clinical Lab Data	AML		50%
	ALL		60%
	Adult AML/ALL	4%	

5.- Enforced expression of MLL-AF4 fusion in cord blood CD34+ cells enhances the hematopoietic repopulating cell function and clonogenic potential but is not sufficient to initiate leukemia.

Montes R., Ayllón V., Gutierrez-Aranda I., Prat I., Hernández-Lamas C., Ponce L., Bresolin S., te Kronnie G., Greaves M., Bueno C., Menendez P.

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INTRODUCCIÓN. La LLA del lactante con reordenamiento MLL-AF4 se asocia con un pronóstico fatal y una latencia muy corta, manifestándose la enfermedad antes del primer año de vida. Actualmente no existen modelos que recapitulen la enfermedad. Dado el origen prenatal de de la traslocación t(4;11) en estas leucemias, así como a su corta latencia, nos planteamos si MLL-AF4 es suficiente para iniciar/desarrollar el proceso leucémico en HSPCs CD34+ derivadas de SCU.

OBJETIVO. Conocer el efecto de la expresión del oncogén MLL-AF4 en células HSPCs CD34+ derivadas de cordón umbilical tanto *in vivo* como *in vitro*.

MATERIAL Y MÉTODOS. Las HSPCs CD34+ se aislaron de SCU mediante separación magnética y con una pureza superior al 91%. Estas células fueron trasducidas mediante vectores lentivirales para lograr la expresión tanto del oncogén MLL-AF4 como del vector vacío control. Para los estudios *in vivo*, las células trasducidas se transplantaron intratibia a ratones inmunodeficientes de la cepa NOD/SCID IL-2Rγ$^{-/-}$ previamente irradiados. Estos animales fueron sacrificados 12-16 semanas post-trasplante, aislándose células de la MO de la tibia inyectada, de la MO de la tibia no inyectada (contralateral), del bazo y de la sangre periférica. En estos órganos se valoró mediante citometría la repoblación hematopoyética humana, así como la reconstitución multilínea del ratón. Se analizarón tanto parámetros bioquímicos-hematológicos en sangre periférica, como la morfología e histología de bazo e hígado. Para los estudios *in vitro*, las células CD34+ trasducidas se utilizaron para ensayos de clonogenicidad en metilcelulosa, y para ensayos de proliferación en cultivo líquido, así como para análisis de ciclo celular y apoptosis. La presencia del oncogén en las muestras se confirmó mediante RT-PCR.

RESULTADOS. Los ratones trasplantados con células que expresan MLL-AF4 mostraban mayor quimerismo humano y movilización a otros órganos. *In vitro*, las células CD34+ MLL-AF4+ mostraban mayor capacidad proliferativa y clonogénica, mostrando además una mayor expresión de genes relacionados con supervivencia celular (Bcl-2 y Bcl-xL). Sin embargo, MLL-AF4 por si sólo no es capaz de inmortalizar (*in vitro*) o trasformar (*in vivo*) células CD34+ neonatales/fetales.

CONCLUSIÓN. A pesar de inducir un mayor nivel de quimerismo humano, así como una mayor capacidad de proliferación y potencial clonogénico, la expresión de MLL-AF4 no fue suficiente para iniciar el proceso leucémico en sí misma, indicando que otros eventos adicionales a esta traslocación podrían ser necesarios para desarrollar la enfermedad, o bien, que las HSPCs CD34+ derivadas de SCU no representan la diana celular apropiada en la LLA MLL-AF4+.

Enforced expression of MLL-AF4 fusion in cord blood CD34$^+$ cells enhances the hematopoietic repopulating cell function and clonogenic potential but is not sufficient to initiate leukemia

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Infant acute lymphoblastic leukemia harboring the fusion mixed-lineage leukemia (MLL)-AF4 is associated with a dismal prognosis and very brief latency. Our limited understanding of transformation by MLL-AF4 is reflected in murine models, which do not accurately recapitulate the human disease. Human models for MLL-AF4 disease do not exist. Hematopoietic stem or progenitor cells (HSPCs) represent probable targets for transformation. Here, we explored in vitro and in vivo the impact of the enforced expression of MLL-AF4 in human cord blood-derived CD34$^+$ HSPCs. Intrabone marrow transplantation into NOD/SCID-IL2R$\gamma^{-/-}$ mice revealed an enhanced multilineage hematopoietic engraftment, efficiency, and homing to other hematopoietic sites on enforced expression of MLL-AF4. Lentiviral transduction of MLL-AF4 into CD34$^+$ HSPCs increased the in vitro clonogenic potential of CD34$^+$ progenitors and promoted their proliferation. Consequently, cell cycle and apoptosis analyses suggest that MLL-AF4 conveys a selective proliferation coupled to a survival advantage, which correlates with changes in the expression of genes involved in apoptosis, sensing DNA damage and DNA repair. However, MLL-AF4 expression was insufficient to initiate leukemogenesis on its own, indicating that either additional hits (or reciprocal AF4-MLL product) may be required to initiate ALL or that cord blood-derived CD34$^+$ HSPCs are not the appropriate cellular target for MLL-AF4-mediated ALL. (*Blood*. 2011;117(18): 4746-4758)

Introduction

The mixed-lineage leukemia (MLL) gene fuses to generate chimeric genes with more than 70 partners in human leukemia.[1,2] Infant pro-B acute lymphoblastic leukemias (ALL) harboring the fusion MLL-AF4 represents a rare leukemia[3,4] and is associated with very brief latency and dismal prognosis, raising the question of how this disease evolves so quickly.[5,6] Over the last decades, major achievements have provided a better understanding about the etiology and pathogenesis of infant MLL-AF4$^+$ pro-B ALL. Studies on identical twins with concordant MLL-AF4$^+$ leukemia and retrospective analyses of the clonotypic MLL-rearranged genomic sequences of leukemic cells from young patients in their neonatal blood spots revealed an in utero origin of the MLL-AF4 fusion.[6-9]

MLL-AF4 leukemogenesis has been particularly difficult to model,[10] and bona fide MLL-AF4 disease human models do not exist. Our understanding of transformation by MLL fusions and their mode of action comes from murine models in which leukemias do not recapitulate the human disease faithfully. Some success has been achieved recently in the Kersey laboratory by ESC knock-in,[11] but the resultant disease differs significantly from that seen in infant ALL in 2 respects: (1) the latency is exceptionally protracted; and (2) the disease is classified as either myeloproliferative or mature/follicular B. Rabbitts's group has developed and used the invertor conditional technology to create a mouse model of MLL-AF4, in which a floxed AF4 cDNA was knocked into MLL. The mice develop exclusively B-cell lineage neoplasias, but of a more mature phenotype than normally observed in childhood leukemia.[12] Similarly, Armstrong's laboratory has recently created a mouse model where conditional expression of MLL-AF4 fusion induces B-precursor ALL and acute myeloid leukemia (AML).[13] In addition, studies from Marschalek's laboratory have recently indicated that the expression of the reciprocal translocation product AF4-MLL in murine hematopoietic stem/progenitor cells (HSPCs) results in the development of a pro-B ALL.[14] These seemingly contradictory findings raise important questions about t(4;11) leukemia. Moreover, the long latency observed in all these mouse models suggests that all of them are missing some essential ingredients to faithfully recapitulate the fast process of leukemogenesis during early human development. It could be argued that the lack of a bona fide MLL-AF4 disease model may be the result of: (1) a cell in a wrong developmental or hierarchical position was targeted in the murine experiments; (2) MLL-AF4 might have a detrimental effect when expressed under the long terminal repeat retroviral promoter at levels much higher than required to be oncogenic; (3) the impact of etiologic exposure factors or other secondary hits has not been properly addressed. For instance, it has been suggested that the remarkably brief latency of infant ALL might be the result of the MLL-AF4–driven preleukemic cells being continuously exposed to the same

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putative transplacental chemical carcinogens that induced the fusion gene itself.[15-17] Clearly, these "etiologic" components are missing from the mouse models in relation to rapid acquisition of essential secondary mutations; or it is plausible that MLL-AF4 exerts its transforming function preferentially in human cells, indicating that questions regarding the MLL-AF4 pathogenesis have to be addressed using ontogenically early human stem cells.

No study to date has explored in vitro or in vivo the oncogenic potential of MLL-AF4 in human CD34+ HSPCs.[18] Here, we have explored, for the first time, the developmental impact of MLL-AF4 on the fate of human neonatal stem cells, which we assume are developmentally close to the "real" target cells in fetal hematopoiesis. We posed the following questions. First, can we elicit enforced expression of MLL-AF4 in HSPCs? Second, does this lead to any in vitro and/or in vivo proliferative or survival advantage as anticipated of a transforming oncogene? And, third, is enforced expression of MLL-AF4 in this cellular context sufficient to generate overt leukemia in vivo?

Successful lentiviral overexpression of MLL-AF4 was achieved in human cord blood (CB)-derived CD34+ HSPCs. Multilineage hematopoietic engraftment, efficiency, and homing to other hematopoietic sites were augmented on enforced expression of MLL-AF4. MLL-AF4 also increased the in vitro clonogenic potential of CB-derived CD34+ progenitors and robustly enhanced their proliferation. Cell cycle and apoptosis analyses suggest that MLL-AF4 conveys a selective proliferation coupled to a survival advantage. However, MLL-AF4 was not sufficient to induce leukemogenesis on its own, indicating that additional hits may be needed to develop leukemia or that CD34+ cells from CB do not constitute the appropriate cellular target for MLL-AF4–mediated ALL.

Methods

CB collection and CD34+ HSPC isolation

Fresh umbilical CB units from healthy newborns were obtained from local hospitals on approval by our local Ethics and Biohazard Board Committee. CB samples were pooled to reduce variability among individual CB units. Mononuclear cells were isolated using Ficoll-Hypaque. After lysing the red cells (StemCell Technologies), CD34+ cells were purified by magnetic bead separation using the human CD34 MicroBead kit and the AutoMACS Pro separator (Miltenyi Biotec) as per the manufacturer's instructions.[15,19] Purity of the CD34+ fraction was assessed by flow cytometry using anti-CD34-phycoerythrin (PE; BD Biosciences), and only CD34+ fractions showing purity more than 90% were used.[20] The CD34− fraction was irradiated (1500 cGy) and used as accessory cells for cotransplantation with transduced CD34+ HSPCs.

Plasmid construction and lentiviral transduction

The MLL-AF4 cDNA, obtained from pMSCV-MLL-AF4 (kindly provided by Dr C. W. So (King's College London, London, United Kingdom) was subcloned into the PmeI site of pRRL-EF1α-PGK-green fluorescent protein (GFP), kindly provided by Prof L. Naldini (ISR, Milano). A truncated MLL exon 10 was fused to AF4 exon 8 to bring it back into frame. The following lentivectors were used: pRRL-GFP (empty vector [EV]) and pRRL-MLL-AF4-GFP (Figure 1A). Vesicular stomatitis virus-G-pseudotyped viral particles were generated on 293T cells by calcium-phosphate transfection and concentrated by ultracentrifugation as described.[21]

Human CD34+ HSPCs (2×10^6 cells) were infected overnight with concentrated viruses in the presence of polybrene (1 μg/mL; Sigma-Aldrich) and the following hematopoietic cytokines: stem cell factor (100 ng/mL), Fms-like tyrosine kinase 3 ligand (100 ng/mL), and IL-3 (10 ng/mL; PeproTech). The following day, the viral supernatant was removed and transduced CD34+ HSPCs were washed and maintained in culture for downstream experiments.[22]

Mice transplantation

NOD/LtSz-scid interleukin-2Rγ$^{-/-}$ mice (NSG)[23] were housed under sterile conditions. The Animal Care Committee of the University of Granada approved all mouse protocols. Mice at 8 to 12 weeks of age were sublethaly irradiated (2.75 cGy) 6 to 16 hours before transplantation. The mice were anesthetized with isoflurane inhalation, and intrabone marrow transplantation (IBMT) was performed as described in detail.[23,24] A total of 3.5×10^5 GFP- or MLL-AF4-infected CD34+ HSPCs along with 5×10^4 irradiated accessory cells were transplanted in a volume of 30 to 40 μL. For pain relief, 1 mg buprenorphine was administered immediately after transplantation.[23,25]

Analysis of engraftment

Mice were killed 12 to 16 weeks after transplantation. Bone marrow (BM) was flushed into the following pools: injected tibia and a pool from the contralateral tibia (CL), femur, and iliac crests. Spleen, liver, and peripheral blood (PB) were also collected and analyzed for human chimerism. Mice health was monitored throughout the entire experiment. Cells from BM, spleen, and PB were stained with anti–HLA-ABC-fluorescein isothiocyanate (BD Biosciences PharMingen) and anti–CD45-peridinin chlorophyll protein-Cy5 (BD Biosciences) to analyze human chimerism by flow cytometry. All engrafted mice were assessed for multilineage analysis using anti–CD33-PE for myeloid cells, anti–CD19-allophycocyanin for B cells, and anti–CD34-PE for immature hematopoietic cells (all from Miltenyi Biotec). Within the CD19+ cell subset, the proportion of CD34+ and CD10+ cells (anti–CD10-PE; Miltenyi Biotec) was analyzed to distinguish between pro-B and pre-B cells.

Analysis of PB hematologic counts and histology of spleen and liver

Hematologic parameters, including absolute counts of white blood cells (WBCs), red blood cells, platelets, and hemoglobin levels, as well as the WBC differential composition were determined in PB the day before killing the animals using the hematologic analyzer Sysmex SX-800i (Roche Diagnostics).[26] Spleen and liver were visualized macroscopically, measured, and weighted. Half of the spleen was used for flow cytometry, and the other half and the liver were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm sections and stained with hematoxylin and eosin for histology analysis.[25,27,28]

In vitro liquid culture, cell cycle, and apoptosis analyses of sorted CD34+ cells

For liquid culture, infected CD34+ cells were sorted (FACSAria, BD Biosciences) based on GFP expression. Purified GFP+ CD34+ HSPCs from both EV- and MLL-AF4-infected cultures were allowed to expand over a 60-day period. To determine the growth kinetics of EV- and MLL-AF4–expressing CD34+ HSPCs, cells were counted twice a week and replated at a density of 1×10^5 cells/cm^2. Cumulative population doublings were calculated at each passage as described.[28]

For cell cycle and apoptosis analyses, EV- and MLL-AF4–infected CD34+ HSPCs were stained with 10μM 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) for 20 minutes. Cells were then sorted based on GFP expression. Purified GFP+ (transduced) and GFP− (nontransduced) CD34+ HSPCs from both EV- and MLL-AF4–infected cultures were fixed in 70% ice-cold ethanol and stored at −20°C. Subsequently, cells were incubated with 2M HCl to depurinate the DNA. Cells were washed with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.2% Tween-20 and incubated with 2 μL of anti–BrdU-fluorescein isothiocyanate (BD Biosciences) for 30 minutes. Cells were finally suspended in propidium iodide (PI) buffer containing 5 μg of PI and 100 μg/mL of RNAase. BrdU staining and cell cycle distribution were analyzed on a FACSCanto-II cytometer using the FACSDiva software (BD Biosciences) to discriminate

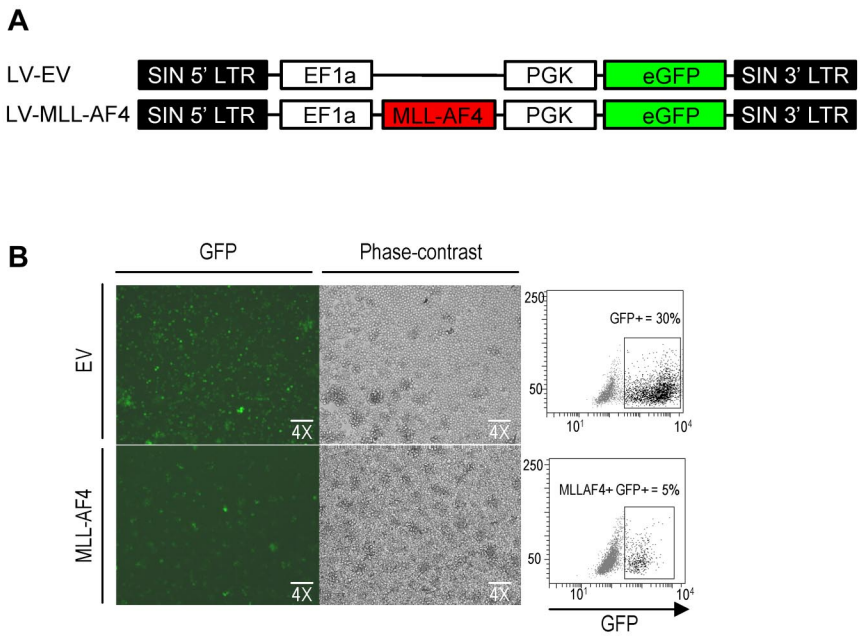


Figure 1. Enforced expression of MLL-AF4 in CD34$^+$ HSPCs. (A) Schematic representation of the lentiviral vectors used. (B) Fluorescence (right) and phase-contrast (left) images of EV-transduced (top) and MLL-AF4-transduced (bottom) CD34$^+$ HSPCs. On the right, images showing the percentage of GFP$^+$ cells in EV-transduced (top) and in MLL-AF4-transduced (bottom) CD34$^+$ HSPCs. (C) Representative genomic PCR and RT-PCR confirming proviral integration (gDNA) and expression of MLL-AF4 transcript (mRNA) in transduced CD34$^+$ HSPCs (n = 3).

among apoptotic cells (Sub-2n), quiescent cells (G_0/G_1), cycling cells (S-phase, BrdU$^+$), and G_2/M cells.[28,29]

Expression of Bcl-2 and Bcl-xL in sorted GFP$^+$ and GFP$^-$ CD34$^+$ HSPCs from both EV- and MLL-AF4–infected cultures was assessed by flow cytometry as described.[30] Briefly, before Bcl-2 (PE-conjugated monoclonal antibody from BD Biosciences) or Bcl-xL (PE-conjugated monoclonal antibody from Santa Cruz Biotechnology) staining, cells were fixed and permeabilized using the Fix&Perm reagent (Caltag Laboratories) as described.[31,32] An isotype-matched nonspecific mouse immunoglobulin was analyzed as negative control.

Cell death/apoptosis analysis by annexin-V and 7-AAD staining

The apoptotic status of EV- and MLL-AF4–transduced CD34$^+$ HSPCs was also assessed using annexin-V apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions.[29,33] Briefly, the cells were washed twice with cold phosphate-buffered saline and stained with annexin-V-PE and 7-amino-actinomycin D (7-AAD). The transduced apoptotic cells were detected by gating the annexin V$^+$ fraction.

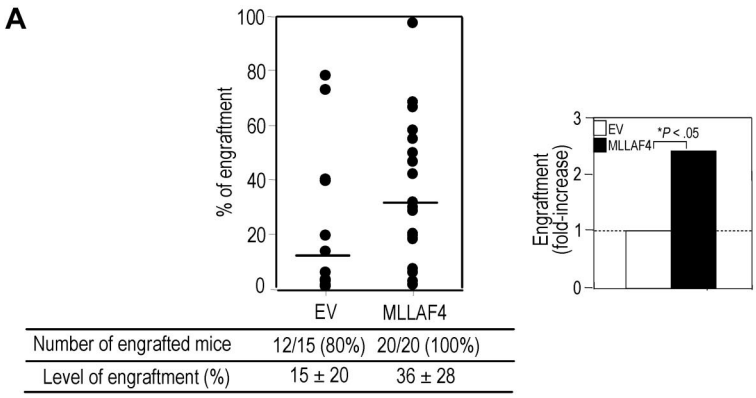
Gene expression analysis by quantitative PCR arrays

We used 2 different RT^2 Profiler PCR Arrays (SA Biosciences, QIAGEN) to analyze the expression levels of 84 genes involved in regulation of apoptosis (Human Apoptosis array, PAHS-012), and 84 genes regulating cell cycle and DNA damage response (Human Cell Cycle array, PAHS-020) in purified GFP- and MLL-AF4–expressing CD34$^+$ HSPCs. A total of 10^5 fluorescence-activated cell sorter (FACS)–sorted GFP or MLL-AF4–transduced CD34$^+$ HSPCs were harvested at day 8 of in vitro culture for total RNA extraction using RNeasy Plus kit (QIAGEN). RNA quality (RIN $\geq$ 9.0) was assessed using a Bioanalyzer (Agilent Technologies) before cDNA synthesis using 200 ng total RNA per sample. The resulting cDNA was used as template to perform quantitative polymerase chain reaction (PCR) analysis.

The raw threshold cycle data were analyzed using SA Biosciences web-based tool (www.sabiosciences.com/pcrarraydataanalysis.php), after which we discarded genes categorized as "C" for their low-quality quantitative PCR data, as recommended by the manufacturer. Genes showing a change in expression more than 1.5-fold were considered differentially expressed between the 2 groups. For the apoptosis array, we grouped the genes in either antiapoptotic or proapoptotic based on published data. For the cell cycle array, we used SA Biosciences functional gene groupings and added 2 extra groups for "DNA damage sensing" and "DNA damage repair," based on published data (see tables of genes with annotations in supplemental Tables 1 and 2, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Several genes, such as *BCL2*, *TP53*, and *ABL1*, appear in both types of arrays because of their pleiotropic function.

CFU assay

Primary human clonogenic progenitor assays were performed by plating 2000 GFP- or MLL-AF4–transduced CD34$^+$ HSPCs into methylcellulose H4434 (StemCell Technologies) containing human growth factors: 50 ng/mL stem cell factor, 10 ng/mL granulocyte-macrophage colony-stimulating

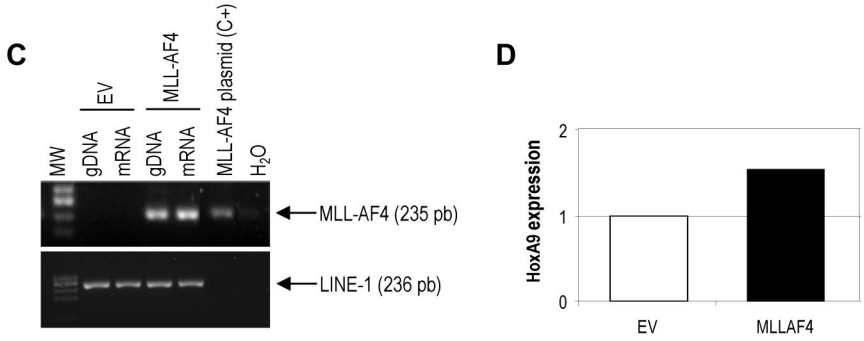


	EV	MLLAF4
Number of engrafted mice	12/15 (80%)	20/20 (100%)
Level of engraftment (%)	15 ± 20	36 ± 28

Figure 2. Levels of human engraftment of MLL-AF4–expressing CD34+ HSPCs into irradiated transplanted NSG mice. (A) Long-term hematopoietic reconstitution of NSG mice (n = 35) after IBMT of EV- or MLL-AF4–expressing CD34+ HSPCs. Engraftment was considered as percentage of hCD45+ more than 1% in the mouse BM. Each dot represents an individual mouse, and the horizontal line indicates the mean of each experimental cohort. The right panel represents the fold increase in the engraftment observed between both groups. (B) Levels of human chimerism in the distinct hematopoietic tissues analyzed. The consistent higher engraftment levels in animals transplanted with MLL-AF4–expressing CD34+ HSPCs, regardless of the tissue analyzed. (C) Representative genomic nested PCR and RT-PCR confirming proviral integration (gDNA) and stable long-term expression of MLL-AF4 transcript (mRNA) in mice killed 12 weeks on IBMT. (D) HoxA9 RT-PCR of CFUs from EV- and MLL-AF4–infected CD34+ HSPCs.

B

	IT	CL	Spleen	PB
EV (% engraftment)	10.6±15.7	13.2±21	4±7.4	3.9±6.7
MLLAF4 (% engraftment)	32.7±26.1	25.5±30.4	21.1±27.6	16.3±21.7
MLLAF4 / EV engraftment ratio	3.1	1.9	5.3	4.2



factor, 10 ng/mL IL-3, and 3 U/mL erythropoietin. Colonies were counted and scored at day 14 of the colony-forming unit (CFU) assay using standard morphologic criteria.[34,35] For secondary replating, all the CFU colonies from each experimental condition were harvested from the methylcellulose and a single-cell suspension was achieved and replated as detailed in "CFU assay."

PCR and RT-PCR

Genomic DNA was isolated from the BM of transplanted mice, CFUs and cells in liquid culture using the DNeasy Blood & Tissue kit (QIAGEN). RNA was isolated with the RNeasy kit (QIAGEN) and treated with DNaseI (Invitrogen). The first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). PCR for GFP and MLL-AF4 was done using 100 ng of template (gDNA or cDNA) and the following primers: MLL-AF4, forward, 5′-CAGAGCAAACA-GAAAAAAGTG-3′ and reverse, 5′-GTTCTGGAAGGGACTGTGGA-3′; and GFP, forward, 5′-GCACCATCTTCTTCAAGGACGAC-3′ and reverse, 5′-TCTTTGCTCAGGGCGGACTG. PCR conditions were 94°C for 3 minutes followed by 35 cycles of 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 45 seconds and a final extension of 72°C for 10 minutes.[36] The resulting amplicons (382 bp for MLL-AF4 and 342 bp for GFP) were resolved on 2% agarose gels. PCR for GFP and MLL-AF4 from BM of transplanted mice was done using a nested PCR approach.[37] These primers were used for the first round of amplification. For the second round of amplification, the following primers were used: MLL-AF4, forward, 5′-GTCAATAAGCAGGAGAATGCAG-3′ and reverse, 5′-CACTGTCACTGTCCTCACTGTC-3′; and GFP, forward, 5′-GAACCG-CATCGAGCTGAAGGGC-3′ and reverse, 5′-GGTTGTCGGGCAGCAG-CACGGG-3′. The resulting amplicons were 235 bp for MLL-AF4 and 236 bp for GFP. LINE-1 or GAPDH was used as housekeeping genes.[36] The following primers were used: Line-1, forward, 5′-GCTGGATAT-GAAATTCTGGGTTGA-3′ and reverse, 5′-AGGAAATACAGAGAACGC-CACAA-3′; GAPDH, forward, 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse, 5′-GAAGATGGTGATGGGATTTC-3′. For human HoxA9 expression, the following primers (forward, 5′-AAGACCGAGCAAAAGAC-GAG-3′ and reverse, 5′-GGGTGAGAGAAGGGAGAAGG-3′) and quantitative reverse-transcribed (RT)-PCR conditions (95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 60 seconds, and 72°C for 60 seconds) were used.

For comparative expression of MLL-AF4 in CB-transduced CD34+ HSPCs, patient primary samples and MLL-rearranged leukemic lines, the following primers (MLL-AF4, forward, 5′-CAGGTCCAGAGCAGAG-CAAAC-3′ and reverse, 5′-GAGCACTTGGAGGTGCAGATG-3′) and quantitative RT-PCR conditions (95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds) were used.

Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using either paired or unpaired Student *t* test, as corresponding. Statistical significance was defined as a P value $< .05$.

Results

Lentiviral transduction of MLL-AF4 into CB CD34$^+$ HSPCs

No study has reported so far an efficient enforced expression of MLL-AF4 in human primary stem cells. Here, purified human CB-CD34$^+$ HSPCs were transduced with an empty (EV; 9.2 kb) or a MLL-AF4–expressing lentiviral vector (MLL-AF4; 15.5 kb; Figure 1A).[21] Gene transfer efficiencies ranged from 5% to 30% as determined by FACS for eGFP expression (confirmed by fluorescence microscopy) 48 hours after infection (Figure 1B). Proviral integration and transgene expression were confirmed by genomic PCR and RT-PCR, respectively, 5 days after the viral infection (Figure 1C). Importantly, in line with the variations in MLL-AF4 expression observed among patient primary samples, the expression of MLL-AF4 also varies slightly between independent CB transductions (supplemental Figure 1A-B). Overall, however, the ectopic expression of MLL-AF4 is slightly higher in CB-CD34$^+$ HSPCs than in patient primary samples (supplemental Figure 1A). Thus, our ability to overexpress this large MLL-AF4 fusion in difficult-to-transduce hCD34$^+$ HSPCs encouraged us to analyze further the in vivo and in vitro impact of MLL-AF4 in CB-CD34$^+$ HSPCs.

Enforced expression of MLL-AF4 augments multilineage hematopoietic engraftment and facilitates homing of CD34$^+$ HSPCs

To explore the effect of MLL-AF4 in regulating human HSPCs, EV- or MLL-AF4–infected CB-CD34$^+$ cells were transplanted via IBMT into sublethally irradiated NSG mice.[23] A total of 35 irradiated NSG mice were transplanted with 350K EV-infected CD34$^+$ cells (n = 15) or MLL-AF4–infected CD34$^+$ cells (n = 20). Animals were monitored for 12 to 16 weeks and, in the absence of signs of disease, they were killed. Human chimerism was defined as more than 1% of hCD45$^+$ HLA-ABC$^+$ cells in the BM. As shown in Figure 2A, EV-CD34$^+$ cells generated a human graft in 80% of the mice. The average level of engraftment was 15% ± 20% (range, 1%-73%). However, MLL-AF4–infected CD34$^+$ cells engrafted in 100% of the mice, and the level of chimerism was 2.7-fold higher compared with EV-transplanted NSG (36% ± 28%; range, 1%-98%; Figure 2A right panel). These data were reproduced when EV- or MLL-AF4–infected CB-CD34$^+$ cells were transplanted via IBMT into nonirradiated mice (supplemental Figure 2).

Direct injection of cells into the tibia provides the opportunity to assess migration of transplanted CD34$^+$ cells in vivo. Accordingly, the in vivo migration ability of transplanted EV- or MLL-AF4-CD34$^+$ cells was assessed by analyzing the level of chimerism in the injected tibiae, noninjected BM (CL), spleen, and PB. Regardless of the ectopic expression of MLL-AF4, CD34$^+$ HSPCs were capable of migrating to and colonizing other hematopoietic sites in all the animals (Figure 2B). However, the levels of chimerism in the CL, spleen, and PB were significantly higher in NSG mice transplanted with MLL-AF4–transduced CD34$^+$ cells. Overall, the hematopoietic engraftment in these sites was between 2 and 5.3 times higher in NSG mice transplanted with MLL-AF4- versus EV-CD34$^+$ HSPCs. These data indicate that enforced expression of MLL-AF4 enhances multilineage hematopoietic engraftment but also facilitates migration.

To ensure that the observed effects in engraftment and migration are linked to MLL-AF4 expression, proviral integration and transgene expression were successfully confirmed by genomic PCR and RT-PCR in BM cells from a cohort of engrafted mice at the time of animal death and tissue removal (Figure 2C). As MLL fusions are positive regulators of homeobox gene expression,[3,38-40] we then analyzed by quantitative RT-PCR the expression of HoxA9 in EV- and MLL-AF4–transduced 14-day-old CFUs. As shown in Figure 2D, the HoxA9 expression was 1.5 times up-regulated in MLL-AF4 progenitors compared with EV progenitors, confirming successful expression of MLL-AF4 in difficult-to-transduce CD34$^+$ HSPCs and suggesting that the enforced expression of MLL-AF4 seems to regulate Hox gene expression.

We next characterized by FACS the composition of the human graft (Figure 3A). Multilineage repopulation was consistently observed in all engrafted mice, regardless of the expression of MLL-AF4 and the tissue analyzed (Figure 3B). The graft was composed predominantly of CD45$^+$CD19$^+$ B-lymphoid cells (60%-80%), followed by CD45$^+$CD33$^+$ myeloid cells (5%-20%) and CD45$^+$CD34$^+$ immature cells (5%-20%; Figure 3A-B). Because MLL-AF4 expression in infants is associated with a pro-B ALL, we analyzed further the phenotype of the CD45$^+$CD19$^+$ B-cell population.[26,41] As shown in Figure 3B, the early B-cell markers CD10 and CD34 were coexpressed by approximately 80% and approximately 50%, respectively, of the B-cell graft indicative of the coexistence of both normal pre-B cells (CD19$^+$CD10$^+$CD34$^+$) and differentiated B cells (CD19$^+$CD10$^-$CD34$^-$). Leukemic blasts in infant MLL-AF4$^+$ ALL are characterized by CD10$^-$CD19$^+$ pro-B phenotype, whereas more than 80% of the BM CD19$^+$ B cells in our lentiviral-based MLL-AF4 xenograft model display a CD10$^+$ pre-B phenotype, suggesting that enforced expression of MLL-AF4 enhances the hematopoietic repopulating cell function of CB-CD34$^+$ HSPCs without blocking or skewing normal developmental stem cell fate.

Expression of MLL-AF4 in CB-CD34$^+$ HSPC is not sufficient for leukemogenesis in vivo

Mouse models have been developed that recapitulate *MLL*-rearranged AML.[13,18,42] However, development of models that faithfully recapitulate MLL-AF4–mediated ALL has proven very difficult, and human MLL-AF4 disease models do not exist. Even though MLL-AF4 expression enhanced in vivo repopulating function, none of the animals transplanted with EV- or MLL-AF4–expressing CD34$^+$ HSPCs showed any sign of disease over a 16-week period. As shown in Figure 3, MLL-AF4 expression did not impair normal developmental stem cell fate. Furthermore, hematopoietic parameters determined at the time of animal killing showed no signs of disease (Figure 4A). No differences were observed between EV- and MLL-AF4–transplanted mice for WBCs, red blood cell, platelet counts, hemoglobin levels, or differential WBC counts (Figure 4A). Consistent with these data, neither splenomegaly nor hepatomegaly was observed in mice transplanted with MLL-AF4–expressing cells (Figure 4B). Pathologic analysis revealed no differences in the cellular composition of EV versus MLL-AF4 livers and spleens (Figure 4C). Thus, MLL-AF4 failed to initiate leukemogenesis on its own.

Enforced expression of MLL-AF4 increased the in vitro clonogenic potential of CD34$^+$ HSPCs

Recently, the in vitro transformation of human mobilized PB-derived CD34$^+$ cells by AML fusion oncogenes, such as PML-RARA, AML1-ETO, MLL-AF9, or NUP98-HoxA9,[43] has been reported. Thus, the human hematopoietic progenitor function was examined by quantitative and qualitative analysis of the CFU

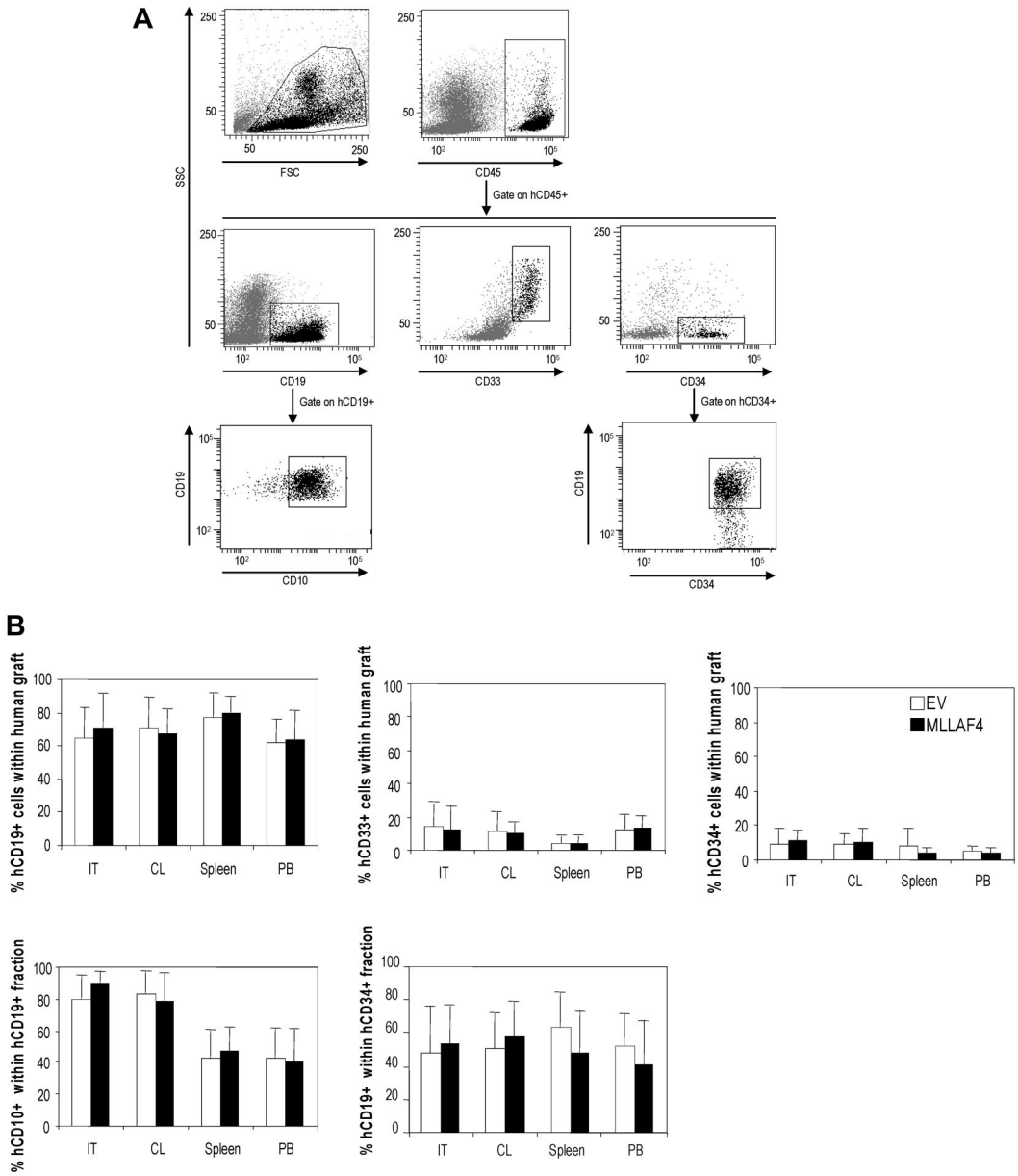


Figure 3. Flow cytometric analysis confirming multilineage engraftment. (A) Representative flow cytometry. The human graft is identified as the CD45⁺ fraction. The CD45⁺ human graft is composed of B-lymphoid cells (CD19⁺), myeloid cells (CD33⁺), and immature cells (CD34⁺). More than 50% of the CD19⁺ B-lymphoid population coexpress CD10 and CD34, indicative of a pre-B cell phenotype. (B) Multilineage and multiorgan human chimerism in the injected tibia, CL, spleen, and PB demonstrating migration of human cells from the injected tibia. No differences in the graft composition between the EV- and MLL-AF4–expressing CD34⁺ HSPCs were found (n = 35).

capacity of EV- and MLL-AF4–transduced CD34⁺ HSPCs. CD34⁺ cultures containing MLL-AF4–expressing HSPCs displayed a slightly higher clonogenic potential than cultures transduced with the EV (157 ± 65 vs 110 ± 50; $P < .05$; Figure 5A). Scoring of primary CFUs revealed no significant differences in CFU types between EV- and MLL-AF4–transduced progenitors (Figure 5A). Importantly, primary CFU cultures were replated and the CFU potential almost disappeared in the secondary read-outs, regardless of the experimental condition, indicating that MLL-AF4 does not confer stable replating efficiency (EV or MLL-AF4, Figure 5A). To confirm survival and hematopoietic progenitor function of transgenic CD34⁺ progenitors (either GFP- or MLL-AF4–expressing), transgene expression was successfully confirmed by RT-PCR in the CFU cultures after 14 days (Figure 5B). Together, the pronounced loss of CFU potential after the secondary replating coupled to similar CFU types scored in assays plated with either EV- or MLL-AF4-expressing CD34⁺ progenitors suggests (in line with the in vivo data) that the enforced expression of MLL-AF4 enhances the in vitro clonogenic potential without impairing normal developmental stem cell fate.

MLL-AF4 conveys selective proliferation and survival advantage to CD34⁺ HSPCs

We next attempted to gain a better understanding about the cellular and molecular mechanisms underlying the MLL-AF4–mediated increase of the in vitro clonogenic potential and the in vivo repopulating cell function. We analyzed the in vitro culture homeostasis of sorted GFP⁺ CD34⁺ HSPCs from both EV- and MLL-AF4–infected cell cultures. MLL-AF4–expressing CD34⁺ HSPCs grew faster and could be maintained significantly longer than GFP⁻CD34⁺ HSPCs

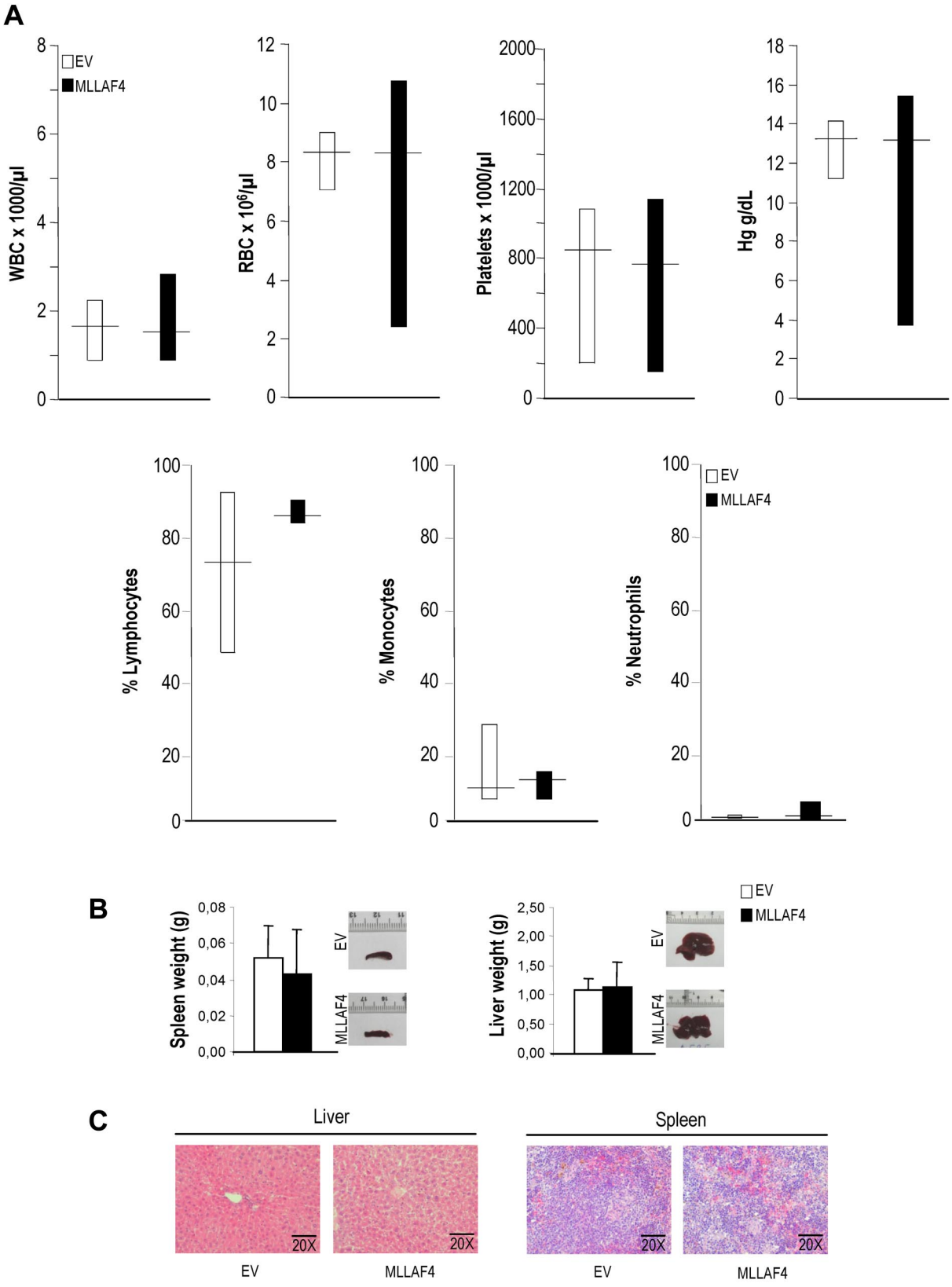


Figure 4. Analysis of hematologic parameters in PB and examination of the liver and spleen reveal no signs of leukemia in mice reconstituted with MLL-AF4–expressing CD34⁺ HSPCs. (A) Top panels: Absolute counts of WBCs, red blood cell, platelets, and hemoglobin in EV versus MLL-AF4 mice groups (n = 35). Bottom panels: Relative percentage of lymphocytes, monocytes, and neutrophils in the PB of EV and MLL-AF4–transplanted mice. The horizontal line indicates the median of each experimental group. (B) Weight and representative images of spleen and liver showing lack of splenomegaly and hepatomegaly in mice transplanted with MLL-AF4–expressing CD34⁺ HSPCs. (C) Hematoxylin and eosin staining (20×), displaying identical cellular composition and morphology of the spleens and livers from EV and MLL-AF4–transplanted mice (n = 35). Pictures were captured (20×/0.50 objective) with an Axiocam MRM digital camera (Zeiss) attached to an AxioImager AI microscope (Zeiss).

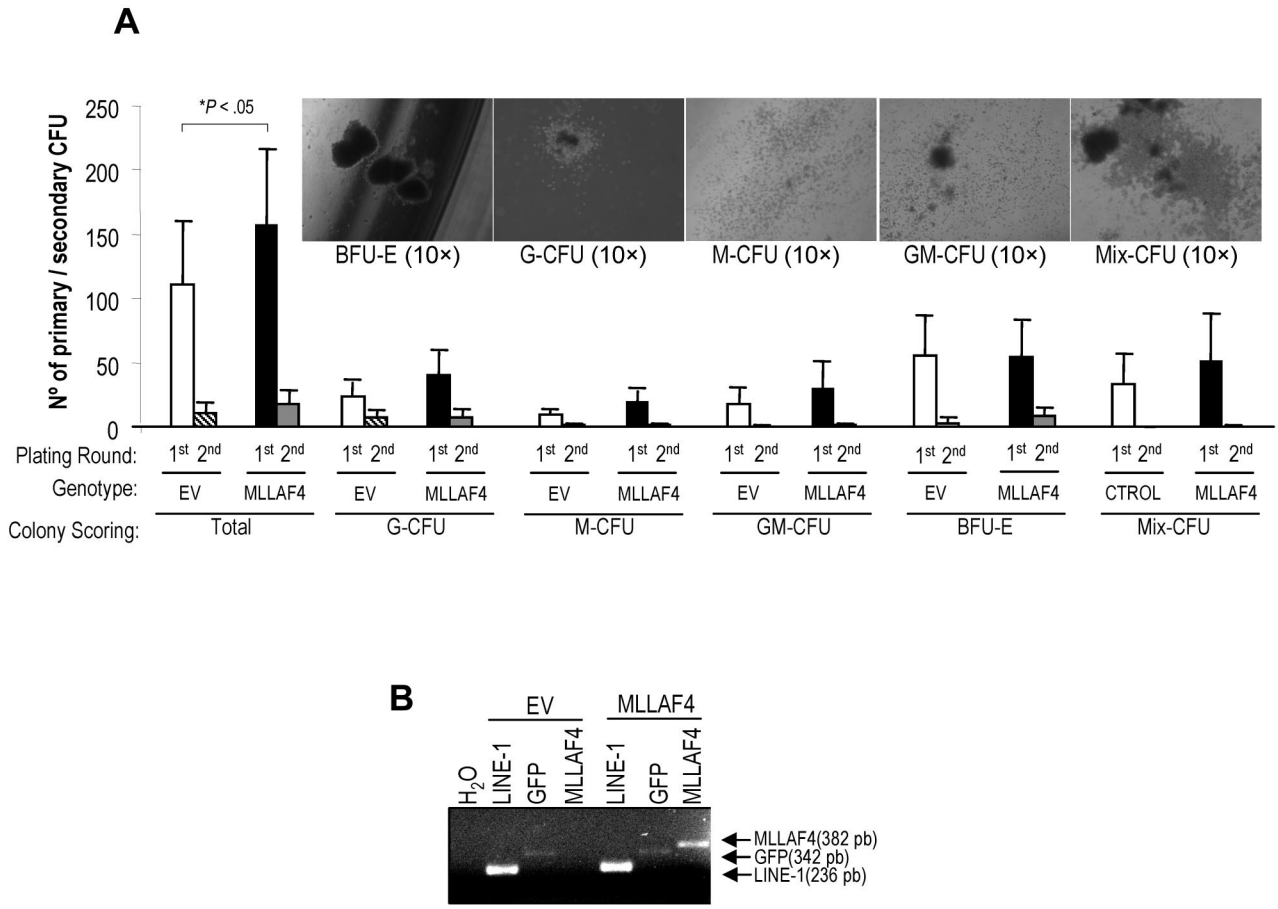


Figure 5. MLL-AF4 expression promotes progenitor clonogenic potential. (A) Primary and secondary CFU (10×) platings showing a higher overall CFU potential (*$P < .05$) in MLL-AF4- versus EV-transduced CD34+ cells (n = 4). The CFU types were not different between EV- and MLL-AF4–transduced CD34+ HSPCs. Error bars represent the SEM from 4 independent experiments. (Inset) Representative phase-contrast pictures of different CFU subtypes, including granulocyte, macrophage, granulocyte-macrophage, mix, and erythroid colonies. Pictures were captured (10×/0.25 PhP objective) with an inverter microscope Olympus CKX41. For analysis and image processing, a Color View Soft Images System software (Olympus V-TV1X-2) was employed. (B) Representative RT-PCR showing the presence of GFP and MLL-AF4 transcripts in CFUs plucked from EV- or MLL-AF4–transduced CD34+ CFU cultures. To ensure the availability of amplifiable template, cDNA from CFUs was subjected to Line-1 RT-PCR.

(Figure 6A). Indeed, the MLL-AF4-expressing CD34+ HSPCs continued to grow after 60 days in culture, whereas GFP-expressing CD34+ HSPCs slowed down their proliferation approximately 20 days earlier, and their growth was exhausted by day 50. These CD34+ cultures lost CD34 expression after 10 to 15 days in culture regardless of the MLL-AF4 expression (data not shown). Importantly, in line with recently published single nucleotide polymorphism experiments on t(4;11) patients,[44,45] karyotype and comparative genomic hybridization arrays analyses of these expanded cell cultures revealed neither chromosomal alterations (numerical or structural) nor minor genomic changes (amplifications or deletions) after 50 days of liquid culture (supplemental Figure 3). Moreover, neither GFP- nor MLL-AF4–expressing cells harvested after 55 days in in vitro culture were capable to engraft or initiate a leukemogenesis process in NSG mice (data not shown), indicating that they are nontransformed MLL-AF4–expressing cells. These data correlate with the observed MLL-AF4–mediated increase of in vitro clonogenic potential and in vivo repopulating cell function as well as with the MLL-AF4 inability to initiate a leukemogenesis process on its own.

We then analyzed cell cycle, proliferation, and apoptosis on sorted GFP+ (transduced) and GFP− (nontransduced) CD34+ HSPCs from both EV- and MLL-AF4–infected cell cultures. As expected, BrdU and PI costaining analysis (Figure 6B) of GFP+ and GFP− cell fractions purified from EV-infected CD34+ cultures revealed no differences in either apoptosis (Sub-2n fraction) or cell cycle distribution (Figure 6C top panel). However, significant differences in apoptosis (Sub-2n fraction) and cell cycle were observed between GFP+ and GFP− cell fractions purified from MLL-AF4–infected CD34+ cultures. MLL-AF4 expression promotes entry in S-phase (24% vs 9%; Figure 6C bottom panel) and diminishes the Sub-2n fraction (19% vs 41%; Figure 6C bottom panel). Apoptosis was also assessed by annexin-V and 7-AAD costaining and revealed lower levels of annexin-V+ apoptotic cells (14.7% vs 34%) in MLL-AF4–transduced than in EV-transduced CD34+ cells (Figure 6D). It was reported that leukemias and cell lines with t(4;11) have abundant levels of the antiapoptotic protein Bcl-2, providing an intrinsic antiapoptotic cell survival mechanism.[46,47] Similarly, it has been very recently demonstrated that the antiapoptotic protein Bcl-xL needs to be stabilized for survival of AML1-ETO harboring cells. We therefore analyzed the expression of Bcl-2 and Bcl-xL on sorted GFP+ and GFP− CD34+ HSPCs from both EV- and MLL-AF4–infected CD34+ cultures and found that the expression of both Bcl-2 and Bcl-xL was highly upregulated in MLL-AF4–expresing CD34+ cells (Figure 6E). These data indicate that MLL-AF4 confers a selective proliferation coupled to a survival advantage when overexpressed on CB-CD34+ cells, explaining, at least in part, the enhanced in vitro

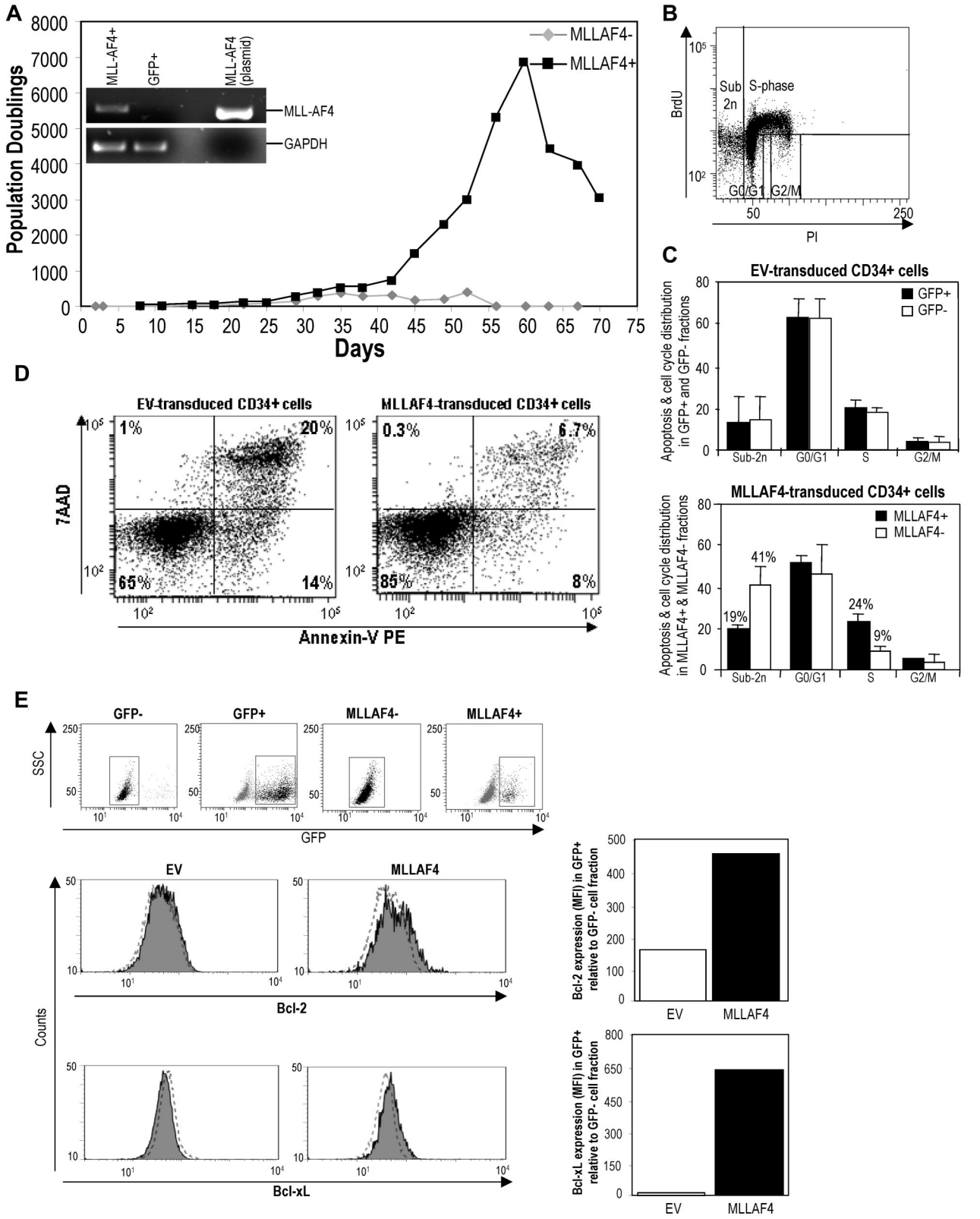


Figure 6. MLL-AF4 conveys a selective proliferation coupled to a survival advantage to CD34⁺ HSPCs. (A) In vitro cell growth, measured as cumulative population doublings, of purified GFP- versus MLL-AF4–expressing CD34⁺ HSPCs (n = 2). (Inset) RT-PCR confirming specific expression of the MLL-AF4 transcript only in the purified MLL-AF4⁺ CD34⁺ HSPCs. (B) Representative BrdU versus PI flow cytometry identifying the apoptotic (Sub-2n) cell fraction and the cells at different stages of the cell cycle: G_0/G_1 phase, S-phase, and G_2/M phase (n = 3). (C) Apoptosis and cell cycle distribution in GFP⁺ (transduced) and GFP⁻ (nontransduced) cell fractions sorted from EV-transduced CD34⁺ HSPCs (left panel) and MLL-AF4–transduced CD34⁺ HSPCs (right panel) (n = 3). (D) Representative flow cytometry panels displaying the apoptosis/cell death of EV- and MLL-AF4-transduced CD34⁺ HSPCs analyzed by 7-AAD and annexin-V costaining. (E) FACS mean fluorescence intensity (MFI) expression levels of the antiapoptotic proteins Bcl-2 and Bcl-xL in GFP⁺ cell fractions sorted from EV-transduced and MLL-AF4–transduced CD34⁺ HSPCs relative to GFP⁻ cell fractions.

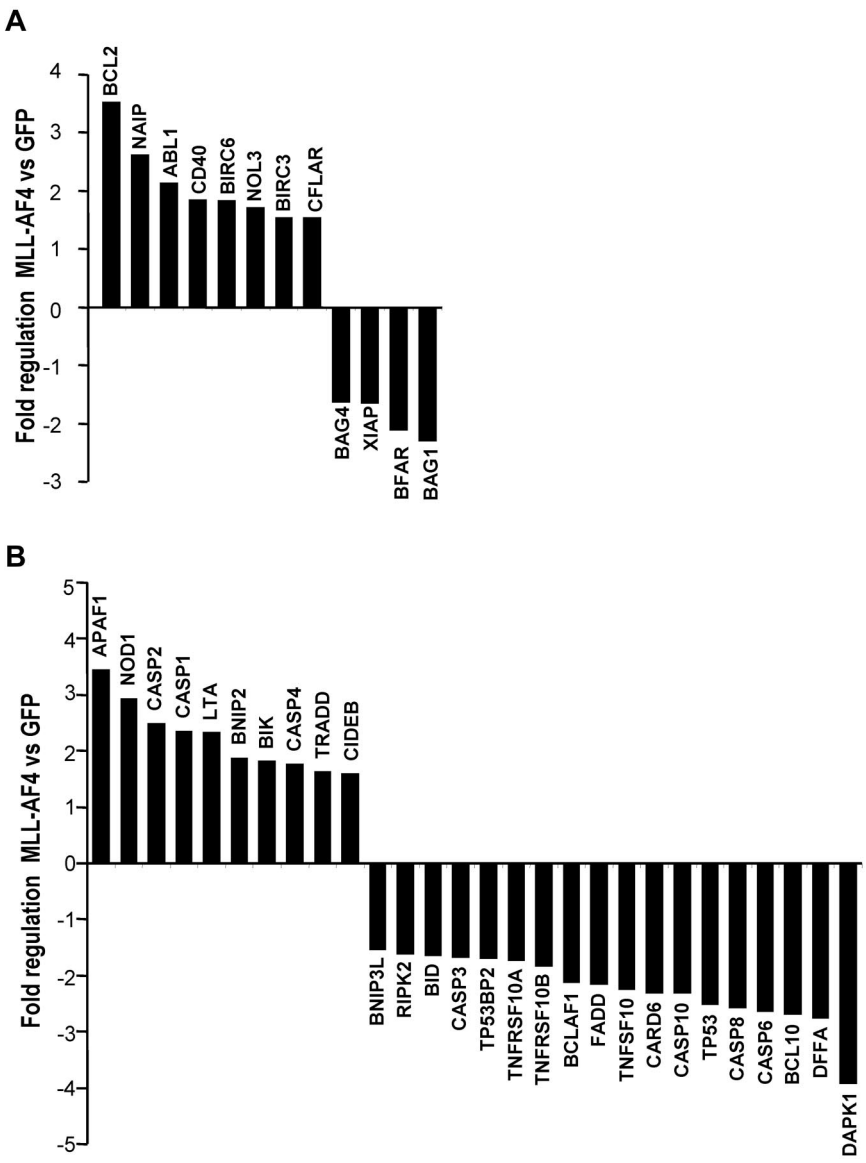


Figure 7. Analysis of differential gene expression profiling between GFP- and MLL-AF4–expressing CD34+ HSPCs. Changes (fold ≥ 1.5) in gene expression in MLL-AF4–transduced CD34+ HSPCs compared with GFP-transduced CD34+ HSPCs were assessed by quantitative PCR arrays. Graphs show fold regulation of antiapoptotic genes (A) and proapoptotic genes (B). Supplemental Table 1 contains a list of all genes analyzed and annotations from the literature supporting the antiapoptotic or proapoptotic function for each gene.

proliferation and clonogenic capacity and in vivo repopulating cell function.

To further analyze the molecular mechanisms behind the increased proliferation and survival of MLL-AF4–expressing CD34+ HSPCs, we used quantitative PCR arrays to determine the expression level of 168 genes involved in the regulation of apoptosis, cell cycle, and DNA damage response. MLL-AF4–expressing CD34+ HSPCs displayed an overall increase in the expression of antiapoptotic genes (8 of the 12 differentially expressed antiapoptotic genes, including Bcl-2; Figure 7A), although there is a decrease in the expression of proapoptotic genes (18 of the 28 differentially expressed proapoptotic genes, including p53; Figure 7B). Overall, the decrease in *p53* and proapoptotic genes coupled to the increase in antiapoptotic genes, such as *Bcl-2* and *Bcl-xL*, may protect cells from apoptosis, contributing to the enhanced proliferation of these nontransformed MLL-AF4–expressing CD34+ cells. Interestingly, MLL-AF4–expressing CD34+ HSPCs differentially express a set of genes involved in the DNA damage response, showing an increased tendency toward genes involved in DNA damage sensing (5 of the 7 differentially expressed genes associated with DNA damage sensing) and a slight decreased tendency toward genes involved in DNA damage repair (supplemental Figures 4, 5).

Discussion

Numerous studies have sought to recapitulate human infant pro-B ALL harboring MLL-AF4 fusion using mouse HSPCs.[10-12,14] Unfortunately, however, in vivo leukemias do not faithfully recapitulate the actual human disease, and the resultant phenotype/latency differs significantly from that seen in infant ALL.[10-12,14] There are several potential reasons for this, including: (1) inappropriate cell type is targeted; (2) expression levels of the fusion gene may not be physiologic[48]; (3) murine systems do not provide appropriate etiologic content for essential secondary mutations[16]; (4) the reciprocal AF4-MLL fusion encoded protein may be necessary[14]; and (5) MLL-AF4–transforming ability is dependent on a human cell context. The recent success in modeling the initiation and progression of acute leukemia mediated by the ectopic expression of MLL-ENL and MLL-AF9 in human CB-derived HSPCs[18] provided a precedent proof of principle for examining the transforming function of

MLL-AF4 in a more relevant target cell with respect to species and developmental status.

We therefore explored the developmental impact of MLL-AF4 in human primary HSPCs from CB because they constitute the postnatal/neonatal stem/progenitor cells ontogenically closest to difficult-to-access prenatal embryonic or fetal stem cells. To circumvent the high expression levels associated with long terminal repeat-driven expression, a long terminal repeat-mutated lentiviral system in which the MLL-AF4 expression is constitutively driven by the EF1α promoter was used. No study has reported so far an efficient enforced expression of MLL-AF4 cDNA in human primary stem cells, possibly because of its large size, which limits its packaging, but we have successfully overexpressed MLL-AF4 in CB-derived $CD34^+$ HSPCs.

Expression of MLL-AF4 had a phenotypic and functional impact. Cell cycle and apoptosis analyses performed on both transduced and nontransduced $CD34^+$ cells suggest that MLL-AF4 confers a selective proliferation coupled to a survival advantage. The lower levels of apoptosis observed in MLL-AF4–expressing $CD34^+$ cells was supported by the higher expression level of Bcl-2 and Bcl-xL antiapoptotic proteins previously reported to be accumulated in MLL-$AF4^+$ leukemias and MLL-$AF4^+$ cell lines, thus providing a potential intrinsic cell survival and proliferation advantage mechanism responsible for the enhanced growth of MLL-AF4–expressing cells in vitro.[46,47] This interpretation is further supported by the gene expression profiles obtained by quantitative PCR arrays, which showed an increase in the expression of antiapoptotic genes in MLL-AF4–expressing $CD34^+$ cells, whereas proapoptotic genes were down-regulated. Moreover, some key genes involved in maintaining genome integrity and DNA damage repair, including *p53*, *PCNA*, *NBN*, *UBA1*, *RBBP8*, and *BCCIP*, were also down-regulated, suggesting that MLL-AF4-expressing $CD34^+$ cells might have limited DNA repair capacity. It is plausible that the expression of these genes may be epigenetically repressed in MLL-AF4–expressing $CD34^+$ HSPCs because whole-genome high-resolution technologies revealed the absence of secondary copy-number alterations or single nucleotide polymorphisms in MLL-$AF4^+$ ALLs.[44,45] MLL has very recently been assigned as a novel effector in the mammalian S-phase checkpoint network, and S-phase checkpoint dysfunction was identified as an underlying mechanism of MLL leukemias.[49] MLL is phosphorylated by ataxia-telangiectasia mutated and Rad3-related in response to genotoxic stress at the S-phase. MLL fusions, however, function as dominant negative mutants that abrogate the ATR-mediated phosphorylation and therefore stabilization of wild-type MLL, compromising the S-phase checkpoint. This molecular mechanism may explain the significant increase of S-phase entry incurred by MLL-AF4 expression in $CD34^+$ HSPCs.

IBMT into immunodeficient mice revealed an enhanced multilineage hematopoietic engraftment level and efficiency as well as homing to other hematopoietic sites on enforced expression of MLL-AF4. Phenotypic characterization of the human graft revealed no differences regardless of the expression of MLL-AF4 and the tissue analyzed. The graft was composed predominantly of B-lymphoid cells, followed by myeloid cells and immature cells. Because MLL-AF4 expression in infants is associated with a pro-B ALL, we further analyzed the phenotype of the $CD45^+CD19^+$ B-cell population and found that the early B-cell markers $CD10^+$ and $CD34^+$ were coexpressed by approximately 80% and approximately 50% of the B-cell graft, respectively, indicative of the coexistence of both normal pre-B cells and differentiated B cells. Of note, leukemic blasts in infant MLL-$AF4^+$ ALL are characterized by $CD10^-CD19^+$ pro-B phenotype, whereas more than 80% of the BM $CD19^+$ B cells in our lentiviral-based MLL-AF4 xenograft model display a $CD10^+$ pre-B phenotype, suggesting that enforced expression of MLL-AF4 enhances the hematopoietic repopulating cell function of CB-$CD34^+$ HSPCs without blocking or skewing normal developmental stem cell fate. The lack of phenotypic differences between mice transplanted with untransduced or MLL-AF4–expressing CB-derived $CD34^+$ HSPCs clearly indicates that MLL-AF4 fails to block the differentiation of the distinct hematopoietic lineages. These data contrast with previous work in murine models showing that MLL-AF4 activation in lymphocyte precursors lead to the development or selection of B cells.[12] Targeting of hematopoietic progenitors at different developmental stage may explain, at least in part, these differences. In addition, these contradictory data also confirm a gap in our understanding between mouse and human disease models and should encourage further leukemia modeling studies using human primary cells.[15,50-52]

Hematopoietic parameters determined at the time of animal killing showed no signs of disease. Furthermore, neither splenomegaly nor hepatomegaly was observed in mice transplanted with MLL-AF4–expressing cells, and pathology analysis revealed no differences in the cellular composition of EV versus MLL-AF4 livers and spleens. In contrast with previous reports of modeling, the initiation and progression of acute leukemia mediated by the ectopic expression of MLL-AF9 and MLL-ENL in HSPCs,[18] our data indicate that MLL-AF4 is insufficient for leukemogenesis on its own when overexpressed in CB-derived $CD34^+$ HSPCs. All transplanted mice were maintained alive for 12 to 16 weeks (mean of ~ 100 days), a period of time long enough to develop a mimic of MLL-$AF4^+$ infant pro-B ALL characterized by its very brief latency.

Lentiviral transduction of MLL-AF4 into $CD34^+$ HSPCs also increased the in vitro clonogenic potential of $CD34^+$ progenitors. Interestingly, however, scoring of primary CFUs revealed no significant differences in CFU types between EV- and MLL-AF4–transduced progenitors. In addition, the CFU potential was almost lost in secondary CFU read-outs, suggesting that the enforced expression of MLL-AF4 enhances the in vitro clonogenic potential without impairing normal developmental stem cell fate.

It has been recently demonstrated that the presence of both reciprocal MLL fusion proteins (MLL-AF4 and AF4-MLL) or AF4-MLL alone[14] is transforming for murine $Lin^-/Sca1^+$ progenitors, suggesting that each of the 2 fusion proteins contributes specific properties or has synergistic effects to the leukemic phenotype. In contrast, a recent work by Kersey's laboratory,[53] based on human MLL-AF4–carrying leukemia cell lines, demonstrated that MLL-AF4 fusion protein is critical for leukemia cell proliferation and survival whereas the inhibition of AF4-MLL fusion gene had no effect on the leukemia cells, suggesting that AF4-MLL may be dispensable at later stages of leukemogenesis but necessary during the onset of the leukemogenesis process. It is worth mentioning that, in a fraction of pro-B ALL, the reciprocal product is not consistently expressed.[54]

There are other, perhaps more plausible, reasons why MLL-AF4 expression did not lead to overt leukemia in our human system. Additional specific mutations, such as Fms-like tyrosine kinase 3 activating mutations,[55] may be essential to develop pro-B/monocyte biphenotypic ALL. In this context, an etiologic component might be missing from both our human cell and the murine models (ie, a genotoxic exposure eliciting both the initiating MLL-AF4 lesion and essential secondary mutations). This

might be especially relevant if MLL-AF4 expression alters DNA repair capacity. Alternatively, CB-derived CD34+ HSPCs may not be the cellular target for MLL-AF4–mediated ALL but rather ontogenically earlier hematopoietic progenitors (fetal CD34+ cells or human ESC-derived CD34+ cells). In addition, considering the in utero origin of MLL-AF4, transduced cells may require being in an ontogenically earlier hematopoietic microenvironment, such as fetal liver, to provide a more permissive milieu for pro-B-ALL development as suggested for GATA1 mutations in AML of Down syndrome.

In conclusion, we report that enforced expression of MLL-AF4 in CB-CD34+ HSPCs enhances the hematopoietic repopulating cell function and clonogenic potential but is not sufficient for leukemogenesis. Expression of MLL-AF4 conveys a selective proliferation coupled to a survival advantage. Our inability to develop a MLL-AF4+ ALL disease model based on hCB-CD34+ HSPCs suggests that secondary mutations or the AF4-MLL reciprocal product might be required to develop overt ALL. Alternatively, CB-derived CD34+ HSPCs may not be the appropriate cellular target for MLL-AF4-mediated ALL.

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Authorship

Contribution: R.M. designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript; V.A. designed and performed experiments, analyzed and interpreted the data, and participated in manuscript preparation; I.G.-A. performed in vivo research; M.C.H.-L., L.P., and I.P. supervised and coordinated CB harvesting and storage; G.t.K. and S.B. provided MLL-AF4+ patient primary samples and performed quantitative RT-PCRs; M.G. supported the initial phase design of this study, analyzed and interpreted the data, and participated in manuscript preparation; C.B. designed and performed experiments, analyzed the data, interpreted the results, supervised the study, and wrote the paper; and P.M. designed the experiments, analyzed the data, interpreted the results, supervised the study, and wrote the paper.

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6.- A human ESC model for MLL-AF4 reveals an impaired early hemato-endothelial specification.

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INTRODUCCIÓN. La LLA pro-B MLL-AF4+ es una enfermedad con muy mal pronóstico que se manifiesta en el primer año de vida. Mientras que el origen prenatal de esta traslocación está bien establecido, poco se sabe sobre su efecto en el desarrollo hematopoyético *in utero*. Pese a que no se dispone de modelos *in vivo* e *in vitro* que reproduzcan este tipo de leucemia, las hESCs suponen *a priori* una herramienta de valor para obtener aproximaciones prenatales que nos permitan conocer aspectos de enfermedad que no son accesibles mediante muestras de pacientes o modelos animales.

OBJETIVO. Utilizar las hESCs como modelo para estudiar el efecto de la expresión de MLL-AF4 sobre la especificación hematopoyética-endotelial de los precursores hemogénicos, y conocer si la expresión de MLL-AF4 es capaz de transformar o proporcionar alguna ventaja proliferativa o de supervivenica a las hESCs como paso previo a un proceso leucémico.

MATERIAL Y MÉTODOS. Las hESCs fueron trasducidas con vectores lentivirales para inducir la expresión de MLL-AF4 y del vector vacío (control). La expresión de MLL-AF4 se confirmó mediante RT-PCR. El impacto de MLL-AF4 en la diferenciación hematopoyética de las hESCs se realizó mediante la formación de hEBs en medio de diferenciación en presencia de citocinas hematopoyéticas y se evaluó la aparición de progenitores hematopoyéticos a diferentes tiempos. Así mismo, se realizaron ensayos de clonogenicidad en metilcelulosa de los hEBS a días 15 y 22. Sobre hESCs, precursores hemogénicos y células hematopoyéticas se estudió el ciclo celular y apoptosis. Análisis de perfiles de expresión génica se realizaron sobre las hESCs. La diferenciación endotelial se realizó sobre gelatina y medio de diferenciación endotelial y se confirmó por inmunohistoquímica la presencia de VE-caderina, eNOS y vWF, así como la absorción de LDL, y la expresión de otros marcadores endoteliales. Para los estudios clonales de los precursores hemogénicos, células individuales de los mismos fueron obtenidas por "*single-cell*" FACS y crecidas en medio de diferenciación hematopoyética y endotelial.

RESULTADOS. La expresión de MLL-AF4 en hESCs es compatible con el mantenimiento de la pluripotencia y activa la expresión de genes Hox, en línea con lo establecido para los reordenamientos de MLL. Con respecto a la diferenciación de hESC, la expresión de MLL-AF4 promueve la especificación hacia precursores hemogénicos, pero no tiene ningún efecto en cuanto a la proliferación selectiva de dichos precursores. Sin embargo, MLL-AF4 bloquea la diferenciación de los precursores hemogénicos hacia células hematopoyéticas, independientemente de la presencia o ausencia de citocinas hematopoyéticas. Además, el cultivo en medio de diferenciación endotelial y el análisis clonal de los

precursores hemogénicos, mostró que los precursores MLL-AF4+ presentan una marcada diferenciación endotelial. En línea con estos resultados, los análisis de expresión génica realizados mostraron que MLL-AF4 induce la expresión de genes y rutas de señalización asociados con función vasculo-endotelial y hematopoyesis temprana. Por otro lado, las hESCs MLL-AF4+ no mostraron ninguna ventaja proliferativa y de supervivencia como paso previo a la transformación oncogénica.

CONCLUSIÓN. La expresión de MLL-AF4 induce un sesgo en la diferenciación hemato-endotelial de progenitores hemogénicos a favor de la aparición de células endoteliales. Además, este trabajo muestra cómo las hESCs suponen una herramienta única para el estudio de desarrollo humano, así como el efecto de genes de fusión de origen prenatal asociados a leucemia, y que pueden regular la especificación hematopoyética embrionaria humana.

NOTA: La relevancia de este artículo mereció, según el editor, el comentario de un experto en el tema, publicado en el misma edición. Este comentario se añade tras el artículo.

MLL-AF4 driven leukemogenesis: what are we missing?

Stam RW.

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ORIGINAL ARTICLE

A human ESC model for MLL-AF4 leukemic fusion gene reveals an impaired early hematopoietic-endothelial specification

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The MLL-AF4 fusion gene is a hallmark genomic aberration in high-risk acute lymphoblastic leukemia in infants. Although it is well established that MLL-AF4 arises prenatally during human development, its effects on hematopoietic development *in utero* remain unexplored. We have created a human-specific cellular system to study early hemato-endothelial development in MLL-AF4-expressing human embryonic stem cells (hESCs). Functional studies, clonal analysis and gene expression profiling reveal that expression of MLL-AF4 in hESCs has a phenotypic, functional and gene expression impact. MLL-AF4 acts as a global transcriptional activator and a positive regulator of homeobox gene expression in hESCs. Functionally, MLL-AF4 enhances the specification of hemogenic precursors from hESCs but strongly impairs further hematopoietic commitment in favor of an endothelial cell fate. MLL-AF4 hESCs are transcriptionally primed to differentiate towards hemogenic precursors prone to endothelial maturation, as reflected by the marked upregulation of master genes associated to vascular-endothelial functions and early hematopoiesis. Furthermore, we report that MLL-AF4 expression is not sufficient to transform hESC-derived hematopoietic cells. This work illustrates how hESCs may provide unique insights into human development and further our understanding of how leukemic fusion genes, known to arise prenatally, regulate human embryonic hematopoietic specification.

***Keywords*:** MLL-AF4; hESC; hematopoiesis; endothelium; hemogenic precursors

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Introduction

The mixed-lineage leukemia (MLL) gene fuses to generate chimeric genes with over 70 partners in human leukemia [1]. Infant pro-B acute lymphoblastic leukemia (ALL) harboring the fusion MLL-AF4 is characterized by a very brief latency and dismal prognosis, raising the question of how this infant cancer evolves so quickly [2, 3]. Moreover, the exceptionally high concordance rate of this leukemia in monozygotic twin infants, approaching 100% [4], suggests that all the necessary genetic events required for leukemogenesis are accomplished prenatally [5].

MLL-AF4-induced leukemogenesis has been particularly difficult to model and *bona fide* MLL-AF4 disease human models do not exist to date. Our understanding of transformation by MLL fusions and their mode of action comes from murine models in which leukemias do not recapitulate the human disease faithfully [6-8]. These findings raise important questions about MLL-AF4+ leukemia and suggest that these mouse models may be

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missing some essential ingredients of leukemogenesis during early human development. It could be argued that the lack of a *bona fide* MLL-AF4 disease model may be due to: (i) a cell in a wrong developmental stage was targeted in the murine approaches; (ii) the impact of other secondary hits has not been properly addressed; or (iii) MLL-AF4 exerts its transforming function preferentially in human cells, indicating that questions regarding the MLL-AF4 pathogenesis have to be addressed using ontogenically primitive human stem cells. Among these, postnatal (cord blood (CB)-derived) $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) or prenatal (fetal- or embryonic-derived) cells represent potential ontogenically early target cells in MLL-AF4 pathogenesis.

Very recently, Montes *et al*. [9] explored for the first time the *in vitro* and *in vivo* developmental impact of MLL-AF4 on the fate of human neonatal $CD34^+$ HSPCs. The expression of MLL-AF4 in human CB-derived HSPCs augmented the *in vivo* multilineage hematopoietic engraftment and homing, the *in vitro* clonogenic potential and enhanced their proliferation. However, MLL-AF4 was not sufficient for leukemogenesis on its own, indicating that additional hits are required to develop leukemia or that CB-HSPCs do not constitute the appropriate target for MLL-AF4-mediated ALL.

Human embryonic stem cells (hESC) are envisioned to become a powerful tool for modeling different aspects of human diseases that cannot otherwise be addressed by patient sample analyses or mouse models [10, 11]. The fact that leukemogenesis manifests as altered cell differentiation suggests that hematopoietic-directed differentiation of hESCs could become a promising human-specific strategy to study the onset of hematopoiesis, particularly the emergence of the earliest events leading to the specification of both normal and abnormal hematopoietic tissue [12]. During hESC differentiation, a population of primitive hemogenic precursors arises that is uniquely responsible for hematopoietic and endothelial development [13-15]. Interestingly, MLL fusions have also been implicated in endothelial cell maturation [16] and endothelial dysfunction has recently been linked to disease outcome in childhood leukemias [17]. We have thus explored the developmental impact of MLL-AF4 on the fate of hESCs and hESC-derived hemogenic precursors. We posed the following questions. First, what is the developmental impact of MLL-AF4 on the specification of hESCs to hemogenic precursors? Second, does MLL-AF4 expression alter subsequent hematopoietic commitment of these hESC-derived hemogenic precursors? And, third, is enforced expression of MLL-AF4 in this cellular context sufficient to confer *in vitro* and/or *in vivo* proliferative or survival advantage as anticipated of a transforming oncogene?

In line with the well-established activation of clustered homeobox (Hox) genes by MLL fusions, our data show that MLL-AF4 also upregulates global Hox gene expression in hESCs. Functionally, MLL-AF4 influences the fate of hESCs and hESC-derived hemogenic precursors, as it first promotes the specification of hemogenic precursors from hESCs while later on it impairs further hematopoietic commitment of these precursors in favor of an endothelial cell fate. Importantly, MLL-AF4 expression is not sufficient to transform hESC-derived hematopoietic cells *in vitro* or *in vivo*. This work illustrates how hESCs can provide unique insights into human development and further our understanding of the early events regulating human embryonic hemato-endothelial specification.

Results

The expression of MLL-AF4 is compatible with hESC pluripotency and induces an activation pattern of Hox gene family expression

The leukemic fusion gene MLL-AF4 arises prenatally [5]. To date, no study has explored the effects of leukemic fusion genes known to arise *in utero* during hESC-derived hematopoietic development. Here, MLL-AF4 cDNA was subcloned in a lentiviral vector expressing the Neomycin resistance cassette (NEO) (Figure 1A). Human ESCs were transduced with either the empty lentivector (NEO) or the MLL-AF4-expressing lentivector (MLL-AF4). After 3-4 weeks of G418 selection, typical neo-resistant hESC colonies emerged (Figure 1B). Successful and stable ectopic expression of MLL-AF4 in these hESCs was confirmed by RT-PCR (Figure 1C) and western blot (Figure 1D) more than 10 weeks after G418 selection. MLL-AF4 hESC cultures were then analyzed for pluripotency markers and functional assays. MLL-AF4-expressing hESCs retained high expression of both the pluripotency markers Oct4, Nanog and Rex-1 (Supplementary information, Figure S1A), and the hESC-associated antigens Tra-1-60, Tra-1-81, SSEA-3 and SSEA-4 (Supplementary information, Figure S1B). Functionally, MLL-AF4 and NEO hESCs formed teratomas with identical efficiency (100%), latency (50-65 days) and histological composition (Supplementary information, Figure S1C). Importantly, the ectopic expression of MLL-AF4 compares quite well between experimentally transduced cells and MLL-AF4-expressing leukemic cell lines (SEM and RS4;11) (Supplementary information, Figure S2).

As MLL fusions are positive regulators of homeobox gene expression [18-20], we next performed microarray

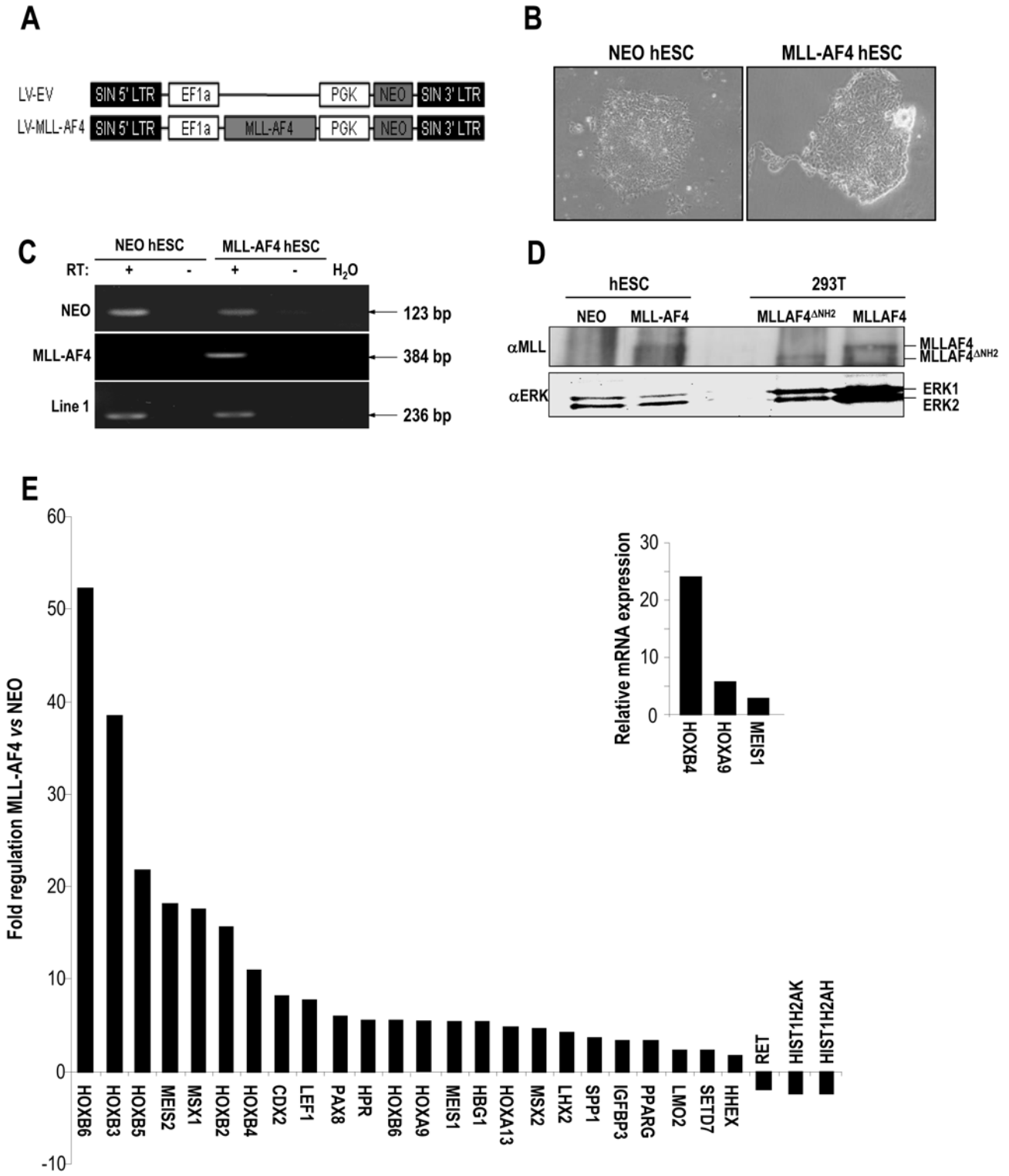


Figure 1 Ectopic expression of MLL-AF4 in hESCs activates Hox gene expression. **(A)** Schematic representation of the lentiviral vectors used. **(B)** Phase contrast morphology of colonies from NEO- and MLL-AF4 hESCs. **(C)** RT-PCR confirming expression of the MLL-AF4 transcript in transduced hESCs. Line 1 was used as a housekeeping gene. **(D)** Western blot detection of the MLL-AF4 protein in transgenic hESCs. 293T cells transduced with MLL-AF4 and MLL-AF4ΔNH2 were used as positive and negative control, respectively. ERK1 and ERK2 blot was used as loading control. **(E)** Hox gene expression profiling in MLL-AF4 vs NEO hESC using microarrays. The inset shows qPCR validation of the indicated Hox genes/co-factors.

gene expression in NEO and MLL-AF4 hESCs to specifically analyze the impact of MLL-AF4 expression on the Hox gene family transcriptome in hESCs. As detailed in Figure 1E, 88.9% of the Hox family genes differentially expressed between MLL-AF4 and NEO hESCs resulted to be upregulated in MLL-AF4 hESCs, including the consistently upregulated targets of MLL fusions HOXA9 and MEIS1. Together, these data confirm successful expression of MLL-AF4 in hESCs that is compatible with pluripotency and activates Hox gene expression.

Augmented specification of hemogenic precursors from MLL-AF4 hESCs

We have established that the expression of MLL-AF4 is compatible with hESC pluripotency and upregulates global Hox gene expression. Next, we tested whether this prenatal leukemic fusion may impact the hemato-endothelial cell fate of hESCs using *in vitro* hematopoietic and endothelial differentiation from hESCs as surrogate assays for early developmental events. During human embryoid body (hEB) differentiation, a population of primitive hemogenic precursors arises, which is uniquely responsible for hematopoietic and endothelial development [14, 15]. We thus investigated first the effect of MLL-AF4 on the emergence of hemogenic precursors (CD45-CD31$^+$CD34$^+$) throughout hEB development (Figure 2A and 2B). To ensure that any developmental effect is linked to MLL-AF4 expression, we confirmed stable transgene expression upon hEB differentiation by RT-PCR (Figure 2C). We found that the frequency of hemogenic precursors in MLL-AF4 hEBs was consistently higher (1.7-4.3-fold) throughout hEB development (Figure 2D). Importantly, MLL-AF4 expression accelerated the kinetics of emergence of hemogenic precursors. As shown in Figure 2D, by day 4 of hEB development, hemogenic precursors emerged in MLL-AF4 hESCs but barely did so in NEO hESCs.

The increased frequency and accelerated emergence of hemogenic precursors in MLL-AF4 hESCs may be the result of either (i) MLL-AF4-mediated specification of hESCs towards hemogenic precursors or (ii) MLL-AF4-mediated enhanced proliferation of the emerging hemogenic precursors. To address this, cell cycle distribution and the proportion of sub-G0/G1 apoptotic cells was analyzed within both the hemogenic precursor population and the remaining EB cells (Supplementary information, Figure S3A). No differences in the proportion of cycling cells or apoptotic cells were observed either between MLL-AF4 and NEO hemogenic precursors (cycling cells: 25% vs 28%; apoptotic cells: 7% vs 12%) or between MLL-AF4 hemogenic precursors and the remaining MLL-AF4 EB cells (cycling cells: 25% vs 23%; apoptotic cells: 7% vs 10%) (Supplementary information, Figure S3A). Similarly, MLL-AF4 expression did not confer either growth or survival advantage to undifferentiated hESC cultures, as measured by BrdU incorporation (Supplementary information, Figure S3B). These data suggest that MLL-AF4 expression promotes specification, rather than selective proliferation, of hemogenic precursors from differentiating hEBs.

MLL-AF4 impairs hematopoietic commitment of hESC-derived hemogenic precursors

Hemogenic precursors derived from hESCs are uniquely responsible for endothelial and hematopoietic development [15, 21, 22]. We next assessed whether the MLL-AF4-mediated enhanced specification of hemogenic precursors occurs with a subsequent increase of both hematopoietic and endothelial commitment. The emergence of primitive (CD45$^+$CD34$^+$) and total hematopoietic cells (CD45$^+$) was analyzed throughout hEB development (Figure 2E and 2F). As expected, hematopoietic cells did not emerge prior to day 10 of hEB development (Figure 2E and 2F). Interestingly, the expression of MLL-AF4 abrogated the hematopoietic differentiation as reflected by a robust reduction on the frequency of both CD45$^+$CD34$^+$ (2.5- to 4.2-fold decrease) and total CD45$^+$ cells (2-fold decrease) at day 11 and day 15 of hEB development (Figure 2E and 2F). Importantly, MLL-AF4 expression not only blocked the generation of CD45$^+$ blood cells but also strongly compromised the clonogenic potential of hematopoietic progenitors derived from day 15 and day 22 hEBs (Figure 2G). Hematopoiesis generated from MLL-AF4 hESCs displayed a highly reduced (3-12-fold decrease) clonogenic potential measured by the ability to form colony-forming units (CFUs) in semisolid cultures (Figure 2G). Of note, MLL-AF4 effects were independent of the presence of hematopoietic cytokines, since an identical trend was observed in differentiation experiments carried out without BMP4 and hematopoietic growth factors (Supplementary information, Figure S4). These data indicate that the ectopic expression of MLL-AF4 promotes specification of hemogenic precursors from hESCs but impairs subsequent hematopoietic commitment of these precursors.

MLL-AF4 hemogenic precursors display enhanced endothelial cell fate

According to our results so far, MLL-AF4 expression promotes specification of hemogenic precursors from hESCs but impairs subsequent hematopoietic commitment of such precursors (Figure 2). Two distinct scenarios are plausible: the expression of MLL-AF4 in hemogenic precursors may (i) block both subsequent

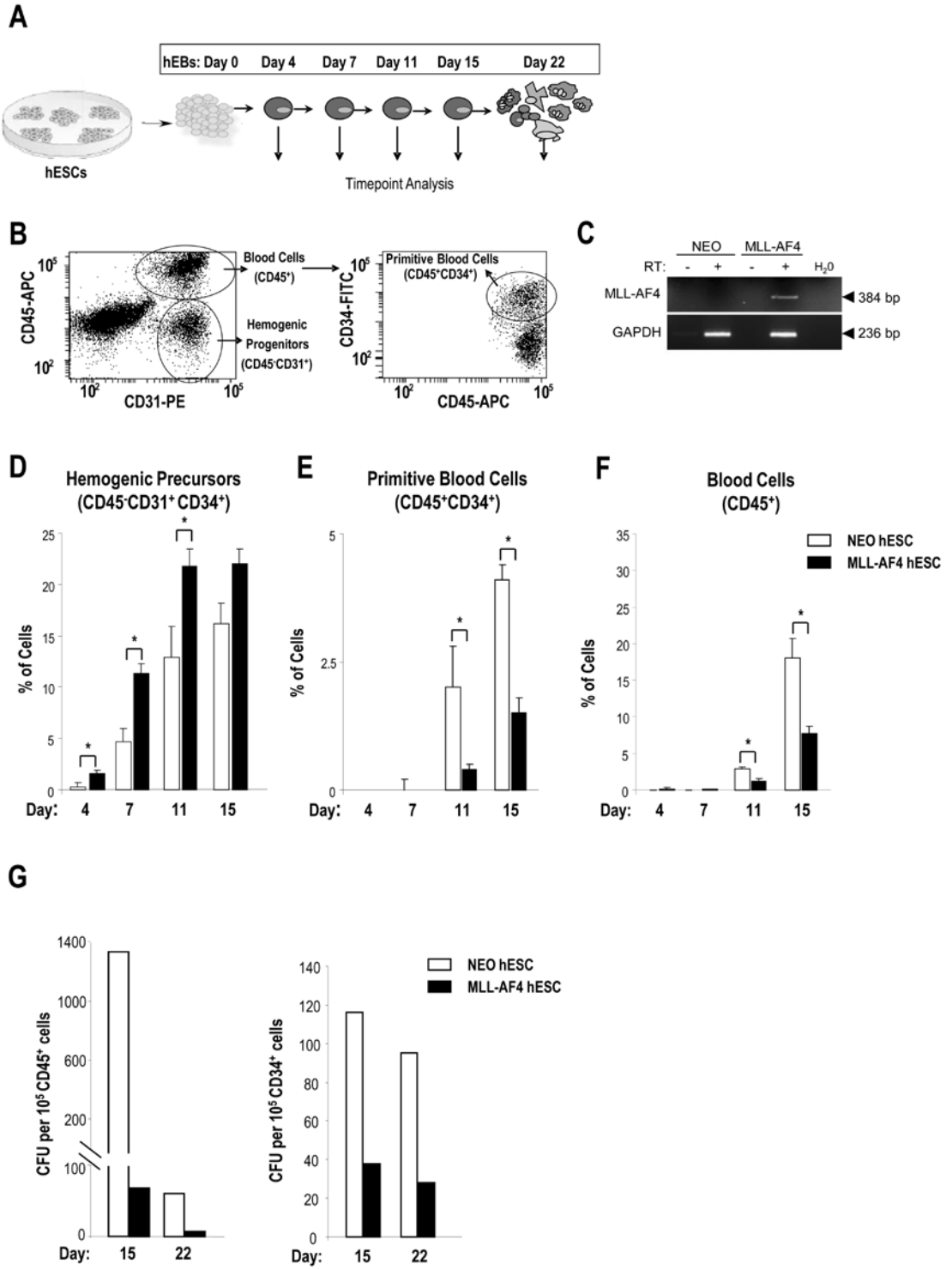


Figure 2 MLL-AF4 promotes hESC specification towards hemogenic precursors but impairs subsequent hematopoietic differentiation of such hemogenic precursors. **(A)** Schematic of the hematopoietic differentiation of hESCs and endpoint analyses. **(B)** Representative flow cytometry dot plots displaying how hemogenic progenitors ($CD45^-CD31^+$), primitive blood cells ($CD45^+CD34^+$) and total blood cells ($CD45^+$) are identified. **(C)** RT-PCR confirming stable MLL-AF4 expression upon differentiation (day 11 hEBs). **(D)** Specification into hemogenic precursors is significantly enhanced in MLL-AF4 hESCs throughout EB development. Subsequent differentiation of MLL-AF4 hemogenic precursors into primitive **(E)** and mature blood cells **(F)** is significantly decreased. **(G)** CFU read out from d15 and d22 hEBs confirming a reduced hematopoietic potential in MLL-AF4 cells. Data are presented as mean ± SEM for six independent experiments.

endothelial and hematopoietic differentiation or (ii) skew the hemato-endothelial commitment in favor of an endothelial cell fate. To explore these two possibilities, we analyzed the ability of both NEO and MLL-AF4 hemogenic precursors to differentiate into mature endothelial cells. NEO and MLL-AF4 hEBs were dissociated at day 11 of development and the hemogenic precursors were MACS-sorted and cultured for 5-7 days in conditions conductive to endothelial maturation (Figure 3A). After 5-7 days in endothelial culture conditions, the expression of the mature endothelial markers VE-cadherin, vWF and eNOS, and the uptake of LDL were examined in both NEO and MLL-AF4 cultures.

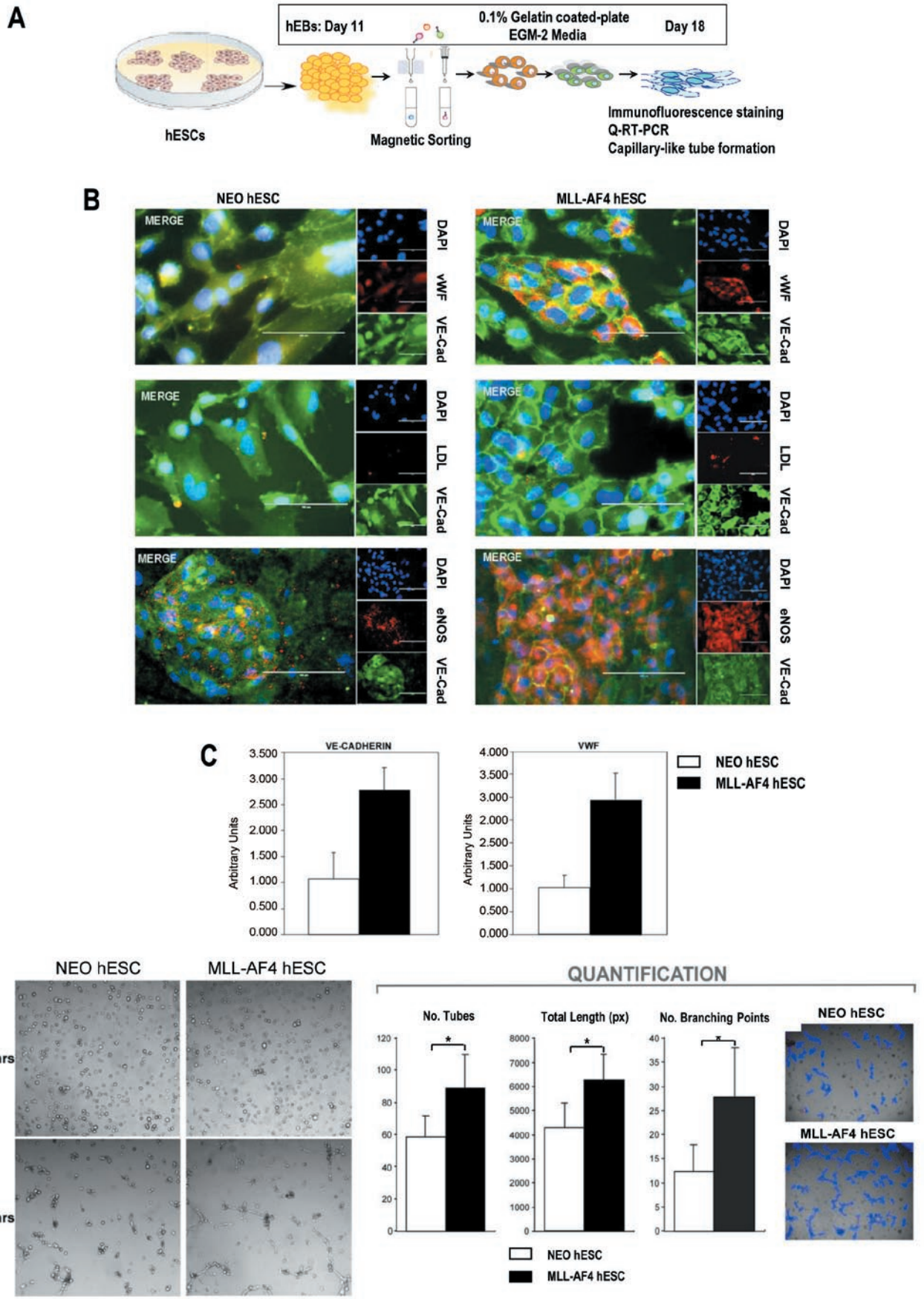
Both NEO and MLL-AF4 hemogenic precursors cultured in endothelial conditions became attached and spindle shaped. However, MLL-AF4 cells expressed stronger levels of VE-cadherin than NEO cells. More importantly, while NEO cells showed a predominant cytoplasmic localization for VE-cadherin, MLL-AF4 cells displayed a clear localization of VE-cadherin at the cell surface, identifying adherent junctions between cells, reminiscent of a distinctive endothelial barrier (Figure 3B). Similarly, the expression of vWF and eNOS was more robust in MLL-AF4 than in NEO endothelial cells (Figure 3B). In addition, MLL-AF4 endothelial cells also possessed higher LDL uptake capacity than NEO endothelial cells, also indicative of endothelial maturation (Figure 3B). A more quantitative assessment of this favored endothelial specification of MLL-AF4 hemogenic precursors was approached by quantitative PCR (qPCR) for VE-cadherin and vWF. MLL-AF4 endothelial cells consistently expressed 3-fold higher levels of both VE-cadherin and vWF than NEO endothelial cells (Figure 3C).

A key evidence of the acquisition of functional endothelial properties was evaluated by culturing NEO and MLL-AF4 hemogenic precursors on Matrigel to assess their capacity to form a network of capillary-like tubes. In line with the immunocytochemical and gene expression data, MLL-AF4 hemogenic precursors displayed an enhanced and faster ability than NEO precursors to align and form capillary-like structures (Figure 3D). As early as 4 h after seeding the cells in Matrigel, MLL-AF4 cultures, but not NEO cultures, contained capillary-like structures (Figure 3D, left panels). Computational quantification revealed that MLL-AF4 endothelial cells are more functional and mature than NEO endothelial cells since they displayed significantly higher number of capillary tubes, longer endothelial tubes and more branching points (Figure 3D, right panels). This data indicates that MLL-AF4 rather than blocking both endothelial and hematopoietic commitment of the hemogenic precursors, seems to skew the hemato-endothelial potential of these hemogenic precursors towards an endothelial cell fate.

Clonal analysis confirms that MLL-AF4 skews hemogenic precursor commitment towards endothelial cell fate

We next addressed whether MLL-AF4 skews the hemato-endothelial potential of the hemogenic precursors at the single cell level. Single hemogenic precursors isolated from day 11 NEO and MLL-AF4 hEBs were deposited into individual wells of 96-well plates and inspected daily. Individual wells targeted for NEO or MLL-AF4 single precursor cell deposition were visually inspected at 2 h and again after 16 h post-clonal isolation and only wells containing one cell were selected for further analysis (Figure 4A). This inspection was performed by two independent observers blinded to the other's results [15]. Both observers demonstrated > 99% concordance of potential wells that contained more than one cell and these wells were then excluded from the subsequent analysis. After 12-15 days, the outgrowth in each well resulting from single hemogenic precursor proliferation was identified by phase contrast morphology (Figure 4B) and by DAPI staining (Figure 4C) and, analyzed *in situ* for expression of CD45 and VE-cadherin, representing hematopoietic and endothelial cell fate, respectively (Figure 4C) [15].

Of the 936 and 960 wells containing, respectively, single NEO or MLL-AF4 hemogenic precursors, 44 wells (4.7%) and 41 wells (4.3%) demonstrated clonal outgrowth, respectively, consistent with the reported difficulty of sustaining single cells differentiated from ESCs in culture (Figure 4D) [15, 23]. The resulting progeny of the single hemogenic precursor clones was then examined for hematopoietic and/or endothelial cell fate by *in situ* analysis of individual wells (Figure 4C). Consistent with the data from bulk cultures and hEB hematopoietic and endothelial differentiation (Figures 2 and 3), expression of MLL-AF4 strongly facilitated clonal endothelial over hematopoietic cell fate of single hemogenic precursors (Figure 4D). Progeny of NEO hemogenic precursor clones was exclusively hematopoietic in 23% of the wells (10 out of 44 wells) or exclusively endothelial in 54% of the wells (24 out of the 44 wells), while the remaining 10 clones (23% of the wells) were capable of giving rise to both endothelial and hematopoietic cells (Figure 4C and 4D). In sharp contrast, progeny of MLL-AF4 hemogenic precursors was exclusively endothelial in 97.5% of the clones (40 out of 41 wells) (Figure 4C and 4D) and no hematopoietic outgrowth from single MLL-AF4 hemogenic precursors could be detected. This clonal analysis confirms that MLL-AF4 expression robustly skews hemogenic precursor commitment towards endothelial cell fate.


A
hEBs: Day 11
0.1% Gelatin coated-plate
EGM-2 Media
Day 18
hESCs
Magnetic Sorting
Immunofluorescence staining
Q-RT-PCR
Capillary-like tube formation
B
NEO hESC
MLL-AF4 hESC
MERGE
DAPI
vWF
VE-Cad
LDL
eNOS
C
VE-CADHERIN
VWF
Arbitrary Units
NEO hESC
MLL-AF4 hESC
D
NEO hESC
MLL-AF4 hESC
0 hrs
4 hrs
QUANTIFICATION
No. Tubes
Total Length (px)
No. Branching Points
NEO hESC
MLL-AF4 hESC

MLL-AF4 acts as a global transcriptional activator and upregulates master genes associated to vascular-endothelial functions and early hematopoiesis

As we had performed microarray analysis of gene expression in undifferentiated NEO and MLL-AF4 hESCs, we looked back at our data in order to identify patterns of gene expression that could help explain at the molecular level the developmental effect of MLL-AF4 in hESCs. Global data analysis identified 1 267 genes differentially regulated (P value < 0.05; 2-fold regulation) between NEO and MLL-AF4 hESCs (Figure 5 and Supplementary information, Table S1). Overall, MLL-AF4 functioned as a transcriptional activator because its expression induced upregulation of 1 015 differentially expressed genes (80.1%) and downregulation of only 252 genes (19.9%) (Figure 5A). Analysis of the altered genes using the Ingenuity Analysis Program (IPA) software revealed that several gene functions (Figure 5B) and signaling pathways (Figure 5C) displayed a significantly altered gene expression profile in MLL-AF4 hESCs. Among the altered gene functions, tissue development, cancer, cardiovascular system development and function as well as hematopoietic system development are the most significantly upregulated cell functions upon the expression of MLL-AF4 in hESCs (Figure 5B and Supplementary information, Table S1). Similarly, among the altered signaling pathways, cardiogenesis, cardiomyocyte differentiation and VEGF signaling represent the most significantly upregulated signaling pathways in MLL-AF4 hESCs (Figure 5C and Supplementary information, Table S1).

Those genes differentially upregulated in MLL-AF4 hESCs, classified by the IPA software as involved in cardiovascular/vascular-endothelial system development and function (Figure 5D) and hematopoietic system development and function (Figure 5E) were analyzed in more detail. We found upregulation of many key genes associated with early hematopoiesis including SCL/TAL1, RUNX1/AML1, GATA2, CD34, BMP4 and HOXB4 and with vascular-endothelium, such as VEGFA, VEGFC, CD34, KDR, TIE1, TEK, SELE, NRP1, NRP2 and different members of the collagen family. Supplementary information, Table S1 contains the complete list of differentially regulated genes classified by the IPA software belonging to other gene functions and canonical pathways. This gene expression profiling (GEP) indicates that MLL-AF4 hESCs seem transcriptionally primed to differentiate towards hemogenic precursors (hemangioblast) prone to subsequent endothelial maturation, as reflected by the marked upregulation of master genes associated to vascular-endothelial functions and early hematopoiesis.

Expression of MLL-AF4 is not sufficient for either in vitro or in vivo transformation of hESC-derived hematopoietic cells

Our findings so far support the idea that MLL-AF4 neither transforms undifferentiated hESC nor promotes hematopoiesis from hESCs. We next wanted to rule out the possibility that the expression of the MLL-AF4 specifically confers *in vitro* or *in vivo* proliferative and survival advantage to hESC-derived hematopoietic cells, as anticipated for a transforming leukemic oncogene. As shown in Figure 6A, MLL-AF4-expressing hESC-derived $CD45^+$ hematopoietic cells did not display proliferative or survival advantage. Functionally, MLL-AF4 did not confer stable *in vitro* replating efficiency of hematopoietic cells in CFU assays (Figure 6B). Although hESC-derived hematopoietic cells have been extensively reported to barely repopulate immunodeficient mice, we finally attempted to ensure that MLL-AF4 does not affect the *in vivo* behavior of hEB-derived hematopoietic cells. Expectedly, MLL-AF4 did not confer *in vivo* function and engraftment potential to hESC-derived hematopoietic cells (Figure 6C), confirming that MLL-AF4 expression does not transform hESC-derived hematopoietic cells *in vitro* or *in vivo*, similar to that reported in neonatal $CD34^+$ HSPCs [9].

Figure 3 MLL-AF4 hemogenic precursors display an enhanced endothelial cell fate as compared to NEO hemogenic precursors. **(A)** Schematic of the endothelial differentiation and phenotypic and functional characterization of sorted hemogenic precursors. **(B)** NEO and MLL-AF4 hemogenic precursors isolated from day 11 EBs were cultured in EGM-2 media for 5-7 days and analyzed by immunohistochemistry for vWF, VE-cadherin and eNOS expression as well as LDL uptake (n = 3). Representative single stainings are shown on the right. A "merge" staining shows marker expression co-localization. **(C)** qPCR analysis of NEO and MLL-AF4 cells for the indicated endothelial-specific genes. This analysis was performed on NEO and MLL-AF4 hemogenic precursors after 6 days of culture in gelatin-coated plates, as indicated in **A**. **(D)** After 6 days of culture, NEO and MLL-AF4 hemogenic precursors were cultured in Matrigel at a density of 12×10^3 cells/well to assess their capacity to form capillary-like structures (n = 3). Images were captured 0 and 4 h after cell plating. Representative images are presented in the left panel. Quantification of the number of endothelial tubes, total length of tubes, and number of branching points was obtained with the software developed by Wimasis. Software-processed images are presented on the right as an example. Data are presented as mean ± SEM.

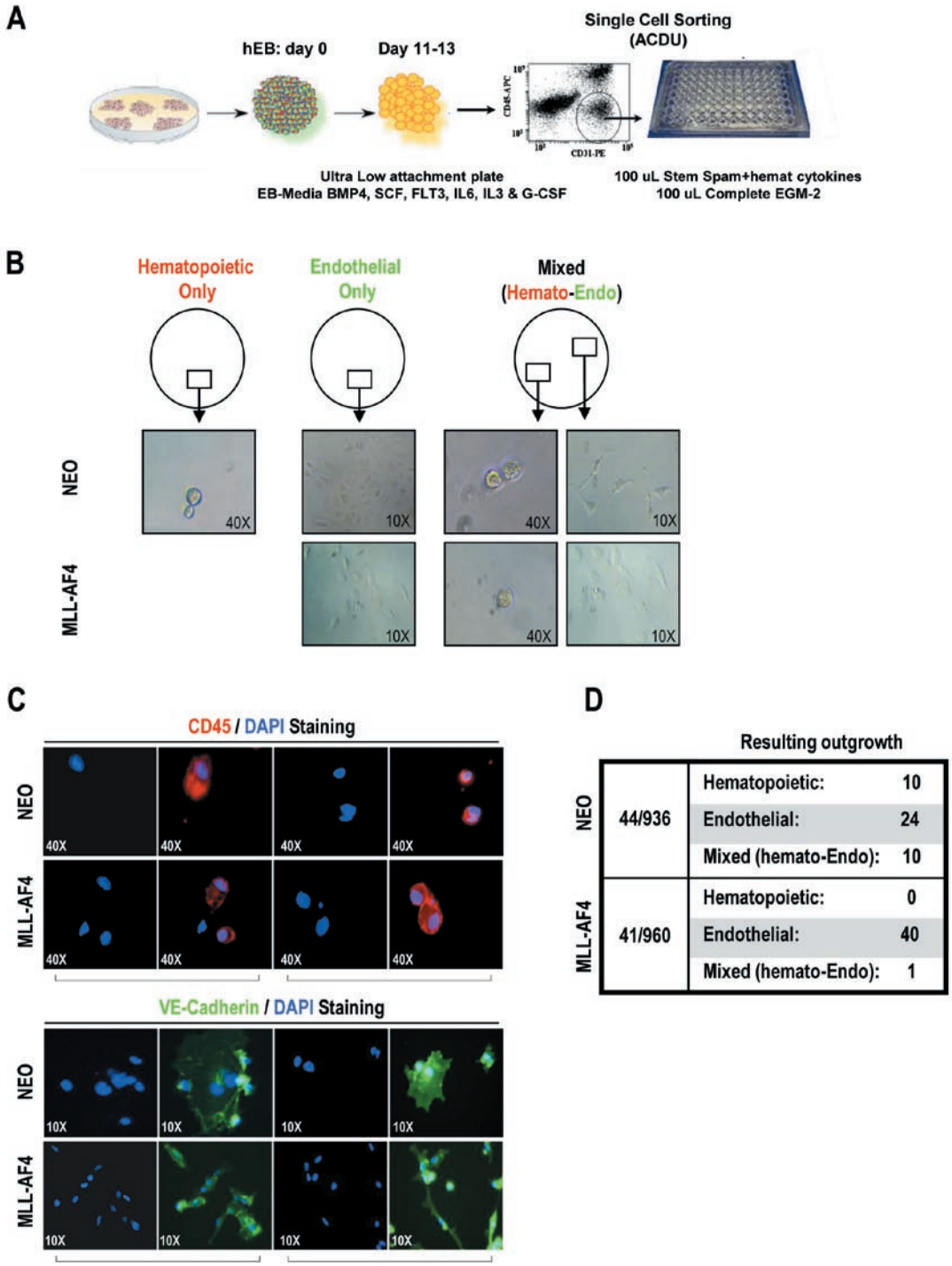


Figure 4 Clonal analysis confirms an enhanced endothelial commitment of MLL-AF4 hemogenic precursors. **(A)** Schematic of EB development and FACS sorting of single hemogenic precursors into 96-well plates. **(B)** Phase contrast images showing hematopoietic outgrowth only, endothelial outgrowth only or mixed (hemato-endothelial) outgrowth. Hematopoietic cells are identified as small round refractive cells loosely attached to the gelatin-coated plastic whereas endothelial cells are larger spindle-shaped cells strongly attached to the gelatin-coated plastic. **(C)** The cells resulting from single hemogenic precursor proliferation in each individual well were analyzed *in situ* for CD45 (hematopoietic cell fate) and VE-cadherin (endothelial cell fate). CD45 expression is shown in red, VE-cadherin in green and DAPI nucleus staining in blue. **(D)** Summary table illustrating the resulting outgrowth within individual wells containing single NEO or MLL-AF4 hemogenic precursors. At 10 to 15 days after sorting, single wells were observed to contain (i) hematopoietic progeny only, (ii) endothelial progeny only or (iii) both hematopoietic and endothelial cells.

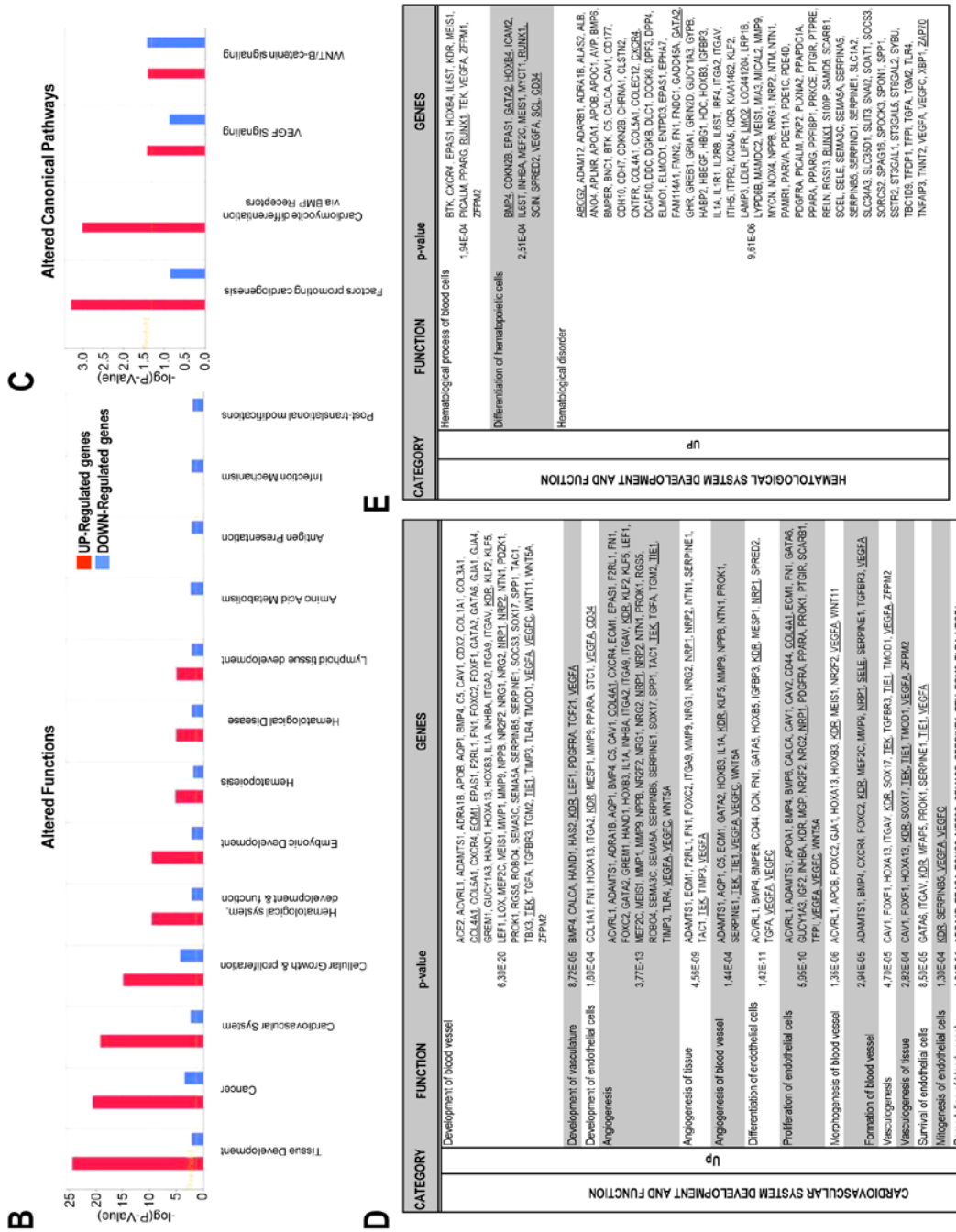

A
hESC-NEO
hESC-MLLAF4
Replicate: 1 2 1 2
+2 0 -2
B
Altered Functions
-log(P-Value)
25 20 15 10 5 0
Tissue Development
Cancer
Cardiovascular System
Cellular Growth & proliferation
Hematological system, development & function
Embryonic Development
Hematopoiesis
Hematological Disease
Lymphoid tissue development
Amino Acid Metabolism
Antigen Presentation
Infection Mechanism
Post-translational modifications
UP-Regulated genes
DOWN-Regulated genes
C
Altered Canonical Pathways
-log(P-Value)
3.0 2.5 2.0 1.5 1.0 0.5 0.0
Factors promoting cardiogenesis
Cardiomyocyte differentiation via BMP Receptors
VEGF Signaling
WNT/B-catenin signaling
D
CARDIOVASCULAR SYSTEM DEVELOPMENT AND FUNCTION
CATEGORY
FUNCTION
p-value
GENES
Up
Development of blood vessel
8.72E-05
ACE2, ACVRL1, ADAMTS1, ADRA1B, APOB, AQP1, BMP4, C5, CAV1, CDX2, COL1A1, COL3A1, COL4A3, COL5A1, CXCR4, EGR1, EPAS1, F2RL1, FN1, FOXC2, FOXF1, GATA2, GATA6, GJA1, GJA4, GREM1, GUCY1A3, HAND1, HOXA13, HOXB3, IL1A, INHBA, ITGA2, ITGA9, ITGAV, KDR, KLF2, KLF5, LEF1, LOX, MEF2C, MEIS1, MMP1, MMP9, NPPB, NR2F2, NRG1, NRG2, NRP1, NRP2, NTN1, PDZK1, PROK1, RGS5, ROBO4, SEMA3C, SEMA5A, SERPINB5, SERPINE1, SOCS3, SOX17, SPP1, TAC1, TBX3, TEK, TGFA, TGFBR3, TGM2, TIE1, TIMP3, TLR4, TMOD1, VEGFA, VEGFC, WNT11, WNT5A, ZFPM2
Development of vasculature
8.72E-05
BMP4, CALCA, HAND1, HAS2, KDR, LEF1, PDGFRA, TCF21, VEGFA
Development of endothelial cells
1.80E-04
COL1A1, FN1, HOXA13, ITGA2, KDR, MESP1, MMP9, PPARA, STC1, VEGFA, CD34
Angiogenesis
3.77E-13
ADVRL1, ADAMTS1, ADRA1B, AQP1, BMP4, C5, CAV1, COL4A1, CXCR4, EGR1, EPAS1, F2RL1, FN1, FOXC2, GATA2, GREM1, HAND1, HOXB3, IL1A, INHBA, ITGA2, ITGA9, ITGAV, KDR, KLF2, KLF5, LEF1, MEF2C, MEIS1, MMP1, MMP9, NPPB, NR2F2, NRG1, NRG2, NRP1, NTN1, PROK1, RGS5, ROBO4, SEMA3C, SEMA5A, SERPINB5, SERPINE1, SOX17, SPP1, TAC1, TEK, TGFA, TGFBR3, TGM2, TIE1, TIMP3, TLR4, TMOD1, VEGFA, VEGFC, WNT11, WNT5A
Angiogenesis of tissue
4.58E-09
ADAMTS1, ECM1, F2RL1, FN1, FOXC2, ITGA9, MMP9, NRG1, NRG2, NRP1, NRP2, NTN1, SERPINE1, TAC1, TEK, TIMP3, VEGFA
Angiogenesis of blood vessel
1.44E-04
ADAMTS1, AQP1, C5, ECM1, GATA2, HOXB3, IL1A, KDR, KLF5, MMP9, NPPB, NTN1, PROK1, SERPINE1, TEK, TIE1, VEGFA, VEGFC, WNT5A
Differentiation of endothelial cells
1.42E-11
ADVRL1, BMP4, BMPER, CD44, DCN, FN1, GATA5, HOXB5, IGFBP3, KDR, MESP1, NRP1, SPRED2, TGFA, VEGFA, VEGFC
Proliferation of endothelial cells
5.95E-10
ADVRL1, ADAMTS1, APOA1, BMP4, BMPR, CALCA, CAV1, CAV2, CD44, COL4A1, ECM1, FN1, GATA6, GUCY1A3, IGF2, INHBA, KDR, MGP, NR2F2, NRG2, NRP1, PDGFRA, PPARA, PROK1, PTGIR, SCARB1, TFP1, VEGFA, VEGFC, WNT5A
Morphogenesis of blood vessel
1.39E-06
ADVRL1, APOB, FOXC2, GJA1, HOXA13, HOXB3, KDR, MEIS1, NR2F2, VEGFA, VWF11
Formation of blood vessel
2.94E-05
ADAMTS1, BMP4, CXCR4, FOXC2, KDR, MEF2C, MMP9, NRP1, SELE, SERPINE1, TGFBR3, VEGFA
Vasculogenesis
4.70E-05
CAV1, FOXF1, HOXA13, ITGAV, KDR, SOX17, TEK, TGFBR3, TIE1, TMOD1, VEGFA, ZFPM2
Vasculogenesis of tissue
2.82E-04
CAV1, FOXF1, HOXA13, KDR, SOX17, TEK, TIE1, TMOD1, VEGFA, ZFPM2
Survival of endothelial cells
8.50E-05
GATA6, ITGAV, KDR, MFAP5, PROK1, SERPINE1, TIE1, VEGFA
Mitogenesis of endothelial cells
1.30E-04
KDR, SERPINB5, VEGFA, VEGFC
Remodeling of blood vessel
1.31E-04
ADRA1B, EPAS1, FOXC2, MEF2C, SEMA3C, SERPINE1, TEK, TGM2, TLR4, VEGFA
Growth of blood vessel
1.50E-04
BMP6, FOXC2, KDR, ROBO4, SERPINE1, VEGFA, VEGFC
Down
Cardiovascular process of organ
2.87E-02
MFGE8, PVALB, UCN
Branching of endothelial cell lines
1.51E-02
LAMA4
Blood pressure of organism
2.79E-02
DIO3, HBA1/HBA2, NOS1, UCN
Vascularization
3.15E-02
IGF1R, MFGE8, NOS1, UCN
E
HEMATOLOGICAL SYSTEM DEVELOPMENT AND FUNCTION
CATEGORY
FUNCTION
p-value
GENES
Up
Hematological process of blood cells
1.94E-04
BTK, CXCR4, EPAS1, HOXB4, IL6ST, KDR, MEIS1, PICALM, PPARG, RUNX1, TEK, VEGFA, ZFPM1, ZFPM2
Differentiation of hematopoietic cells
2.51E-04
BMP4, CDKN2B, EPAS1, GATA2, HOXB4, ICAM2, IL6ST, INHBA, MEF2C, MEIS1, MYCT1, RUNX1, SOIN, SPRED2, VEGFA, SOL, CD34
Hematological disorder
9.61E-06
ABCG2, ADAM12, ADARB1, ADRA1B, ALAS2, ALB, ANO4, APLNR, APOA1, APOB, APOC1, AVP, BMP6, BMPER, BNC1, BTK, C5, CALCA, CAV1, CD177, CDH10, CDH7, CDKN2B, CHRNA1, CLSTN2, CNTFR, COL4A1, COL5A1, COLEC12, CXCR4, DCAF10, DDC, DGKB, DLC1, DOCK8, DPF3, DPP4, ELMO1, ELMOD1, ENTPD3, EPAS1, EPHA7, FAM114A1, FMN2, FN1, FNDC1, GADD45A, GATA2, GHR, GREB1, GRIA1, GRIN2D, GUCY1A3, GYPB, HABP2, HBEGF, HBG1, HDC, HOXB3, IGFBP3, IL1A, IL1R1, IL3RB, IL6ST, IRF4, ITGA2, ITGAV, ITIH5, TPR2, KCNA5, KDR, KIAA1462, KLF2, LAMP3, LDLR, LIFR, LIMS2, LOC441204, LRP1B, LYPD6B, MAMDC2, MEIS1, MMA3, MICAL2, MMP9, MYCN, NOX4, NPPB, NRG1, NRP2, NTM, NTN1, PAMR1, PARVA, PDE11A, PDE1C, PDE4D, PDGFRA, PICALM, PKP2, PLXNA2, PPAPDC1A, PPARA, PPARG, PPIBP1, PRKCE, PTGIR, PTPRE, RELN, RGS13, RUNX1, S100P, SAMD5, SCARB1, SCEL, SELE, SEMA3C, SEMA5A, SERPINA5, SERPINB5, SERPINDI, SERPINE1, SLC1A2, SLC9A3, SLC39D1, SLIT3, SNAI2, SOAT1, SOCS3, SORCS2, SPAG16, SPOCK3, SPON1, SPP1, SSTR2, ST3GAL1, ST3GAL5, ST6GAL2, SYBU, TBC1D9, TFDP1, TFPI, TGFA, TGM2, TLR4, TNFAIP3, TNNT2, VEGFA, VEGFC, XBP1, ZAP20

Figure 5 Gene expression profiling and signaling pathways are altered in MLL-AF4 hESCs. **(A)** Heatmap showing a total of 1 267 genes differentially regulated between MLL-AF4 and NEO hESCs (*P* value < 0.05; 2-fold regulation). In all, 81% (1 015) of the genes differentially expressed are upregulated in MLL-AF4 hESCs. **(B-E)** After gene expression microarray analysis, the groups of genes differentially expressed (*P* value < 0.05; 2-fold regulation) in MLL-AF4 vs NEO hESCs were compared and the lists of functions and canonical pathways significantly altered were generated using the Ingenuity Pathways Analysis (IPA) 8 software. IPA software-based data mining generated a list of significantly modulated (up and down) gene functions **(B)** and canonical pathways **(C)** between MLL-AF4 and NEO hESCs. A more profound analysis was then performed for all the genes classified by the IPA software as involved in cardiovascular/vascular-endothelial system development and function **(D)** and hematopoietic system development and function **(E)**. Master genes strongly associated with early hematopoiesis (i.e., SCL, RunX1, GATA2, CD34, BMP4, HOXB4) and vascular-endothelium (i.e., VEGFA, VEGFC, CD34, KDR, TIE1, TEK, SELE, NRP1, NRP2, COLs) likely to contribute to hemogenic precursor specification and subsequent endothelial development are underlined. Supplementary information, Table S1 contains the complete list of differentially regulated genes classified by the IPA software in the other gene functions and canonical pathways.

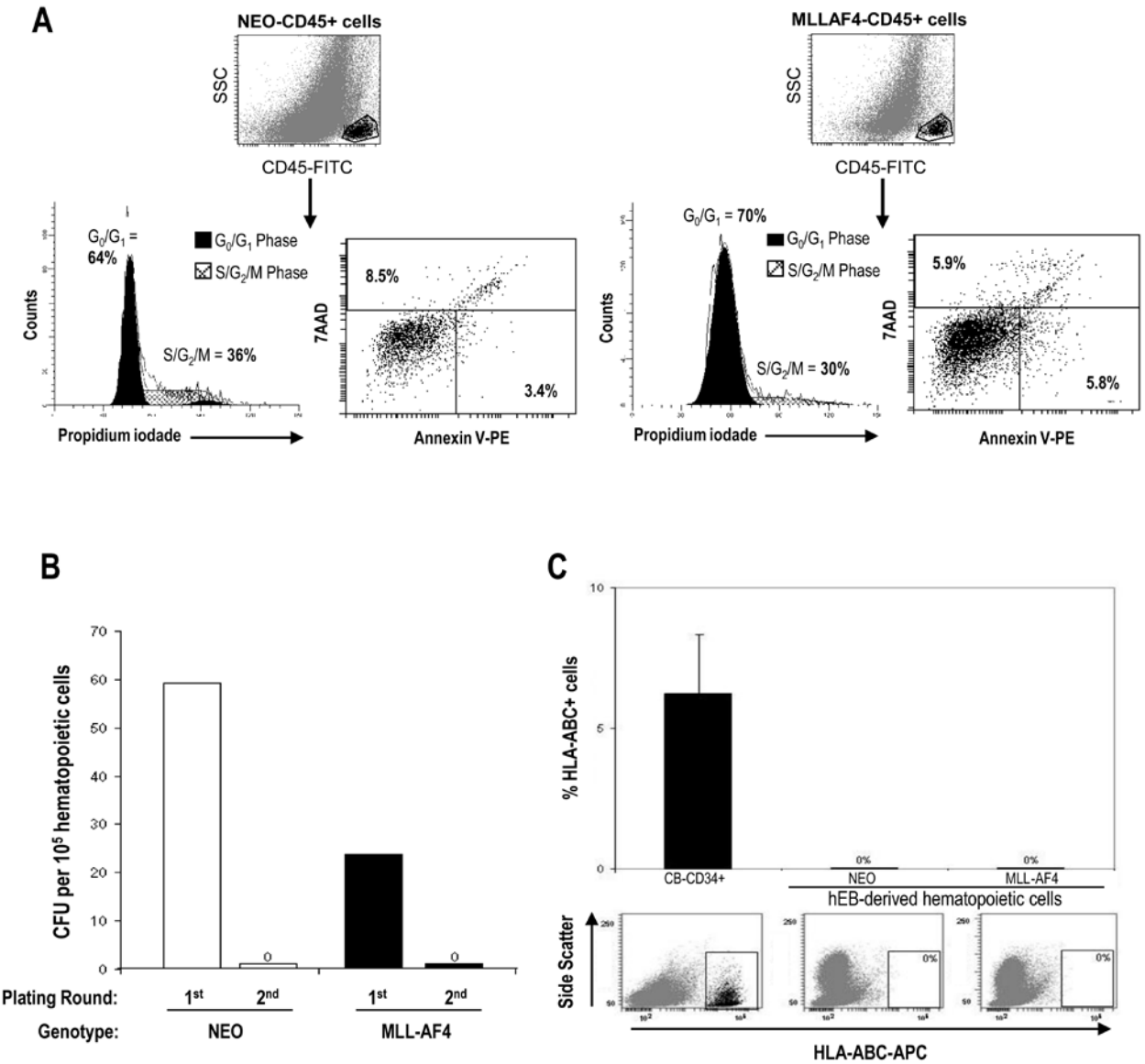


Figure 6 MLL-AF4 does not transform hESC-derived CD45$^+$ hematopoietic cells *in vitro* nor *in vivo*. MLL-AF4-expressing hESC-derived CD45$^+$ hematopoietic cells do not show either proliferative/survival advantage **(A)** or stable *in vitro* replating efficiency in hematopoietic CFU assays **(B)** or *in vivo* hematopoietic engraftment potential into NSG mice **(C)**.

Discussion

MLL-AF4+ pro-B ALL is a dismal infant leukemia that manifests in the first year of life [24]. Mounting evidence indicates that MLL-AF4 is the initiating leukemogenic event with an *in utero* origin [5, 25]. However, an understanding of potential changes in early hematopoietic development mediated by MLL-AF4 is lacking, despite of the recent advances by Krivtsov *et al*. [8] and Bursen *et al*. [26], current mouse models do not accurately recapitulate either the disease phenotype or latency [6, 8, 9]. Furthermore, studies using primary cells from MLL-AF4 patients are incapable of addressing the developmental genesis of the hematopoietic system. The lack of *bona fide* putative MLL-AF4 oncogene models suggests that these approaches may be missing some essential aspects impacting hematopoietic cell fate during early human development. Postnatal (CB-derived) or prenatal (hESC-derived) stem cells represent ontogenically primitive target cells to address the developmental impact of MLL-AF4. Very recently, Montes *et al*. [9] explored for the first time the effect of MLL-AF4 on the fate of human neonatal HSPCs. MLL-AF4-expressing CB-HSPCs displayed an enhanced *in vivo* hematopoietic engraftment and *in vitro* clonogenic potential, but MLL-AF4 expression was not sufficient for leukemogenesis, indicating that either additional hits are required to develop leukemia or that CB-derived HSPCs do not represent the appropriate target for MLL-AF4 to induce transformation.

hESCs and hESC-derivatives enable the study of unique aspects of early human development that cannot otherwise be addressed by patient sample analyses or mouse models [11, 12]. The fact that leukemogenesis manifests as a blockage or altered cell differentiation suggests that hematopoietic differentiation of hESCs could become a promising human-specific strategy to study the onset of hematopoiesis, particularly the emergence of the earliest events leading to the specification of the hematopoietic tissue [12]. Previous studies made use of the BCR-ABL fusion gene to promote hematopoietic proliferation in mouse ESCs [27, 28] and only a very recent study has studied the impact of Nup98-HoxA10 fusion oncogene on hESC-derived hematopoiesis [29]. However, this is the first study exploring the developmental impact of a leukemic fusion known to arise *in utero* on hESC hemato-endothelial development.

Despite being a leukemic oncogene, MLL-AF4 expression in hESCs or hESC-derived hematopoietic cells did not transform either *in vitro* or *in vivo*. *In vitro,* MLL-AF4+ hESC-derived hematopoietic cells did not display any proliferative and/or survival advantage and failed to confer replating efficiency in hematopoietic CFU assays, whereas *in vivo*, MLL-AF4 failed to endow hESC-derived hematopoiesis with engraftment potential upon xenotransplantation into NSG mice. This suggests that either additional hits or the reciprocal AF4-MLL [26] may be required for leukemogenesis, or that hESCs or hESC-derived hematopoietic derivatives are not the appropriate cellular targets for MLL-AF4-mediated transformation. It cannot be ruled out that other embryonic precursors (i.e., mesodermal precursors) or even fetal HSPCs may represent potential target cells in which MLL-AF4 originates and/or exerts its oncogenic function.

Importantly, MLL-AF4 did impact early hemato-endothelial specification from hESCs. It promoted the specification, rather than expansion, of early hemogenic precursors from differentiating hESCs. However, MLL-AF4 induced later developmental defects in the hematopoietic lineage as shown by a highly reduced production of both $CD45^+$ and $CD45^+CD34^+$ hematopoietic cells and hematopoietic clonogenic potential. As the process of *in vitro* hematopoietic differentiation from hESCs closely mirrors early events in embryonic hematopoietic development, our results provide the first indication showing how the leukemic fusion gene MLL-AF4 impairs embryonic blood formation when experimentally overexpressed in hESCs. This is in agreement with the traditional view that hematopoietic dysfunction in newborn ALL carrying MLL-AF4 fusion might already occur at the embryonic/fetal stage, supporting the existence of concordant leukemia in monozygotic twins and suggesting that MLL-AF4 originates early *in utero*. Unexpectedly, MLL-AF4 expression led to an enhanced mature endothelial cell fate of the hemogenic precursors. Because hESC-derived hemogenic precursors are uniquely responsible for endothelial and hematopoietic development [15, 21, 22, 30], these data indicate that MLL-AF4 does not block both endothelial and hematopoietic commitment of the hemogenic precursors but instead, it skews the hemato-endothelial potential of these hemogenic precursors towards a pronounced endothelial cell fate.

The leukemia fusion oncogene BCR/ABL, as well as lymphoma-specific genetic aberrations, has been found in endothelial cells from chronic myeloid leukemia and B-cell lymphoma patients. This suggests that endothelial cells may be part of the neoplastic clone [31, 32] and that hemangioblasts/hemogenic precursors rather than HSPCs appear to be target cells for the first oncogenic hit, which could occur during the first steps of ESC differentiation and/or in hemangioblasts persisting in adults. In line with this, bone marrow-derived mesenchymal stem cells (BM-MSCs) from 100% infant pro-B ALL were recently found to harbor and express the MLL-AF4 fusion gene, suggesting that MLL-AF4 might arise in a population of

pre-hematopoietic precursors [33]. However, whether a potential endothelial dysfunction in MLL-AF4+ pro-B ALL patients exists remains to be clinically assessed. Interestingly, it has recently been demonstrated the existence of a common precursor of MSCs and endothelial cells, the mesenchymoangioblast, using hESCs directed towards mesendodermal differentiation [34]. The existence of such a common embryonic precursor for MSCs and endothelium, and the reported expression of MLL-AF4 in both leukemic blasts and MSCs in the BM of infant patients suggest that MLL-AF4 fusion might arise and display a developmental impact on early pre-hematopoietic mesodermal or hemangioblastic precursors.

MLL fusions are well-known positive regulators of homeobox gene expression [18, 19]. Interestingly, Stam *et al*. [19] have reported the existence of two distinct subgroups among t(4;11)-positive infant ALL cases characterized by the absence or presence of HOXA expression, with those patients lacking HOXA expression being at extreme high risk of disease relapse. In contrast, Trentin *et al*. [35] have recently questioned the function of activated Hox gene expression in t(4;11)-positive infant ALL. These patients were sub-divided into Hox gene expression high vs low and no biological/clinical relevance could be demonstrated. In line with this, MLL-AF4 expression in our experimental system robustly upregulated global Hox gene expression that was not oncogenic in a variety of assays, suggesting that activated Hox gene expression might be on its own not important for ALL.

Additionally, the enhanced specification of MLL-AF4 hESCs towards early hemogenic precursors (hemangioblasts) prone to subsequent endothelial maturation is reflected by gene expression analysis revealing a marked upregulation of key genes associated to neovasculature functions (VEGFA, VEGFC, CD34, KDR, TIE1, TEK, SELE, NRP1, NRP2 and several members of the collagen family) and early hematopoiesis (SCL/TAL1, RUNX1/AML1, GATA2, CD34, BMP4 and HOXB4), suggesting that MLL-AF4 hESCs seem transcriptionally primed to differentiate towards early hemogenic precursors as compared to NEO hESCs. Importantly, Guenther *et al*. [36] recently reported a list of 42 direct targets of MLL-AF4. Despite constituting different experimental approaches (non-transformed hESCs vs fully transformed leukemia cell lines), we have compared our GEP with the MLL-AF4 direct targets proposed by Guenther *et al*.[36] and found that 8 (ERG, TNRC18, ADAM10, HOXA10, HOXA9, MEIS1, MEF2C, ZEB2) out of these 42 (20%) MLL-AF4 direct targets were also upregulated in our experimental system.

We have also compared our GEP with the GEP reported by Trentin *et al*. [35] and Stam *et al*. [19]. As shown in Supplementary information, Figure 5SA, few genes were commonly found regulated not only among the three studies, but also between Trentin *et al*. [35] and Stam *et al*. [19] (< 19 genes). Interestingly, Meis1, a key Hox co-factor implicated in leukemogenesis, was found upregulated in the three studies. The groups of genes differentially upregulated in the three studies were compared using IPA software. IPA software-based data mining generated a list of significantly upregulated gene functions and canonical pathways between MLL-AF4+ hESC/infant B-ALL and NEO hESCs/normal BM. As shown in Supplementary information, Figures 5SB, 5SC and S6, genes differentially upregulated in both MLL-AF4+ hESCs and MLL-AF4+ infant B-ALL were classified as involved in hematopoiesis, tissue development, VEFG signaling and cardiovascular/vascular-endothelial system development. It is crucial to be aware that these two GEP studies based on primary patient samples are conceptually distinct. Trentin *et al*. [35] compared MLL-AF4+ infant B-ALL with normal bone marrow whereas Stam *et al*. [19] compared MLL-AF4+ infant B-ALL with other MLL germline ALL, indicating that because gene expression data has been normalized against different primary samples, caution must be taken when comparing independent GEP studies. Additionally, caution is also required when comparing independent GEP studies mediated by MLL-AF4 because secondary oncogenic events already present in patient samples [19, 35] may contribute along with MLL-AF4 to the gene expression signatures observed in these clinical samples, whereas in our transgenic hESC model we studied the impact of gene expression of MLL-AF4 on its own (in a non-leukemic background).

Our experimental approach may constitute a new system to study the cellular and molecular mechanisms underlying MLL-AF4-mediated human early embryonic development. Because MLL-AF4 expression does not alter pluripotency, the unlimited replicative potential of hESCs enables the production of MLL-AF4-expressing embryonic hematopoietic, endothelial and MSC cells for studies that were previously unfeasible. Long-term, large-scale culture of MLL-AF4 hESC-derived hematopoietic cells or hemogenic precursors provides an unprecedented system for drug screening and toxicity studies. It also offers a unique *in vitro* system to test the ability of potential cooperating oncogenic events (reciprocal AF4-MLL or FLT3-activating mutations) or causal genotoxic compounds to induce leukemic transformation *in vitro*, characterized by the outgrowth of malignant B-lymphoid/monocyte clones [29, 37, 38]. Taken together, our results provide the first indication showing how the leukemic fusion gene MLL-AF4 when overexpressed in hESCs impairs embryonic blood formation, establishing

a potential novel experimental system to further study the developmental impact of MLL-AF4.

Materials and Methods

Plasmid construction and lentiviral transduction

The MLL-AF4 cDNA (MLL exon 10 was fused to AF4 exon 8; kindly provided by Professor Eric So, King's College London) was subcloned into the PmeI site of pRRL-EF1α-PGK-NEO vector (kindly provided by Professor L Naldini, ISR, Milano, Italy) [9]. The following lentivectors were used: pRRL-EF1α-PGK-NEO (control; NEO) and pRRL-EF1α-MLLAF4-PGK-NEO (MLL-AF4). Viral particles pseudotyped with VSV-G were generated on 293T cells by standard calcium-phosphate transfection protocol and concentrated by ultracentrifugation as previously described [33]. hESCs were infected overnight on the day of passage with concentrated virus in the presence of polybrene at 8 μg/ml (Sigma-Aldrich). At the following day, the viral supernanant was removed and infected hESCs were washed with fresh media and maintained in culture. After 3 days, transduced cells were selected with G418 (Invitrogen) at 50-100 μg/ml for 3 weeks. MLL-AF4 expression was confirmed in selected cells before being used for further experiments.

Human ESC culture

H9 and AND-1 hESCs were maintained undifferentiated in a feeder-free culture as previously described [14, 39]. Briefly, hESCs were cultured in Matrigel (BD Biosciences, Bedford, MA, USA)-coated T25 flasks in human feeder conditioned medium (CM) supplemented with 8 ng/ml basic fibroblast growth factor (bFGF; Miltenyi, Germany) [40]. Media was changed daily, and the cells were split weekly by dissociation with 200 U/ml of collagenase IV (Invitrogen, Edinburgh, Scotland). Human ESC cultures were visualized daily by phase contrast microscopy. Approval from the Spanish National Embryo Ethical Committee was obtained to work with hESCs.

RNA isolation, RT-PCR and qPCR analysis

Total RNA was isolated from undifferentiated hESCs, hEBs or hemogenic precursors using the Total RNA Purification Kit (Norgen, Canada) followed by RNase-free DNase treatment (Invitrogen). cDNA synthesis was done with 0.5 μg of total RNA using the First-Strand cDNA Synthesis Kit (Amersham, PA, USA). The resulting cDNA was used for conventional and qPCR. In qPCR experiments, the expression of each target gene was normalized to the expression of β-actin. qPCR was performed using power SYBR Green PCR Master Mix (Applied Biosystems) and the 7500 Real Time PCR System. Primer sequences used in this study are shown in Supplementary information, Table S2. For comparative expression of MLL-AF4 in transduced human stem cells and MLL-rearranged leukemic lines (SEM and RS4;11), the following primers (MLLAF4-Fw, 5′-CAGGTCCAGAGCAGAGCAAAC-3′ and MLLAF4-Rw, 5′-GAGCACTTGGAGGTGCAGATG-3′) and qRT-PCR conditions (95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s) were used.

Flow cytometry characterization of hESCs

Flow cytometry analysis was carried out as previously described [41]. Briefly, hESC cultures were dissociated with trypsin-EDTA and the single cell suspension was stained (2-5 × 10^5 cells/ml) with TRA-1-60-PE, TRA-1-81-FITC, SSEA3-PE and SSEA-4-FITC (all from BD Biosciences). The cells were then washed and stained with 7-AAD (BD Biosciences) for 15 min at room temperature. Cells were analyzed using a FACS Canto-II flow cytometer.

Western blot analysis

hESC cultures (3-5 × 10^5 cells) were dissociated with trypsin-EDTA for 10 min and the single cell suspension was subsequently lysed in 50 μl of RIPA buffer containing protease inhibitors for 30 min. 30 μl of the whole-cell lysate was mixed with 10 μl of loading buffer and resolved on 8% SDS-PAGE and transferred to PVDF membranes using a semi-dry transfer apparatus (at 15 V for 1 h). The membrane was blocked for 30 min with the blocking reagent BM Chemiluminiscence Western Blotting Kit (Roche). For MLL detection, the membrane was incubated overnight with anti-MLL antibody (1:100 dilution; clone N4.4; Millipore) followed by 2 h incubation with anti-mouse-HRP (Millipore). The MLL-AF4 fusion protein (250 KDa) was detected with the BM Chemiluminiscence Western Blotting Kit (Roche).

In vivo teratoma formation

Animal protocols were approved by the Local University Hospital Council On Animal Care and Experimentation. *In vivo* pluripotency was tested as previously described [42]. Briefly, hESCs were harvested and implanted beneath the testicular capsule of 8-week-old immunodeficient male mice (The Jackson Lab, Bar Harbor, MA, USA). Teratoma growth was determined by palpation every week, and the mice were sacrificed ~8-10 weeks after implantation. Teratomas were fixed, embedded in paraffin, and sections were stained with hematoxylin and eosin as described [42].

Hematopoietic differentiation from hESCs

Undifferentiated hESC cells at confluence were treated with collagenase IV and scraped off from the Matrigel attachments. They were then transferred to low-attachment plates (Corning, NY, USA) to allow hEB formation by overnight incubation in differentiation medium consisting of KO-DMEM supplemented with 20% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Medium was changed the next day (day 1) with the same differentiation medium supplemented with hematopoietic cytokines: 300 ng/ml SCF, 300 ng/ml Flt-3L, 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml G-CSF and 25 ng/ml BMP-4 [14, 30, 37, 43]. hEBs were dissociated using collagenase B (Roche Diagnostic, ON, Canada) for 2 h at 37 °C followed by 10 min incubation at 37 °C with Cell Dissociation Buffer (Invitrogen) at days 4, 7, 11 and 15 of development. A single cell suspension achieved by gentle pipetting and passaged through a 70-μm cell strainer was stained with anti-CD34-FITC, anti-CD31-PE, anti-CD45-APC (all from Miltenyi, Germany) and 7-AAD. Live cells identified by 7-AAD exclusion were analyzed using a FACS-Canto II flow cytometer (BD Biosciences). Hemogenic precursors with hemangioblastic properties were identified as $CD34^+CD31^+CD45^-$. Immature and mature blood cells were identified as $CD45^+CD34^+$ and $CD45^+CD34^-$, respectively (Figure 2B) [14, 15, 30, 37].

Colony forming unit assay

Human clonogenic progenitor assays were performed by plating 50 000 cells from day 15 and day 22 hEBs into methylcellu-

lose H4230 (Stem Cell Technologies, Vancouver, Canada) supplemented with recombinant human growth factors: 50 ng/ml SCF, 3 units/ml erythropoietin, 10 ng/ml GM-CSF and 10 ng/ml IL-3. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere and at day 14 colonies were counted with CFU assay using standard morphological criteria [14, 43, 44]. For secondary re-plating, all the CFU colonies from each experimental condition (NEO and MLL-AF4) were harvested from the methylcellulose and a single cell suspension was achieved and re-plated as above [9].

Mice transplantation and analysis of engraftment

NOD/LtSz-scid IL-2R$\gamma^{-/-}$ mice (NSG) were housed under sterile conditions. The Animal Care Committee of the University of Granada approved all mouse protocols. Briefly, CB-derived $CD34^+$ HSPCs (n = 3, 3 × 10^4 cells in 50 μl) as well as NEO (n = 12) and MLL-AF4 (n = 15) day 15 hEB hematopoietic differentiating cells were transplanted intrahepatically (n = 20; 4-12 × 10^4 cells in 50 μl) into newborn NSG mice or intra-bone marrow (n = 7; 7-8 × 10^5 cells in 30 μl) as previously described in detail [45]. Mouse health was monitored throughout the entire experiment. Mice were killed 6-8 weeks post-transplant and BM, spleen and liver were collected and analyzed for human chimerism. Cells from BM, spleen and liver were stained with anti-HLA-ABC-FITC, anti-CD31-PE and anti-CD45-APC (Beckton Dickinson) to analyze human chimerism by flow cytometry. Engrafted mice were assessed for multilineage analysis using anti-CD33-PE for myeloid cells, anti-CD19-APC for B-cells and anti-CD34-PE for immature hematopoietic cells (all from Miltenyi) [45].

GEP and data analysis

hESC samples were collected during the exponential cell growth phase and stabilized in RNAlater solution (Ambion, Austin, TX, USA) until RNA extraction. RNA was isolated using Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA) and its quality was checked in the Agilent 2100 Bioanalyzer platform. 500 ng of each total RNA sample was labeled with Cy3 using the Quick-Amp Labeling kit and hybridized with the Gene Expression Hybridization Kit to a Whole Human Genome Oligo Microarray (G4112F; 45 000 probes corresponding to 25 000 genes; Agilent Technologies) following the manufacturer's instructions. Each sample was labeled and hybridized as independent triplicates. Primary data was examined using GeneSpring 11.0 software (Silicon Genetics, Redwood City, CA, USA). A gene was considered differentially expressed if it was more than 2-fold regulated (up or down) in MLL-AF4 as compared to NEO hESCs. A t-test and a Benjamini Hochberg multi-testing correction were performed to better judge the significance of the regulated genes. Only genes satisfying the threshold of P value < 0.05 and a fold-change expression > 2 were included and assigned as significant. Pools of genes that were differentially expressed were clustered according to their expression pattern dynamics into hierarchical tree clustering algorithms using the Pearson's centered correlation distance definition as similarity measure and Centroid's as the linkage rule. Analysis of gene functions and canonical pathways significantly altered by MLL-AF4 was performed using the Ingenuity Pathway Analysis (IPA) software 8.0 (Ingenuity Systems, Inc., Redwood City, CA, USA). Several genes were confirmed by qPCR. Microarray data has been deposited and is available at Gene Expression Omnibus (GSE29869; http://www.ncbi.nlm.nih.gov/geo/).

Cell cycle analysis of hESCs, hemogenic precursors and $CD45^+$ hematopoietic cells

Undifferentiated hESC cultures were stained with 10 μM BrdU (Sigma) for 20 min as previously described [9]. The cells were then harvested, fixed in 70% ice-cold ethanol, and stored overnight at –20 °C. Subsequently, the cells were washed with PBS followed by 30 min incubation with 2 M HCl to depurinate the DNA. After washing with PBS containing 0.1% BSA and 0.2% Tween 20, the cells were incubated with 2 μl of anti-BrdU-FITC (BD Biosciences) for 30 min. After washing, the cells were resuspended in PI buffer containing 5 μg of PI and 100 μg/ml of RNAase in PBS.

For cell cycle analysis of hESC-derived hemogenic precursors and $CD45^+$ hematopoietic cells, the hEBs were dissociated at day 11 and day 22 of development, respectively. Cells were then washed with PBS and incubated with anti-CD31-FITC and anti-CD34-FITC or anti-CD45-FITC, respectively, for 15 min. After washing, the cells were suspended in PI buffer as described above. BrdU staining of undifferentiated hESCs and cell cycle distribution of hemogenic precursors was analyzed on a FACS Canto-II cytometer discriminating among apoptotic cells (Sub-G0/G1), quiescent cells (G0/G1), cycling cells (S-phase, BrdU+) and G2/M cells [46, 47]. The apoptotic status of NEO and MLL-AF4 hESC-derived $CD45^+$ hematopoietic cells was assessed using the Annexin-V apoptosis detection kit (BD Biosciences), according to the manufacturer's instructions [9]. Briefly, day 22 EBs were dissociated and washed twice with cold PBS before staining with anti-CD45-FITC, Annexin-V-PE and 7-AAD. Apoptotic cells were detected by gating the Annexin V+/7-AAD+ fraction.

Endothelial differentiation from hESCs, uptake of acetylated LDL and in situ immunocytochemistry

$CD34^+$ cells were isolated from hEBs at day 11 of development by magnetic-activated cell sorting (MACS) using the hCD34 MicroBead kit and the AutoMACS Pro separator (Miltenyi Biotech), as per manufacturer's instructions [37, 46]. To promote endothelial differentiation, isolated $CD34^+$ cells were seeded on 0.1% gelatin-coated plates at 1.2 × 10^4 cells/cm^2 in complete EGM-2 media (Lonza, Walkersville) for 5-7 days. For uptake of acetylated LDL, cells were incubated with 10 μg/ml of Dil-Ac-LDL (Molecular Probes) at 37 °C for 4 h. After fixation and permeabilization, cells were stained with rabbit anti-human VE-cadherin (Cayman, MI, FL, USA), mouse anti-human eNOS (BD Biosciences), mouse anti-human vWF (DAKO) and mouse anti-human CD45 (BD Biosciences) followed by FITC-conjugated anti-rabbitt or Cy3-conjugated anti-mouse antibodies (Jakson Immunoresearch), respectively.

For capillary-like tube formation assay, MACS-isolated $CD34^+$ cells were expanded for 5-7 days in complete EGM-2 media and 1.2 × 10^4 cells/well were then seeded on top of 25 μl of Matrigel previously spreaded onto a 96-well plate. Pictures were captured (5× objective) at the indicated times with an Axiocam MRM digital camera (Zeiss) attached to an AxioImager A1 microscope (Zeiss). For quantification, all the images were analyzed with the WimTube software (www.wimasis.com; Wimasis SL, Munich, Germany). This object recognition tool identifies cellular tubes, branching points and loops automatically without manual adaptation.

Clonal experiments with FACS-sorted single hemogenic precursors

Single $CD34^+CD31^+CD45^-$ hemogenic precursors FACS-isolated from day 11 NEO hEBs or MLL-AF4 hEBs were deposited into individual wells of 96-well plates and inspected daily. Ten 96-well plates (~960 single cells) were used per condition. Single cell sorting was carried out using a FACSAria sorter equipped with an Automatic Cell Deposition Unit (ACDU). Sorted single cells were allowed to expand and differentiate in 200 μl of a 50:50 mixture of hematopoietic (100 μl of StemSpam supplemented with BMP4, SCF, FLT3 and IL3) and endothelial (100 μl of complete EGM-2 media) supportive media. After 12-15 days, the resulting clonal outgrowth in each well was analyzed *in situ* by phase contrast morphology and immunocytochemical staining for CD45 (hematopoietic) and VE-cadherin (endothelial). The nuclei were counterstained with DAPI [15].

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)

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RESEARCH HIGHLIGHT

MLL-AF4 driven leukemogenesis: what are we missing?

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Pre-leukemic MLL-AF4 fusions arise prenatally and typically lead to overt acute lymphoblastic leukemia (ALL) at or shortly after birth. In a recent study, Bueno and colleagues explored the effects of MLL-AF4 expression in human embryonic stem cells (hESCs), with a focus on early hemato-endothelial development.

Infant pro-B acute lymphoblastic leukemia (ALL) harboring MLL-AF4 fusion proteins instigated by chromosomal translocation t(4;11), represents an aggressive, high-risk type of childhood leukemia, characterized by a very brief disease latency and undisputedly originates *in utero*. Despite of recent advances and multiple important breakthroughs, MLL-AF4-driven leukemogenesis remains difficult to model and mouse models that accurately recapitulate the disease phenotype and latency are still lacking. Among several remarkable studies recently published, Montes *et al*. [1] demonstrated that enforced expression of MLL-AF4 in cord blood-derived hematopoietic stem cells (HSCs) increased the clonogenic potential of $CD34^+$ progenitors and promoted proliferation, but appeared insufficient to induce leukemia. This study undeniably questions whether MLL-AF4 fusion proteins are capable of driving leukemogenesis on their own or whether additional genetic events are required such as *RAS* mutations [2]. However, recent whole genome sequencing analysis in primary *MLL*-rearranged infant ALL samples revealed the presence of remarkably few somatic mutations [3]. Contributing to the complexity of the matter, Bursen *et al*. [4] recently showed that introducing the reciprocal fusion protein AF4-MLL (resulting from the same balanced t(4;11) translocation), but not MLL-AF4, into murine hematopoietic stem/progenitor cells induced ALL in mice without the requirement of MLL-AF4. Nonetheless, these experiments have not yet been performed in human HSCs. Moreover, MLL-AF4 and AF4-MLL knockdown experiments have shown that t(4;11)-positive cell lines display addiction to MLL-AF4 (which appeared essential for leukemic cell proliferation and survival), but not to AF4-MLL [5]. Thus, the AF4-MLL fusion protein may well be important or essential in the early transformation process and the MLL-AF4 fusion is certainly required for the maintenance of the leukemia. However, although the studies by Montes *et al*. [1] and Bursen *et al*. [4] seem to support that MLL-AF4 by itself is not sufficient to induce leukemogenesis in HSCs, others were able to induce lymphoid leukemias using MLL-AF4 knockin models in mice [2, 6]. Yet, in these latter studies both the disease phenotye and latency of the observed leukemias appeared to deviate from the highly immature pro-B cell phenotype characteristically found in humans.

While the above described contradictions make it difficult to draw solid conclusions on the oncogenic potential of the MLL-AF4 fusion protein itself, there is another important question to be asked and answered: Are these MLL-AF4-driven leukemogenesis studies targeting the right cells? In a recent paper published by *Cell Research*, Bueno *et al*. [7] elegantly attempted to address this question by creating a human-specific cellular system to study early hemato-endothelial development in MLL-AF4-expressing human embryonic stem cells (hESCs). A recent report showed that bone marrow-derived mesenchymal stem cells from primary t(4;11)-positive pro-B infant ALL patients harbor and express the MLL-AF4 fusion gene [8]. Thus, MLL-AF4 may well arise prenatally in pre-hematopoietic mesodermal or hemangioblastic precursors sprouting from differentiating hESCs [9] rather than in more committed HSCs. From this perspective, Bueno *et al*. [7] introduced MLL-AF4 expression in hESCs and monitored the consequences. Interestingly, enforced MLL-AF4 expression in hESCs led to the accelerated emergence and elevated frequencies of hemogenic precursors. Moreover, in these hESCs, MLL-AF4 appeared to act as a global transcriptional activator, positively regulating homeobox gene expression, which is

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in line with what is usually (but not always) observed in primary t(4;11)-positive infant ALL samples [10]. Nevertheless, the MLL-AF4 fusion protein was not able to transform hESC-derived hematopoietic cells, but instead strongly impaired subsequent hematopoietic commitment in favor of an endothelial cell fate. Unfortunately, the latter brings us back to the same persistent conundrum: In order to successfully induce MLL-AF4-positive leukemia, did this system adopted by Bueno *et al*. require additional genetic hits such as the presence of the AF4-MLL fusion or *RAS* mutations? Or, despite the relevant rationale behind targeting hESC-derived pre-hematopietic precursors, did these cells not reflect the correct equivalents from which t(4;11)-positive pro-B ALL in infants originate? Perhaps MLL-AF4-positive infant ALL does arise in HSCs or early HSC progenitors, but not in those obtained from cord blood or the bone marrow. Given the strong body of evidence supporting that MLL-AF4 fusions arise during embryonic development [11, 12] when hematopoiesis still mainly takes place in the liver, the correct target cells, e.g., hematopoietic or specific lymphoid-monocytic stem cells [13], should possibly be searched for in the fetal liver.

Nonetheless, the study presented by Bueno *et al*. [7] provides unique insights into how MLL fusions regulate human embryonic hematopoietic specification and represents an intriguing experimental system to study the impact of MLL fusions from a developmental point of view. Hopefully this or similar approaches will further be exploited to unravel the riddle of MLL-AF4-driven leukemogenesis.

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7.- Prognostic significance of FLT3 mutational status and expression levels in MLL-AF4+ and MLL-germline acute lymphoblastic leukemia.

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INTRODUCCIÓN. La leucemia con reordenamiento MLL-AF4 está siendo particularmente difícil de modelar, sugiriendo la existencia de eventos secundarios a la aparición prenatal de la traslocación MLL-AF4, como cooperantes necesarios a la aparición de la enfermedad. Con respecto a la activación de FLT3 en LLAs con reordenamiento de *MLL*, unos autores postulan que son las mutaciones de FLT3 (puntuales o ITD) las responsables de esta activación, mientras que otros la atribuyen a una sobrexpresión en ausencia de mutaciones.

OBJETIVO. Analizar la presencia de mutaciones de *FLT3* y los niveles de expresión de FLT3 en una serie de muestras de pacientes con LLA. Determinar si *FLT3* mutado o sobrexpresado constituye un factor pronóstico a tener en cuenta en el desarrollo de la enfermedad.

MATERIAL Y MÉTODOS. Se tomaron muestras de MO de 54 pacientes con LLA-B y LLA-T al diagnóstico así como de donantes sanos que fueron usados como control negativo para mutación de *FLT3* y calibrador de la expresión de *FLT3*. RT-PCR se realizó para detectar la mutación ITD. Para las mutaciones puntuales (D835Y o D836del) el ensayo RFLP sobre la amplificación del exón 20 de *FLT3* (dominio tirosina kinasa). En ambos casos se realizó un análisis de secuencia para confirmar. La cuantificación de la expresión génica de *FLT3* se realizó mediante qPCR. La probabilidad de supervivencia total y de superviviencia libre de enfermedad se realizó mediante el método Kaplan-Meier, mientras que el modelo de regresión Cox se utilizó para valorar el valor predictivo de múltiples variables al diagnóstico en relación a la supervivencia total y libre de enfermedad en un análisis multivariable.

RESULTADOS. Se estudiaron 54 casos de LLA-B y LLA-T de nuevo diagnóstico, agrupándose las LLA-B de la siguiente forma atendiendo al estudio citogenético: MLL-AF4+, TEL-AML1+, BCR-ABL+, E2A-PBX1+, o de citogenética normal. No se detectaron ni mutación/deleción en los codones D835/I836 del dominio tirosín kinasa, ni mutación ITD en el dominio juxtamembrana en ninguno de los casos. La expresión génica de *FLT3* mostró que ésta variaba dependiendo del grupo citogenético estudiado, correspondiendo la expresión más alta a los pacientes con LLA-B MLL-AF4+. Así mismo, cuando los pacientes fueron estudiados de manera independiente según la expresión de *FLT3*, únicamente en los pacientes MLL-AF4+, la expresión de *FLT3* tenía valor pronóstico, con una supervivencia total (0% *vs* 71%;$p<0.002$) y una supervivencia libre de enfermedad a 5 años (0% *vs* 43%;$p=0.03$) significativamente menor cuando la expresión de *FLT3* era alta (por encima de la media).

CONCLUSIÓN. Las LLAs no presentan mutaciones de *FLT3*, indicando que la sobrexpresión del receptor es la vía más probable de su activación constitutiva. Por otro lado, los pacientes con LLA MLL-AF4+ son los que presentan mayor sobrexpresión del receptor, siendo en este grupo el único de los estudiados donde el nivel de expresión de *FLT3* supone un factor pronóstico de relevancia.

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ORIGINAL ARTICLE

Prognostic significance of FLT3 mutational status and expression levels in MLL-AF4 + and MLL-germline acute lymphoblastic leukemia

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There is barely any information about the prognostic significance of *FLT3* expression and mutational status in cytogenetically distinct subgroups of acute lymphoblastic leukemia (ALL). We analyzed the presence of FLT3-tyrosine kinase domain (TKD) and FLT3-internal tandem duplication (ITD) mutations as well as *FLT3* expression levels in 54 newly diagnosed patients with B-ALL ($n = 49$) or T-ALL ($n = 5$). All B/T-ALL samples tested negative for the presence of FLT3-TKD or FLT3-ITD. None of the T-ALL and E2A-PBX1 + B-ALL overexpressed *FLT3*. In contrast, mainly MLL-AF4 + B-ALL but also ETV6-RUNX1 +, BCR-ABL + or B-ALL displaying normal cytogenetics exhibited significantly higher *FLT3* expression levels than normal bone marrow, supporting that aberrantly increased transcription of *FLT3*, rather than activating *FLT3* mutations, contributes to the pathogenesis of these B-ALL. Using the median *FLT3* expression as cut-off value we found that high-level FLT3 expression is associated with an extremely poor 1-year overall survival (OS; 0 vs 71%; $P = 0.002$) and disease-free survival (DFS; 0 vs 43%; $P = 0.03$) in MLL-AF4 + B-ALL but not in MLL-germline B-ALL. Cox regression analysis with OS/DFS as end points showed that age > 14 years and high-level *FLT3* expression were independent prognostic factors when all ALL patients were analyzed together. Importantly, when the MLL-AF4 + B-ALL subgroup was analyzed separately, high-level *FLT3* expression was the only independent prognostic factor for OS and treatment outcome. These findings indicate that high *FLT3* expression identifies MLL-AF4 + ALL patients at very high risk of treatment failure and poor survival, emphasizing the value of ongoing/future clinical trials for FLT3 inhibitors.

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Keywords: FLT3 mutations; FLT3 expression; MLL-AF4; B-ALL; T-ALL; fusion genes

INTRODUCTION

MLL-AF4 leukemogenesis has been particularly difficult to model and *bona fide* MLL-AF4 disease human models do not exist. Our current understanding of transformation by MLL-AF4 and its mode of action comes from murine models in which leukemias do not recapitulate the human disease faithfully. Existing murine models of MLL-AF4 acute leukemia have shown an extraordinary long latency period before overt disease develops, suggesting that additional genetic insults are required to generate leukemia.[1–4] Gene expression profiling showed that the FMS-related tyrosine kinase-3 (*FLT3*) is highly expressed in MLL-rearranged acute lymphoblastic leukemia (ALL),[5] leading to the characterization of *FLT3* mutations as potential secondary cooperating events.[6] Accordingly, Ono *et al.*[7] and Yamaguchi *et al.*[8] have reported that constitutively activating *FLT3* mutations cooperate with MLL-ENL and MLL-AF4, respectively, to induce leukemia in murine models.

Although multiple studies have shown that activating mutations of *FLT3* are common in AML,[9–11] there is controversy about the presence of such mutations among different subtypes of ALL. Several groups reported the presence of *FLT3*/tyrosine kinase domain (TKD) mutations in 3–20% of MLL-rearranged ALLs,[6,12–14] whereas Stam *et al.*[15,16] together with Bardini *et al.*,[17,18] using high-resolution genomic analysis, showed that *FLT3* mutations in MLL-rearranged ALLs do not occur. Despite this emerging controversy, *FLT3* is consistently highly expressed in MLL-rearranged ALL, and therefore it is not fully resolved whether the constitutive activation of *FLT3* in MLL-rearranged ALL is due to the presence of activating *FLT3* mutations, or simply due to a transcriptional increased expression of *FLT3* in an attempt to provide blast cells with survival and proliferative advantage. The latter has been recently suggested by Guenther *et al.*,[19] by showing through elegant studies that *FLT3* may be a direct target of MLL-AF4.

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Interestingly, there is little information about the prognostic significance of *FLT3* expression and mutational status in cytogenetically distinct subgroups of ALLs. For this reason, we aimed to analyze the presence of *FLT3* TKD mutations and *FLT3*-internal tandem duplication (ITD) as well as *FLT3* expression levels in 54 newly diagnosed patients with B-ALL ($n=49$) or T-ALL ($n=5$). Our data reveal that *FLT3* mutations do not occur in cytogenetically different subgroups of ALL. However, we report a significant clinical relevance of *FLT3* expression in MLL-AF4 B-ALL but not in MLL-germline ALL. High *FLT3* expression identifies those MLL-AF4+ B-ALL patients at very high risk of treatment failure and extremely poor survival, emphasizing the value of ongoing and future clinical trials for FLT3 inhibitors.

MATERIALS AND METHODS

Patients

Bone marrow samples from 54 newly diagnosed untreated patients with B-ALL ($n=49$) or T-ALL ($n=5$) were studied. Diagnosis of the different B-cell malignancies was based on clinical, morphologic, immunophenotypic, cytogenetic and molecular criteria according to the WHO classification.[11,20] Acute leukemias were cytogenetically grouped as follows: MLL-AF4+ B-ALL ($n=17$), ETV6-RUNX1+ B-ALL ($n=13$), BCR-ABL^{P190}+ B-ALL ($n=8$), E2A-PBX1+ B-ALL ($n=4$), cytogenetically normal B-ALL ($n=7$) and T-ALL ($n=5$). Table 1 summarizes the diagnosis, cytogenetics and age for each patient. Median age for each group was 18 ± 17 for MLL-AF4+ B-ALL, 5.3 ± 3 for ETV6-RUNX1+ B-ALL, 44 ± 20 for BCR-ABL^{P190}+ B-ALL, 23 ± 8 for E2A-PBX1+ B-ALL, 6.7 ± 3 for cytogenetically normal B-ALL and 27 ± 19 for T-ALL. Ten normal bone marrow (NBM) samples were obtained from healthy volunteers and used as negative controls for *FLT3* mutations and as calibrator for *FLT3* gene expression. In all cases, informed consent according to the Local Ethics Committee of the University Hospital was given prior to entering the study.

RNA extraction and cDNA synthesis

RNA extraction and reverse transcription was performed using the Europe against Cancer Group (EAC) protocol.[21] Briefly, 1 μg of total RNA was added to a 20-μl volume containing random hexamers as primers and 100 U of SuperScript RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The mixture was incubated at 42 °C for 60 min, followed by 3 min at 99 °C and 2 min at 4 °C. Aliquots were stored at −80 °C prior to further analysis.

Determination of *FLT3* mutation status

FLT3-ITD was examined by amplification of the juxtamembrane domain spanning exons 14 and 15, as previously described.[22] To obtain the size and the relative level of mutations, real-time PCR was performed using a fluorescently-labeled primer with 6-FAM. Products were analyzed by Genescan analysis on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) (Supplementary Figure S1A). To detect point mutations in codon D835 or deletions of codon I836, the restriction fragment length polymorphism-mediated PCR assay was used by amplifying the exon 20 of the *FLT3* tyrosine kinase domain [22] (Supplementary Figure S1B). In all cases, a confirmatory sequencing analysis of the amplified products was performed with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Quantification of *FLT3* gene expression

The quantification of *FLT3* gene was performed using the Step One Plus Real-Time PCR System and TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The assay IDs were: Hs00245445_m1 (*ABL1*, as control gene) and Hs00174690_m1 (*FLT3*). Relative quantification was calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{FLT3} - Ct_{ABL1}$ and $\Delta\Delta Ct = \Delta Ct_{PATIENT} - \Delta Ct_{HEALTHY\ BM}$ as previously described.[23] In addition, *FLT3* gene expression of 10 BM samples from healthy volunteers was used as a calibrator.

Statistical Analysis

The association between variables was analyzed by the χ^2-square and the Fisher's exact tests for categorical variables and by the Student's *t*-test or the Mann–Whitney *U*-test for continuous variables. In addition, a Kruskal–Wallis test was also used to compare the relative gene expression between different ALL cytogenetic subgroups. The probability of disease-free (DFS) and overall survival (OS) was estimated according to the Kaplan–Meier method,[24] and the log-rank test was used to evaluate differences between survival distributions. OS was calculated from the date of diagnosis to the date of death or last follow-up, and DFS was calculated from the date of complete remission achievement to the date of relapse, death or last follow-up of the patient in continuous complete remission. The Cox regression model was used to assess the predictive value of multiple variables at diagnosis in relation to OS and DFS in multivariate analysis. The parameters were considered either as continuous or as categorical variables and only those reaching a P value <0.1 in the respective univariate analysis were included.[24,25] All P values were considered significant when <0.05. These analyses were performed using the SPSS 15.0 statistical software package (SPSS Inc., Chicago, IL, USA).

RESULTS

Absence of *FLT3*-TKD/ITD mutations in cytogenetically distinct groups of B- and T-ALL

By means of real-time PCR followed by Genescan analysis of PCR products and restriction fragment length polymorphism-mediated PCR assay, we determined the *FLT3* mutational status of 54 newly diagnosed untreated patients with B-ALL ($n=49$) or T-ALL ($n=5$). Neither point mutation/deletion in codons D835/I836 of the juxtamembrane TKD nor ITD of the *FLT3* locus could be detected in any case, regardless of the clinical subgroup (Table 1 and Supplementary Figure S1).

FLT3 expression varies among cytogenetically distinct groups of ALL

We then analyzed the *FLT3* gene expression relative to *ABL1* and normalized against the *FLT3* expression in NBM. The expression of *FLT3* varied between cytogenetically distinct groups of ALLs ($P<0.0001$) (Figure 1). The lowest *FLT3* expression level was found in E2A-PBX1+ B-ALLs (mean: 1.4 ± 0.7) and T-ALLs (mean: 0.9 ± 0.6). In contrast, patients with ETV6-RUNX1+, BCR-ABL^{P190}+ or cytogenetically normal B-ALL displayed a *FLT3* expression level 8.8-, 7.7- and 16-fold higher than NBM (Figure 1). Interestingly, MLL-AF4+ B-ALLs showed the highest *FLT3* expression (37-fold higher than NBM), confirming that very high constitutive expression of *FLT3* is a hallmark event in MLL-AF4+ ALLs. The increased expression of *FLT3* gene in the absence of *FLT3* mutations suggests that constitutively activated *FLT3* expression is a result of aberrantly increased transcription of *FLT3* and it is not due to *FLT3* activating mutations.

FLT3 expression level is a robust prognostic factor in MLL-AF4+ B-ALL but not in MLL-germline ALL

We analyzed whether *FLT3* expression is a prognostic factor for OS and DFS in B-ALL patients using the median *FLT3* expression as the cut-off value to divide all patients into two subgroups expressing high or low levels of *FLT3* (Figure 2). When all the ALL cases were analyzed together (regardless of the cytogenetic and phenotypic subgroup) we found that high-level *FLT3* expression was associated with a slightly lower OS ($P=0.224$) but did not associate with significantly poor treatment outcome (DFS; $P=0.491$) (Figures 2a and b). The 5-year OS estimate is 48% for patients expressing high levels of *FLT3* compared with 65% for patients displaying low-level *FLT3* expression (Figure 2a). However, the 5-year DFS estimate is 58% for high-level *FLT3* patients compared with 60% for low-level *FLT3* patients (Figure 2b).

Interestingly, when B-ALL patients were analyzed as MLL-AF4+ vs MLL-germline cohorts, *FLT3* expression was not a prognostic factor for MLL-germline ALL but for MLL-AF4+ B-ALLs (Figures 2c and d). MLL-AF4+ patients with high-level *FLT3* expression displayed a dramatically poor OS and DFS. The 1-year OS estimate

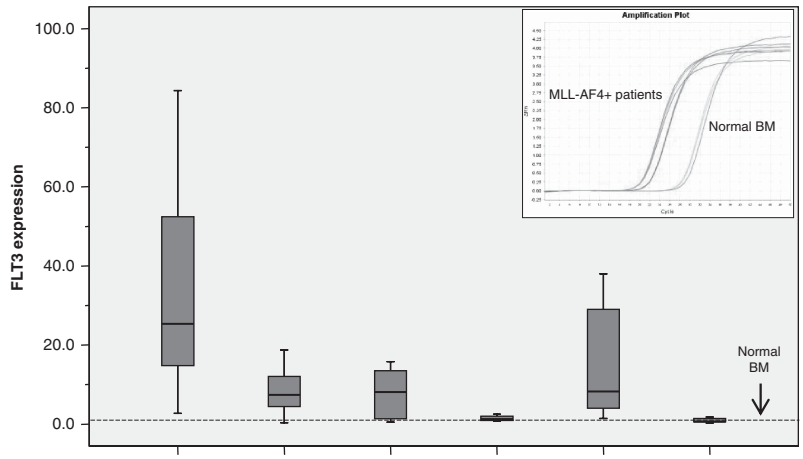
Table 1. FLT3 gene expression and FLT3 mutations in cytogenetically distinct B-ALL and T-ALL

Patient ID	*Diagnosis*	*Cytogenetics (fusion gene)*	*Age (years)*	*FLT3 gene relative expression**	*FLT3 gene mutation (ITD or TKD)***
1	B-ALL	t(4;11) *MLL-AF4*	29	110	Negative
2	B-ALL	t(4;11) *MLL-AF4*	1	24.5	Negative
3	B-ALL	t(4;11) *MLL-AF4*	15	66.6	Negative
4	B-ALL	t(4;11) *MLL-AF4*	4	14.5	Negative
5	B-ALL	t(4;11) *MLL-AF4*	0.1	84.3	Negative
6	B-ALL	t(4;11) *MLL-AF4*	45	37.9	Negative
7	B-ALL	t(4;11) *MLL-AF4*	61	62.1	Negative
8	B-ALL	t(4;11) *MLL-AF4*	31	15.5	Negative
9	B-ALL	t(4;11) *MLL-AF4*	0.3	3.53	Negative
10	B-ALL	t(4;11) *MLL-AF4*	28	44	Negative
11	B-ALL	t(4;11) *MLL-AF4*	23	25.5	Negative
12	B-ALL	t(4;11) *MLL-AF4*	4	33.3	Negative
13	B-ALL	t(4;11) *MLL-AF4*	40	25	Negative
14	B-ALL	t(4;11) *MLL-AF4*	35	14.8	Negative
15	B-ALL	t(4;11) *MLL-AF4*	42	2.7	Negative
16	B-ALL	t(4;11) *MLL-AF4*	0.5	4.1	Negative
17	B-ALL	t(4;11) *MLL-AF4*	0.5	65	Negative
18	B-ALL	t(12;21) *ETV6-RUNX1*	2	9.7	Negative
19	B-ALL	t(12;21) *ETV6-RUNX1*	13	12	Negative
20	B-ALL	t(12;21) *ETV6-RUNX1*	5	18.7	Negative
21	B-ALL	t(12;21) *ETV6-RUNX1*	8	12.2	Negative
22	B-ALL	t(12;21) *ETV6-RUNX1*	2	0.3	Negative
23	B-ALL	t(12;21) *ETV6-RUNX1*	5	25.5	Negative
24	B-ALL	t(12;21) *ETV6-RUNX1*	3	7.4	Negative
25	B-ALL	t(12;21) *ETV6-RUNX1*	6	4.8	Negative
26	B-ALL	t(12;21) *ETV6-RUNX1*	5	6.1	Negative
27	B-ALL	t(12;21) *ETV6-RUNX1*	8	2.1	Negative
28	B-ALL	t(12;21) *ETV6-RUNX1*	5	3.9	Negative
29	B-ALL	t(12;21) *ETV6-RUNX1*	3	4.4	Negative
30	B-ALL	t(12;21) *ETV6-RUNX1*	4	7.8	Negative
31***	B-ALL	t(9;22) *BCR-ABL*	58	0.5	Negative
32***	B-ALL	t(9;22) *BCR-ABL*	65	5.8	Negative
33***	B-ALL	t(9;22) *BCR-ABL*	30	15	Negative
34***	B-ALL	t(9;22) *BCR-ABL*	58	0.7	Negative
35***	B-ALL	t(9;22) *BCR-ABL*	60	11.7	Negative
36***	B-ALL	t(9;22) *BCR-ABL*	22	10.4	Negative
37***	B-ALL	t(9;22) *BCR-ABL*	20	1.8	Negative
38***	B-ALL	t(9;22) *BCR-ABL*	34	16	Negative
39	B-ALL	t(1;19) *E2A-PBX1*	23	2.4	Negative
40	B-ALL	t(1;19) *E2A-PBX1*	14	1.5	Negative
41	B-ALL	t(1;19) *E2A-PBX1*	35	0.75	Negative
42	B-ALL	t(1;19) *E2A-PBX1*	22	1	Negative
43	B-ALL	Normal cytogenetics	5	26	Negative
44	B-ALL	Normal cytogenetics	8	1.4	Negative
45	B-ALL	Normal cytogenetics	7	8.2	Negative
46	B-ALL	Normal cytogenetics	8	6.5	Negative
47	B-ALL	Normal cytogenetics	12	1.5	Negative
48	B-ALL	Normal cytogenetics	2	38	Negative
49	B-ALL	Normal cytogenetics	5	32	Negative
50	T-ALL	Normal cytogenetics	55	1.43	Negative
51	T-ALL	Normal cytogenetics	28	0.56	Negative
52	T-ALL	Normal cytogenetics	3	0.61	Negative
53	T-ALL	t(11;19) *MLL-ENL*	18	1.76	Negative
54	T-ALL	*SIL-TAL1*	31	0.25	Negative

Abbreviations: B-ALL, B-acute lymphoblastic leukemia; ITD, internal tandem duplication; T-ALL, T-acute lymphoblastic leukemia; TKD, tyrosine kinase domain. *FLT3 gene expression measured relative to normal bone marrow and normalized against ABL housekeeping gene. **FLT3 mutations analyzed were FLT3 ITD or the punctual mutations in the kinase domain (TKD) D835 and I836. ***All B-ALL patients harboring the t(9;22) do express the BCR-ABL isoform p190.

is 0% for patients expressing high levels of *FLT3* compared with 71% for patients displaying low-level *FLT3* expression ($P = 0.002$; Figure 2c). Similarly, the 1-year DFS estimate is 0% for patients expressing high levels of *FLT3* compared with 43% for patients displaying low-level *FLT3* expression ($P = 0.032$; Figure 2d). In contrast, for MLL-germline ALLs the 5-year OS and DFS estimates are 83% and 92%, respectively, for patients expressing high levels of *FLT3* compared with 63% and 54%, respectively, for patients displaying low-level *FLT3* expression (OS: $P = 0.22$; DFS: $P = 0.1$; Figures 2e and f). Together, *FLT3* expression is a robust negative prognostic factor only in MLL-AF4+ B-ALL cases.

Relevant clinical–biological features were analyzed and compared between the *FLT3*-high and -low ALL subgroups (Supplementary Table S1). For MLL-germline ALL cases, none of the clinical–biological features studied differed between *FLT3*-high and -low subgroups ($P > 0.1$; Supplementary Table S1). In contrast, within the MLL-AF4+ ALL group patients expressing high levels of *FLT3* exhibited a significantly lower rate of complete remission



Cytogenetics:	MLL-AF4 (n=17)	TEL-AML1 (n=13)	BCR-ABL (n=8)	E2A-PBX1 (n=4)	Normal Cytogenetics (n=7)	T-ALL (n=5)
% cases FLT3+:	17/17 (100%)	12/13 (93%)	5/8 (62%)	0/4 (0%)	5/7 (72%)	0/5 (0%)
FLT3 fold-expression:						
Median:	25.3	7.3	8.1	1.2	8.2	0.6
Mean±SD:	37±31	8.8±6.9	7.7±6.4	1.4±0.7	16.2±15	0.9±0.6

Figure 1. Expression levels of *FLT3* mRNA by quantitative real-time PCR. Box plots showing *FLT3* gene expression levels by neoplastic cells from cytogenetically distinct B-ALL and T-ALL. *FLT3* levels are shown as fold-expression relative to NBM and normalized against *ABL1* housekeeping gene. The boxes extend from the 25th to 75th percentiles and the line in the middle represents median values. The expression of *FLT3* varied between cytogenetically distinct groups of ALLs. The lowest *FLT3* expression level was found in E2A-PBX1 + B-ALL and T-ALL. In contrast, MLL-AF4 + B-ALLs showed a *FLT3* expression level 37-fold higher than NBM, confirming that very high constitutive activation of *FLT3* is a hallmark event in MLL-AF4 + ALL. At the bottom, median and media ± s.d. of *FLT3* expression is shown for the different cytogenetic subgroups of B-ALL and T-ALL normalized against NBM samples is shown.

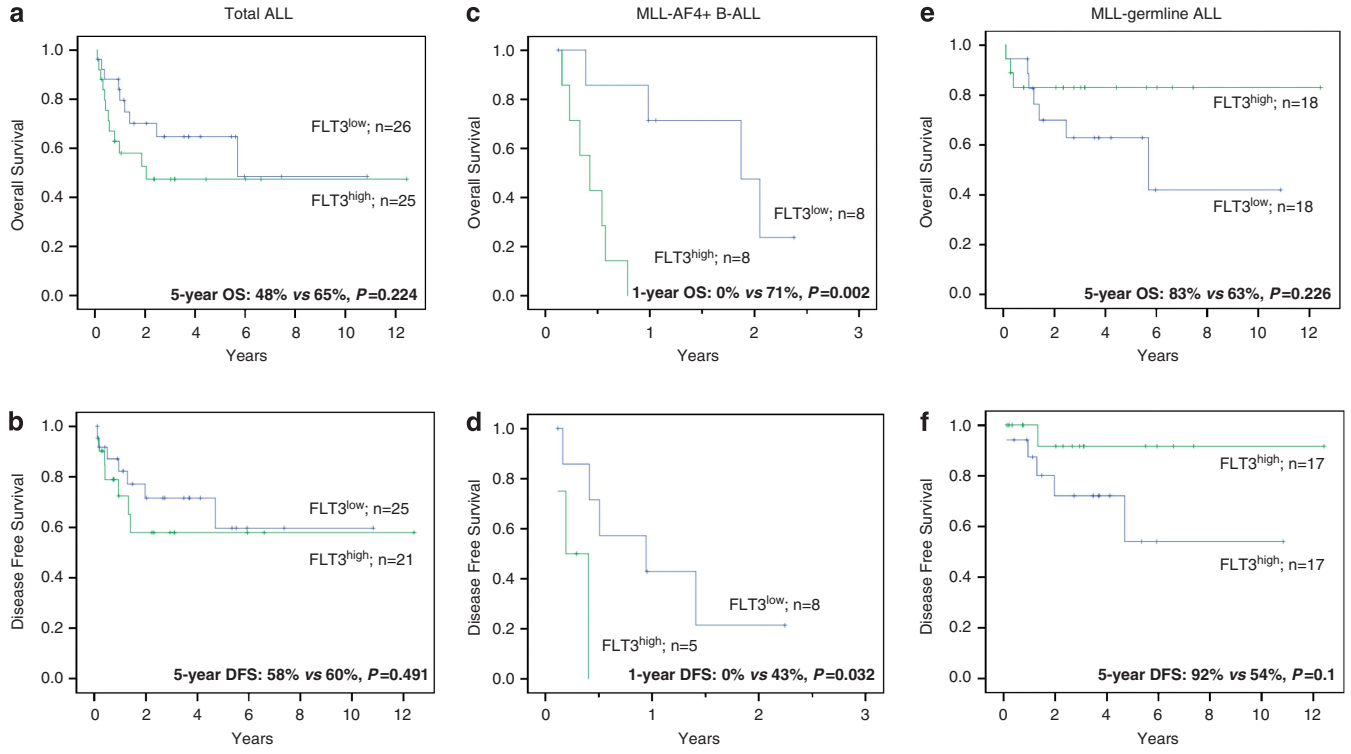


Figure 2. OS and DFS curves in MLL-AF4 + and MLL-germline ALL reveal that the expression of *FLT3* is a robust prognostic factor only in patients with MLL-AF4 + B-ALL. 5-year OS (**a**) and 5-year DFS (**b**) curves for all ALL patients ($n = 51$). OS (**c**) and DFS (**d**) curves for MLL-AF4 + B-ALL patients ($n = 15$). OS (**e**) and DFS (**f**) curves for MLL-germline B-ALL patients ($n = 36$). Two MLL-AF4 + cases were not included owing to the lack of clinical follow-up data (diagnosed in March 2012).

(57% vs 100%; $P = 0.038$) and lower survival (50% vs 100%; $P = 0.029$).

Prognostic value of *FLT3* gene expression in the context of other risk factors in ALL

We first assessed to what extent *FLT3* expression and other clinico-biological risk factors influence OS in the entire cohort of ALLs. The variables that were significantly associated with decreased OS were: age >14 years ($P<0.0001$), WBC count $>50\times10^9$/l ($P<0.0001$), $>50\%$ blast cells in PB ($P = 0.003$), platelet count $<56\times10^9$/l ($P = 0.01$), B-ALL cytogenetic subtype (ETV6-RUNX1 negative ($P = 0.002$) and MLL-AF4-positive ($P<0.0001$)) and high expression of *FLT3* (75th percentile, $P = 0.008$) (Table 2). When these variables were entered into a multivariate analysis using a Cox regression model, only age >14 years ($P<0.0001$) and high *FLT3* expression ($P = 0.007$) retained independent prognostic values for a shorter OS (Table 2), which was also confirmed when they were analyzed as continuous variables ($P = 0.001$; data not shown).

Because FLT3 expression level was a very robust prognostic factor for both OS and DFS in the MLL-AF4+ B-ALL subgroup but not in MLL-germline ALL (Figure 2), we analyzed the influence of *FLT3* expression on OS and DFS in the context of other clinico-biological risk factors specifically in the MLL-AF4+ ALL subgroup ($n = 15$). Interestingly, univariate analysis with OS and DFS as end points showed that high expression of *FLT3* (median, $P = 0.002$ for OS and $P = 0.03$ for DFS) was the only independent prognostic factor for shorter OS and poorer treatment outcome (Table 3).

Table 2. Influence of clinical–biological characteristics and FLT3 expression in 51 ALL patients according to OS

Variable	*OS* (n = *51*)			
	N	*5-year, %**	*Univariate*	*Multivariate*
Age (years)[a]				
⩽14	26	87	<0.0001	<0.0001
>14	25	25		
WBC at diagnosis ($\times10^9$/l)[a]				
⩽50	28	80	<0.0001	0.27
>50	22	26		
BM blasts at diagnosis (%)[b]				
⩽90	23	64	0.22	—
>90	24	56		
PB blasts at diagnosis (%)[b]				
⩽50	23	79	0.003	0.20
>50	23	40		
Platelet at diagnosis ($\times10^9$/l)[b]				
⩽56	25	45	0.011	0.27
>56	25	70		
Hemoglobin (g/dl)[b]				
⩽9.3	25	59	0.88	—
>9.3	26	57		
ALL subtype				
ETV6-RUNX1 negative	38	37	0.002	0.44
ETV6-RUNX1 positive	13	100		
ALL subtype				
E2A-PBX1 negative	47	53	0.13	—
E2A-PBX1 positive	4	100		
*ALL subtype**				
BCR-ABL1 negative	43	70	0.14	—
BCR-ABL1 positive	8	63		
*ALL subtype**				
MLL-AF4 negative	36	83	<0.0001	0.52
MLL-AF4 positive	15	36		
*ALL subtype**				
ALL-B	46	73	0.058	0.069
ALL-T	5	40		
FLT3 expression[c]				
Low	39	65	0.008	**0.007**
High (p75)	12	25		

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; OS, overall survival; PB, peripheral blood; WBC, white blood cell. [a]Variables were dichotomized based on high-risk criteria. [b]Variables were dichotomized based on the median value. [c]For FLT3 gene expression, the quartile providing the best separation of survival curves (lowest *P*-value) is shown. In the univariate analysis the log-rank test was used. Multivariate analysis (Cox regression model) was performed by including those clinico-biological features with a *P*-value <0.1 in the univariate analysis. Only variables with a *P*-value <0.05 were considered as having an independent prognostic value. *In adverse ALL subtypes the survival percentage was calculated for 1 year. *P*-value for FLT3 expression is shown in bold.

DISCUSSION

Several studies have shown that activating mutations of *FLT3* are common in AML.[9–11] However, the presence of such mutations among different subtypes of ALL remains controversial. Several groups reported the presence of FLT3-TKD or FLT3-ITD mutations in 3–25% of MLL-rearranged or hyperdiploid ALL,[6,12–14] whereas Stam *et al.*[15,16] and Bardini *et al.*[17,18], using high-resolution genomic analysis, showed that *FLT3* mutations in MLL-rearranged ALL do not occur. Despite this controversy, gene expression studies revealed that *FLT3* is highly expressed in MLL-rearranged ALL, and therefore it still needs to be defined whether the constitutive activation of *FLT3* in MLL-rearranged ALL is a direct effect of the presence of *FLT3*-activating mutations. In the present study, 54 out of 54 (100%) *de novo* cytogenetically distinct B-ALL and T-ALL lacked FLT3-TKD point mutations (D835Y or I836del) and FLT3-ITD. Thesedata are in line with Stam *et al.*,[15,16] suggesting that activating FLT3 mutations do not seem to contribute to ALL pathogenesis. In addition, *FLT3* gene expression was analyzed in cytogenetically distinct subgroups of ALLs and T-ALL. T-ALLs and E2A-PBX1+ B-ALL lacked activation of *FLT3*. In contrast, the remaining cytogenetic subgroups of ALL, especially the MLL-AF4+ pro-B ALL (37-fold increase), exhibited a significantly higher *FLT3* expression levels as compared with NBM. This increased *FLT3* expression in the absence of *FLT3*-activating mutations suggests that the constitutive overexpression of *FLT3* is a result of aberrantly increased transcription of *FLT3* and it is not due to *FLT3*-activating mutations. This is supported by Guenther *et al.*,[19] who showed through elegant studies that *FLT3* may be a direct target of MLL-AF4. We have analyzed the most common *FLT3* mutations found in acute leukemia but have not sequenced the entire *FLT3* coding sequence, and therefore, although unlikely,[15] the presence of novel *FLT3*-activating mutations responsible for constitutive activation of *FLT3* might not be completely excluded.

The confirmation that MLL-AF4+ pro-B ALL patients do not carry *FLT3* mutations but display a constitutive transcriptional activation of *FLT3* may have relevant implications for the development of MLL-AF4+ disease models. Leukemia is generally studied once the full transformation events have already occurred and therefore, the mechanisms by which MLL-AF4 transforms to a

Table 3. Influence of clinical–biological characteristics and FLT3 expression in MLL-AF4+ B-ALL patients according to their OS and DFS

Variable	*OS (n = 15)*				*DFS (n = 12)*			
	n	*1-year, %*	*Univariate*	*Multivariate*	n	*1-year, %*	*Univariate*	*Multivariate*
Age (years)[a]								
⩽31	8	29	0.45	—	6	21	0.30	—
>31	7	43			6	42		
WBC at diagnosis ($\times 10^9$/l)[a]								
⩽170	8	67	0.17	—	7	33	0.96	—
>170	7	14			5	50		
BM blasts at diagnosis (%)[a]								
⩽92	8	67	0.24	—	6	40	0.72	—
>92	7	14			6	30		
PB blasts at diagnosis (%)[a]								
⩽88	7	50	0.65	—	5	67	0.80	—
>88	6	17			5	50		
Platelet at diagnosis ($\times 10^9$/l)[a]								
⩽40	8	29	0.42	—	6	44	1.00	—
>40	7	60			6	25		
Hemoglobin (g/dl)[a]								
⩽9.6	8	17	0.67	—	6	27	0.58	—
>9.6	7	57			6	42		
FLT3 expression[b]								
Low	8	71	**0.002**	—	8	43	**0.032**	—
High (median)	7	0			4	0		

Abbreviations: B-ALL, B-acute lymphoblastic leukemia; BM, bone marrow; DFS, disease-free survival; FLT3, FMS-related tyrosine kinase-3; OS, overall survival; PB, peripheral blood; WBC, white blood cell. [a]Variables were dichotomized based on the median value. [b]For FLT3 gene expression, the quartile providing the best separation of survival curves (lowest *P*-value) is shown. In the univariate analysis the log-rank test was used. Multivariate analysis (Cox regression model) was not performed because FLT3 expression was the only variable with prognostic significance in the univariate analysis. Only variables with a *P*-value <0.05 were considered as having an independent prognostic value. *P*-value for FLT3 expression is shown in bold.

pre-leukemic state followed by rapid transition to overt ALL are not amenable to analysis with patient samples. Most of our understanding of transformation by MLL-AF4 has come from murine models[2–4,26,27] and human stem cell systems.[1,28–33] These have provided important insights into the likely mode of action of MLL-AF4, but the *in vivo* leukemias produced in these studies do not recapitulate the actual human disease phenotype and latency, and therefore MLL-AF4 leukemogenesis remains particularly difficult to model. This suggests that in order to develop a *bona fide* MLL-AF4 model by which to further understand the disease pathogenesis and to screen novel small-molecule compounds, additional oncogenic events such as *FLT3* constitutive activation[8] or the derivative AF4-MLL seem to be required to develop ALL.[26]

The very high *FLT3* mean expression in MLL-AF4+ ALL patients coupled to the high variability (3.5- to 110-fold expression) suggests the possibility that the *FLT3* expression level may have a prognostic significance in MLL-AF4+ ALL. To analyze the prognosis of our ALL cohort in relation to *FLT3* expression, the median *FLT3* expression was used as the cut-off value to divide patients into two subgroups expressing high or low levels of *FLT3*. High-level *FLT3* expression is associated with an extremely poor 1-year OS (0 vs 71%) and DFS (0 vs 43%) in MLL-AF4+ B-ALL but not in any other MLL-germline B-ALL. The presence of BCR-ABL fusion is associated with poor-prognosis B-ALL. In this study, BCR-ABL+ B-ALL also displayed a shorter OS, which was not statistically significant ($P = 0.14$) probably owing to the small cohort (Table 2). The prognostic value of *FLT3* expression was determined in the context of other clinical–biological risk factors in ALL. Cox regression analysis showed that age >14 years and high-level *FLT3* expression were independent prognostic factors for OS when all ALL patients were analyzed together. Importantly, when the MLL-AF4+ B-ALL subgroup was analyzed separately, high-level *FLT3* expression was the only independent prognostic factor for OS and treatment outcome. These findings indicate that high *FLT3* expression identifies MLL-AF4+ ALL patients at very high risk of treatment failure and poor survival. To the best of our knowledge, this is the first study demonstrating the usefulness of *FLT3* expression in improving patient risk stratification in patients suffering from MLL-AF4+ B-ALL. Previously, Stam *et al.*[16] have found that high-level *FLT3* expression is likely associated with a poor treatment outcome in MLL-rearranged ALL. Our work expands current knowledge because: (i) our cohort specifically comprises MLL-AF4+ patients and no other MLL rearrangements, (ii) OS was used as end-point analysis besides DFS for patient risk stratification, (iii) our MLL-AF4+ cohort is composed of both infants and adult patients, indicating that high-level *FLT3* expression and the lack of *FLT3* mutations are hallmark not only in infants but also in adults with MLL-AF4+ pro-B ALL, and (iv) Cox regression multivariate analysis reveals that high-level *FLT3* expression is an independent prognostic factor when compared with other clinical–biological risk factors in MLL-AF4+ ALL. The existence of two distinct subgroups among t(4;11)-positive infant ALL cases characterized by the absence or presence of *HOXA* expression, and that patients lacking *HOXA* gene expression are at high risk of disease relapse were reported.[34] Future work should be conducted to determine whether there is any biochemical link between *HOXA* and *FLT3* gene expression, and to decipher whether MLL-AF4+ ALL patients at extremely high risk of relapse and very short OS represent the same cohort displaying high-level *FLT3* and lacking *HOXA* expression, thus providing important novel

insights into the complex biology of MLL-AF4 + ALL and boosting our progress in finding novel therapeutic approaches. In this sense, our data support that FLT3 may be a therapeutic target in MLL-AF4 ALL, emphasizing the value of ongoing and future clinical trials for FLT3 inhibitors in infants and adults with MLL-AF4 + pro-B ALL.[5]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MCC, MTG-C, MG and PM were involved in theconception and design, collection and/or assembly of the data, financial support, data analysis and interpretation and manuscript writing. CEL-J, C R-M, AM, MES, MA, JDG-S, CB, RM, FR, JNR, PG, MR, RG-D and JLF were involved in provision of patient samples, clinical data collection and interpretation, and also in the collection and/or assembly of the data.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

8.- FLT3 activation cooperates with MLL-AF4 fusion protein to abrogate the hematopoietic specification of hESCs.

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INTRODUCCIÓN. El oncogén de fusión MLL-AF4 en LLA pro-B aparece en estadio prenatal. Pese a la corta latencia de la enfermedad y su fatal pronóstico, no se ha podido desarrollar hasta la fecha un modelo que reproduzca la enfermedad. Diversos estudios han corroborado que la expresión de MLL-AF4 por sí misma no es suficiente para desencadenar el proceso leucémico. Existe la posibilidad de que la acción de ciertos eventos secundarios posteriores a la traslocación t(4;11) colaboren con la misma siendo responsables de la aparición de la leucemia. En este sentido, uno de los candidatos es la activación constitutiva del receptor FLT3, altamente expresado en LLA pro-B MLL-AF4+.

OBJETIVO. Determinar el impacto de la activación constitutiva del receptor FLT3 (mutado o sobre-expresado) en el desarrollo y diferenciación hematopoyética a partir de hESCs, por sí sólo o en combinación con MLL-AF4.

MATERIAL Y MÉTODOS. Las hESCs fueron trasducidas con vectores lentivirales para forzar la expresión de MLL-AF4, FLT3-WT, FLT3-TKD, FLT3-ITD y del vector vacío (control). Dicha expresión fue confirmada por RT-PCR, qPCR y RLFP-PCR. Los marcadores de indiferenciación característicos de hESCs se determinaron por citometría de flujo y la expresión de los factores de trascripción asociados a pluripotencia por RT-PCR. La capacidad pluripotente de las líneas de hESCs transgénicas se confirmó *in vivo* mediante la formación de teratomas en ratón. La activación de FLT3 se valoró mediante citometría de flujo, estudiando la fosforilación de proteínas de la cascada de señalización (AKT, STAT5, ERK). La diferenciación hematopoyética de las hESCs se realizó mediante formación de hEBs y cultivo con citocinas hematopoyéticas. El ensayo en metilcelulosa determinó la capacidad clonogénica de las células hematopoyéticas derivadas de las hESCs trasgénicas. El perfil de expresión génica mostró que genes se encontraban activados o silenciados en los progenitores hemogénicos y en las células hematopoyéticas derivadas de hESCs trasgénicas. También se realizaron análisis de ciclo celular y apoptosis sobre los precursores hemogénicos y las células hematopoyéticas.

RESULTADOS. Mientras que la activación de FLT3 aumenta la especificación de las hESCs hacia hematopoyesis, la combinación de MLL-AF4 y la activación de FLT3 bloquea tanto la aparición de precursores hemogénicos como de células hematopoyéticas definitivas, sin provocar trasformación, como se muestra en los ensayos de clonogenicidad. El perfil trascripcional confirmó la inhibición de la hematopoyesis mediada por la activación de FLT3 en las hESCs MLL-AF4+.

CONCLUSIÓN. En hESCs, la activación de FLT3 en combinación con la expresión de MLL-AF4, bloquea la aparición tanto de precursores hemogénicos como de células hematopoyéticas CD45+ y CFUs, sin producirse trasformación oncogénica, por lo que posiblemente son otros eventos secundarios, como pueden ser modificaciones epigenéticas, los responsables de la leucemogénesis mediada por MLL-AF4.

NOTA: La relevancia de este artículo mereció, según el editor, el comentario de un experto en el tema, publicado en el misma edición. Este comentario se añade tras el artículo.

The ongoing conundrum of MLL-AF4 driven leukemogenesis.

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Regular Article

LYMPHOID NEOPLASIA

FLT3 activation cooperates with MLL-AF4 fusion protein to abrogate the hematopoietic specification of human ESCs

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Key Points

- *FLT3* activation cooperates with the MLL-AF4 fusion gene to fully abolish blood formation from hESCs.
- *FLT3* activation does not cooperate with the MLL-AF4 fusion oncogene to transform hESCs or hESC-derived hematopoietic progeny.

Mixed-lineage leukemia (MLL)–AF4 fusion arises prenatally in high-risk infant acute pro-B-lymphoblastic leukemia (pro-B-ALL). In human embryonic stem cells (hESCs), MLL-AF4 skewed hematoendothelial specification but was insufficient for transformation, suggesting that additional oncogenic insults seem required for MLL-AF4–mediated transformation. MLL-AF4+ pro-B-ALL expresses enormous levels of *FLT3*, occasionally because of activating mutations, thus representing a candidate cooperating event in MLL-AF4+ pro-B-ALL. Here, we explored the developmental impact of *FLT3* activation alone, or together with MLL-AF4, in the hematopoietic fate of hESCs. *FLT3* activation does not affect specification of hemogenic precursors but significantly enhances the formation of CD45+ blood cells, and CD45+CD34+ blood progenitors with clonogenic potential. However, overexpression of *FLT3* mutations or wild-type *FLT3* (*FLT3*-WT) completely abrogates hematopoietic differentiation from MLL-AF4–expressing hESCs, indicating that *FLT3* activation cooperates with MLL-AF4 to inhibit human embryonic hematopoiesis. Cell cycle/apoptosis analyses suggest that *FLT3* activation directly affects hESC specification rather than proliferation or survival of hESC-emerging hematopoietic derivatives. Transcriptional profiling of hESC-derived CD45+ cells supports the *FLT3*-mediated inhibition of hematopoiesis in MLL-AF4–expressing hESCs, which is associated with large transcriptional changes and downregulation of genes involved in hematopoietic system development and function. Importantly, *FLT3* activation does not cooperate with MLL-AF4 to immortalize/transform hESC-derived hematopoietic cells, suggesting the need of alternative (epi)-genetic cooperating hits. (*Blood*. 2013;0(0):1-12)

Introduction

Newborn cancer is progressively seen as a developmental biology disease.[1] An intriguing newborn cancer is the infant pro-B/monocyte acute lymphoblastic leukemia (ALL) characterized by the hallmark genetic abnormality t(4;11) encoding the fusion mixed-lineage leukemia (MLL)-AF4, which is associated with a dismal prognosis and very brief latency, raising the question of how this disease evolves so quickly.[2-4] Compelling evidence indicates that MLL-AF4 arises prenatally during embryonic/fetal hematopoiesis.[2,3,5,6] To understanding the developmental impact of MLL-AF4, we first need to elucidate which is the target cell for transformation and the mechanisms underlying MLL-AF4–mediated transformation.

MLL-AF4–induced leukemogenesis has been difficult to model, and bona fide MLL-AF4 disease models do not exist.[7-10] Our understanding of MLL fusions comes from murine models, which do not recapitulate the human disease faithfully, suggesting that these mouse models may be missing essential components of leukemogenesis during early human development. It could be argued that the lack of an MLL-AF4 model may be because (i) a cell in a wrong developmental stage was targeted in the mouse; (ii) the impact of other secondary hits has not been properly addressed; (iii) MLL-AF4 requires the reciprocal AF4-MLL fusion protein to cause pro-B ALL as shown in the mouse; and (iv) MLL-AF4 exerts its transforming function preferentially in human cells, indicating that the MLL-AF4 function has to be addressed using ontogenically primitive human stem cells. Among these, neonatal (cord blood [CB]–derived) CD34+ hematopoietic stem/progenitor cells (HSPCs) or prenatal (fetal- or embryonic-derived) cells represent ontogenically early candidate target cells. Thus, human embryonic stem cell (hESC)–derived hematopoietic differentiation constitutes a robust human-specific strategy to study the onset of hematopoiesis, representing a promising tool for modeling developmental mechanisms of human disease and lineage specification that cannot be addressed with patient samples or mouse models.[11,12]

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We have reported that the expression of MLL-AF4 has a functional impact in CB-CD34+ HSPCs[13] and hESC-hematopoietic cells.[14] In CB-CD34+ HSPCs, MLL-AF4 enhanced hematopoietic engraftment and clonogenic potential but was not sufficient for leukemogenesis.[13] In hESCs, MLL-AF4 altered the developmental cell fate, skewing the early hematoendothelial specification of hESCs.[14] This mechanism suggests that the inability to develop an MLL-AF4 model is not the result of the human cell context or cell type targeted, but rather that additional oncogenic lesions are required for leukemogenesis.

Gene expression profiling showed that *FLT3* is highly expressed in MLL-AF4+ pro-B-ALL. Moreover, it was shown that other MLL-rearranged leukemias display *FLT3* mutations (*FLT3*-tyrosine kinase domain [TKD] or *FLT3*-internal tandem duplication [ITD]) in up to 20% of the cases, suggesting that they may represent candidate cooperating events.[15-17] Accordingly, Yamaguchi et al[18] have reported that *FLT3*-TKD cooperates with MLL-AF4 to induce in vitro aggressive proliferation of the mouse cell line 32Dc. Several other groups suggest, however, that *FLT3* mutations are not common in MLL-AF4+ pro-B-ALL, and that an increased transcriptional expression of *FLT3* may act as a secondary cooperating hit.[17,19-21] The latter is supported by Guenther et al[19], who reported that *FLT3* is a direct transcriptional target of MLL-AF4.

We have thus explored the developmental impact of *FLT3* on its own, or in cooperation with MLL-AF4, in the hematopoietic fate of hESCs. We posed the following 3 questions. First, what is the developmental impact of *FLT3* mutations/*FLT3*-WT in the hematopoietic specification of hESCs? Second, do *FLT3* mutations/*FLT3*-WT cooperate with MLL-AF4 during hematopoietic commitment of hESCs? Third, is expression of *FLT3* mutations/*FLT3*-WT together with MLL-AF4 sufficient to confer a proliferative/survival advantage as anticipated for transforming oncogenes?

Materials and methods

Plasmid construction and lentiviral transduction

The MLL-AF4 cDNA (MLL-exon10 fused to AF4-exon8) and the *FLT3*-TKD (D835 mutation), *FLT3*-ITD, and *FLT3*-WT cDNAs were subcloned into the pRRL-EF1α-PGK-green fluorescent protein(GFP)/NEO vector.[14] The following lentivectors were used: pRRL-EF1α-PGK-NEO (empty vector; EV), pRRL-EF1α-MLL-AF4-PGK-NEO (MLL-AF4), pRRL-EF1α-*FLT3*-TKD-PGK-GFP (*FLT3*-TKD), pRRL-EF1α-*FLT3*-ITD-PGK-GFP (*FLT3*-ITD), and pRRL-EF1α-*FLT3*-WT-PGK-GFP (*FLT3*-WT) (Figure 1A). Viral production and infection were performed as described previously.[22] NEO-expressing hESCs were selected with G418 at 100 μg/mL for 3 weeks.[22] In dual transfection experiments, *FLT3*/GFP-expressing viruses were used to infect G-418–resistant MLL-AF4–expressing hESCs. MLL-AF4 and *FLT3* expression and mutational status were confirmed.

Human ESC culture

hESCs (AND-1 line) were maintained undifferentiated in a feeder-free culture as described.[23-25] Briefly, hESCs were cultured in Matrigel-coated T25 flasks in human feeders-conditioned medium supplemented with 4 ng/mL of basic fibroblast growth factor.[26-28] The medium was changed daily, and the cells were split weekly with 200U/mL of collagenase IV. This study was approved by The Spanish National Embryo Ethical Committee to work with hESCs.

Q:1 Q:2

RNA extraction, cDNA synthesis, and MLL-AF4 and *FLT3* gene expression

RNA extraction and reverse transcription were performed using the Europe Against Cancer group protocol.[17] cDNA was used for conventional (MLL-AF4) and quantitative (*FLT3*) polymerase chain reaction (PCR). MLL-AF4 expression was confirmed using the following primers (Fw:5′-CAGGTCCAGAGCAGAGCAAAC-3′ and Rw:5′-GAGCACTTGGAGGTGCAGATG-3′) and PCR conditions (95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds).[17] In quantitative PCR experiments, *FLT3* expression was normalized to the expression of β-actin. Quantitative PCR was performed using SYBRGreen PCR Master mix (Applied BioSystem) and the 7500 reverse transcriptase (RT)-PCR system.

FLT3 gene mutation analysis

The presence of *FLT3*-activating mutations was assessed by restriction fragment length polymorphism PCR, using primers and PCR conditions previously described in detail.[17,29]

Flow cytometry characterization of hESCs

hESCs were dissociated with trypsin-EDTA, and the single-cell suspension was stained (2-5 × 10^5cells/mL) with TRA-1-60-PE, SSEA3-PE, SSEA4-fluorescein isothiocyanate (FITC), and OCT4-FITC (all from BD). Cells were then washed and stained with 7-amino-actinomycin D (7-AAD) for 15 minutes.[25,30] Cells were analyzed using a FACS Canto-II-cytometer.

Expression of pluripotency-associated transcription factors

Transgenic hESCs were subjected to RT-PCR for detection of *OCT4, NANOG, SOX2*, and *REX1* expression. *GAPDH* was used as a housekeeping gene. The primers and PCR conditions used were described elsewhere.[31]

In vivo teratoma formation

Animal protocols were approved by the local university hospital council on animal care and experimentation. Teratoma assay was conducted to confirm in vivo pluripotency.[24,32] hESCs were implanted subcutaneously in 8-week-old immunodeficient mice (The Jackson Laboratory, Bar Harbor, ME). Teratoma growth was determined by palpation, and mice were euthanized 7 weeks after implantation. Teratomas were fixed, embedded in paraffin, and sections stained with hematoxylin/eosin. Immunocytochemistry analysis was performed for smooth muscle actin, pan-cytokeratin, α-fetoprotein, and β-III-tubulin (Dako).

Q:3

Phosphoflow analysis of hESCs

For phosphosignaling, hESCs were incubated with TryplE (Invitrogen) for 5 minutes, washed and resuspended in staining buffer (BDBiosciences). Then, cells were fixed with Cytofix Buffer (BDBiosciences) and permeabilized in chilled Phosphoflow PermBuffer-III for 30 minutes before staining with anti-ERK1/2 (pT302/pY204)-PE, anti-STAT5 (pY694)-PE, and anti-Akt (pS473). Data were analyzed in a FACSCanto-II-cytometer equipped with the FACSDiva software. The mean fluorescence intensity for each hESC line was compared with EV-hESC.

Immunoprecipitation and western blotting

Confluent hESCs were lysed in 1% Triton X-100 lysis buffer supplemented with Complete protease inhibitors cocktail and phosphatase inhibitors (Roche). Lysates were immunoprecipitated using protein-A agarose beads (Roche) and FLT3 antibody (clone S18; Santa Cruz). Immunoprecipitations were performed from 5-8 × 10^6 cells. Detection of tyrosine phosphorylation in FLT3 pull-downs was performed by immunoblotting using 4G10 antibody (Upstate). Signal was detected with the Odyssey infrared imaging system (Li-cor, Lincoln, NE). The MLL-rearranged THP1 line before and after FLT3L treatment was used as control.

Hematopoietic differentiation from hESCs

Undifferentiated hESCs were treated with collagenase IV and lifted of the Matrigel attachments. They were transferred to low-attachment plates to allow embryoid body (EB) formation in differentiation medium

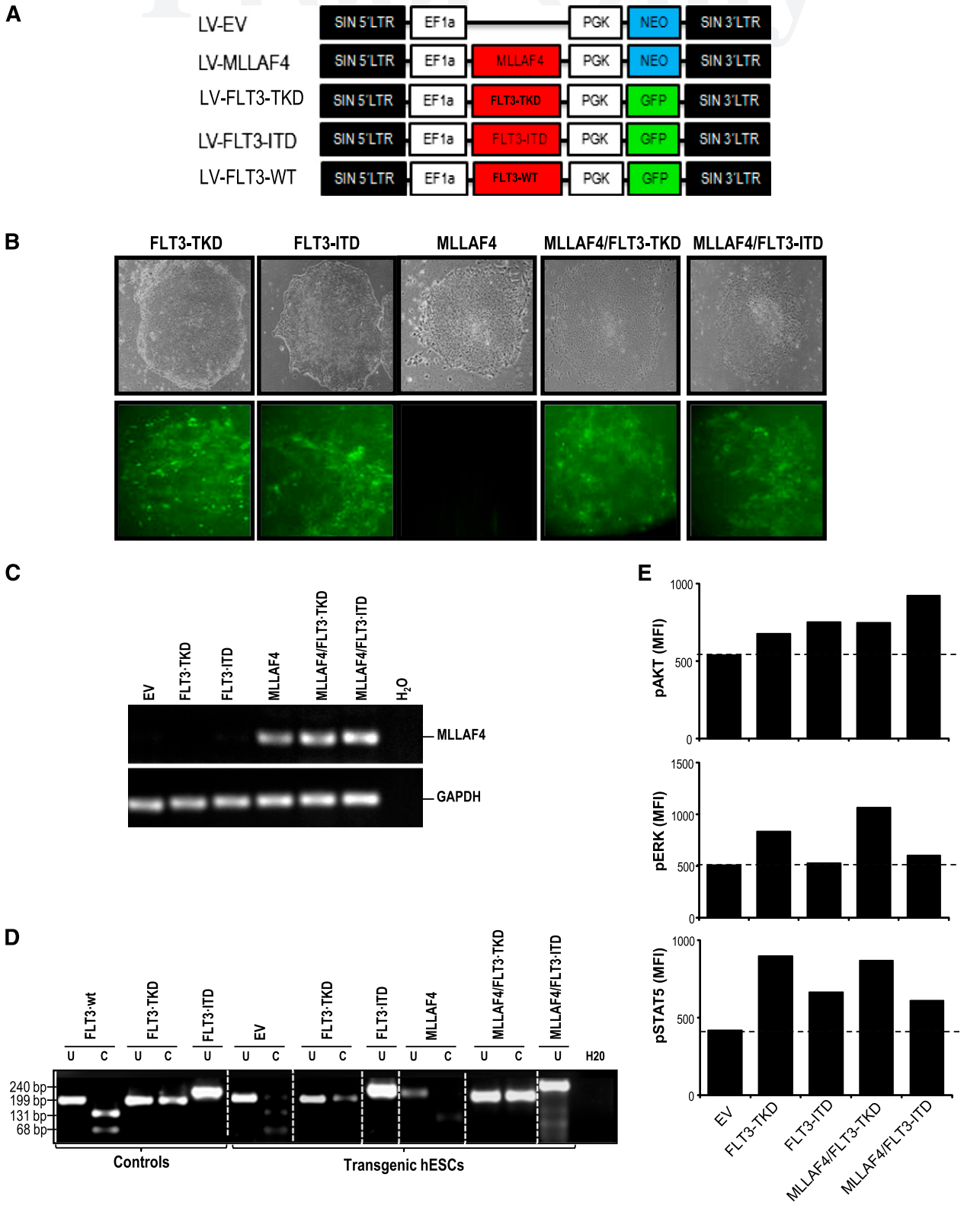


Figure 1. Generation of transgenic hESCs expressing MLL-AF4 and/or *FLT3*-activating mutations. (A) Schematic representation of the lentiviral vectors used. (B) Phase-contrast morphology (top) and fluorescence microscopy (bottom) of representative hESC colonies from *FLT3*-TKD-, *FLT3*-ITD-, MLL-AF4-, MLL-AF4/*FLT3*-TKD-, and MLL-AF4/*FLT3*-ITD-hESCs. (C) RT-PCR confirming expression of the MLL-AF4 transcript in transduced hESCs. GAPDH was used as a housekeeping gene. (D) PCR confirming the presence of either *FLT3*-TKD or *FLT3*-ITD mutations in transgenic hESCs. Vectors containing the *FLT3* mutations were used as positive controls. (E) Phosphosignaling analysis of transduced hESCs, showing increased AKT, ERK, and STAT5 phosphorylation (relative to EV-hESCs) in cells transduced with *FLT3*-activating mutations.

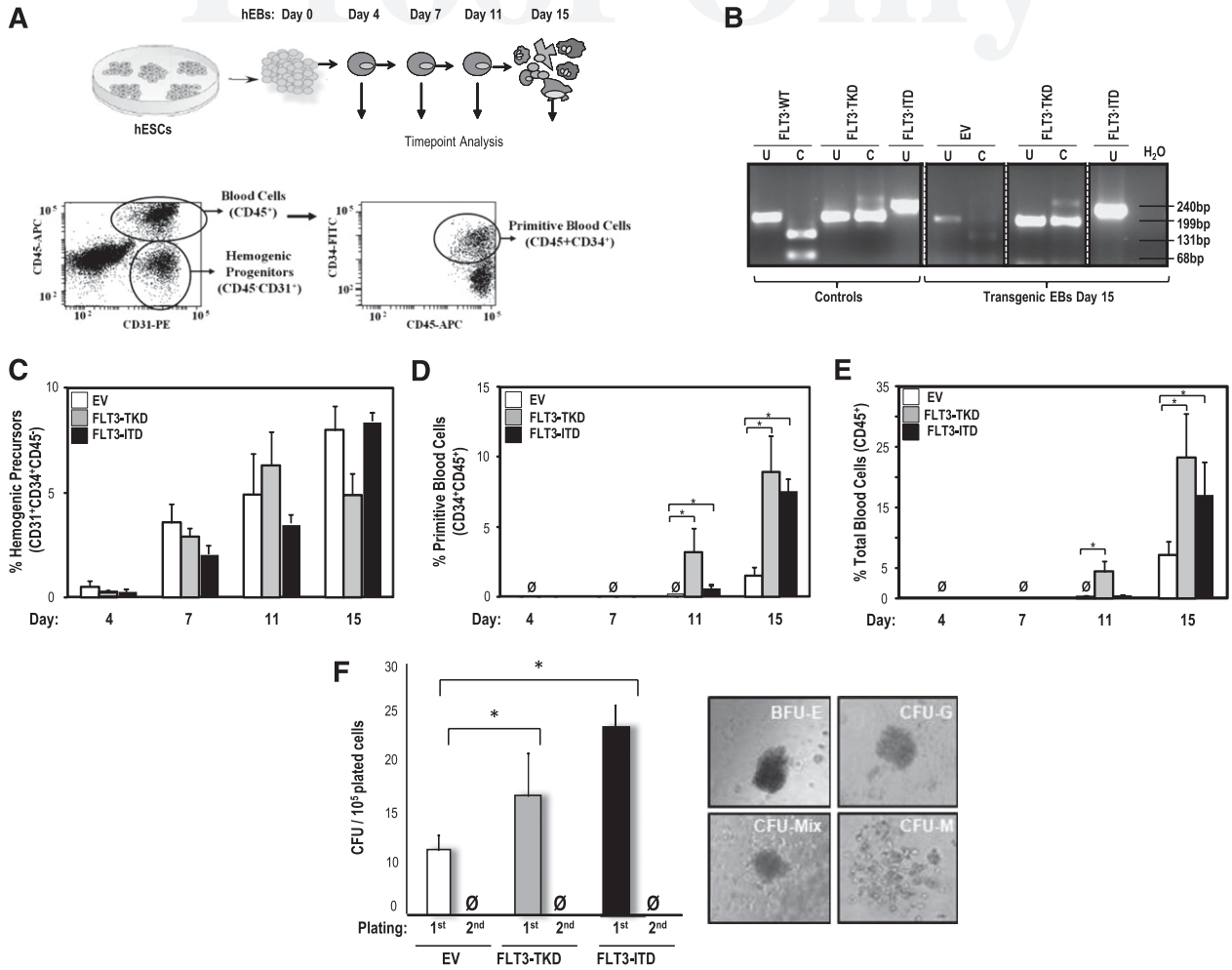


Figure 2. FLT3 activation enhances hematopoietic differentiation from hESCs. (A) Schematic of the hematopoietic differentiation of hESCs and end point analyses (top) and representative flow cytometry dot plots displaying how hemogenic precursors (CD45$^-$ CD31$^+$), primitive blood cells (CD45$^+$ CD34$^+$), and total blood cells (CD45$^+$) are identified (bottom). (B) PCR confirming the presence of either *FLT3*-TKD or *FLT3*-ITD mutations in transgenic day 15 hEBs. (C) Specification into hemogenic precursors is not significantly affected by *FLT3* activation throughout EB development. However, *FLT3* activation enhances hESC differentiation into primitive blood cells (D) and total blood cells (E). (F) CFU readout from d15 hEBs confirms a significant increase in hematopoietic potential in hESCs expressing either *FLT3* mutation. Data are presented as mean± SEM for 6 independent experiments. *FLT3* mutation–expressing hESC-derived hematopoietic cells do not show stable in vitro replating efficiency in secondary CFU assays. Right panels depict representative CFU colonies.

(KO-DMEM+20% fetal bovine serum, 1% nonessential amino acids, 1mM of L-glutamine, 0.1 mM of β-mercaptoethanol). The medium was changed every 3 days and was supplemented with hematopoietic cytokines (300 ng/mL of SCF, 10 ng/mL of IL-3, 10 ng/mL of IL-6, and 50 ng/mL G-CSF, 25 ng/mL BMP-4). EBs were dissociated using collagenase B at days 4, 7, 11, and 15 of development. A single-cell suspension achieved by gentle pipetting and passage through a 70-μm cell strainer was stained with anti-CD31-PE, anti-CD45-APC, and anti-CD34-PECy7 (BDBiosciences) and 7AAD. Live cells (7AAD-) were analyzed using a FACSCanto-II-cytometer. Hemogenic precursors were identified as CD34$^+$CD31$^+$CD45$^-$. Immature and total blood cells were identified as CD45+CD34+ and CD45+, respectively (Figure 2A).[22,25]

Colony-forming unit (CFU) assay

Primary and secondary clonogenic progenitor assays were performed by plating 50 000 cells from day 15 hEBs into methylcellulose H4434 (Stem Cell Technologies), as reported extensively.[22,25]

Gene expression profiling (GEP) and data analysis

CD45+ cells derived from EV-hESCs, MLL-AF4-hESCs, and MLL-AF4/*FLT3*-TKD-hESCs were sorted for GEP.[14,33] RNA was isolated and its quality checked in the Agilent 2100 Bioanalyzer platform. RNA was labeled (Quick-Amp Labeling kit) with Cy3 and was hybridized with the Gene Expression Hybridization kit to a Whole Human Genome Microarray (G4112F;Agilent Technologies) following the manufacturer's instructions.[14,33] Four independent samples per condition were labeled and hybridized. Hierarchical clustering of genes and samples was performed with the 1− correlation metric and the unweighted average distance. A gene was considered differentially expressed when it was > 2-fold up/down-regulated (P value < .01) compared with the control. Analysis of gene functions and canonic pathways significantly altered between experimental conditions was performed using the Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA).[14,34,35] Microarray data have been deposited in the National Center for Biotechnology Gene Expression Omnibus (NCBI-GEO) (GSE40103).

Cell cycle/apoptosis analysis of hemogenic precursors and CD45+ hematopoietic cells

EBs were dissociated at day 15 of development. Washed cells were incubated with anti-CD31-FITC, anti-CD34-FITC, anti-CD34-PECy7, and anti-CD45-APC. Cell cycle distribution and apoptosis status of hemogenic precursors and CD45+ blood cells were analyzed, as described previously in detail.[14]

Results

FLT3 activation alone or in combination with MLL-AF4 expression is compatible with hESC pluripotency and induces phosphorylation of FLT3R and *FLT3*-mediated signal transducing effectors.

To study the cooperation between MLL-AF4 and *FLT3*, *FLT3*-TKD and *FLT3*-ITD were subcloned in a lentivector expressing GFP (Figure 1A). Empty vector (EV)/NEO or MLL-AF4/NEO-hESCs had been established after G418 selection.[14] G-418–resistant EV- and MLL-AF4–expressing hESCs were infected with viruses expressing FLT3-TKD/GFP or *FLT3*-ITD/GFP achieving > 85% transduction efficiency (Figure 1B). Successful and stable expression of MLL-AF4 was confirmed by RT-PCR > 20 passages after G418 selection (Figure 1C). *FLT3* mutations were also confirmed by PCR (Figure 1D). Ectopic expression of *FLT3* mutants or *FLT3*-WT gene induced *FLT3* receptor phosphorylation (supplemental Figure 1A), and activated the 3 major intracellular pathways AKT, ERK, and STAT5.[36] Phosphosignaling analysis showed increased phosphorylation of these signal-transducing effectors in hESCs transduced with *FLT3* mutations (Figure 1E), confirming that *FLT3* expression was functional.

Human ESC cultures were then analyzed for pluripotency markers and functional assays. hESCs transduced with *FLT3* mutations retained expression of the antigens Tra-1-60, SSEA-3, SSEA-4, and OCT-4 (supplemental Figure 1B) and the pluripotency markers *SOX2, NANOG, OCT4*, and *REX1* (supplemental Figure 1C). Functionally, all hESCs formed teratomas with 100% efficiency (supplemental Figure 1D), indicating that *FLT3* activation is compatible with hESC pluripotency.

FLT3-activating mutations enhance hematopoietic differentiation of hESCs.

We tested whether *FLT3*-TKD and *FLT3*-ITD affect the hematopoietic cell fate of hESCs. During hEB differentiation, a population of primitive hemogenic precursors arises, which is responsible for hematopoietic and endothelial development.[14,22, 25,37] We investigated the effect of *FLT3*-TKD and *FLT3*-ITD on the emergence of hemogenic precursors (CD45-CD31+CD34+) throughout hEB development (days 4, 7, 11, and 15) (Figure 2A). We confirmed by PCR stable expression of ectopic *FLT3* mutations on hEB differentiation (Figure 2B). Hemogenic precursors gradually emerge with time in all hESC lines studied, and their specification is not significantly affected by *FLT3* mutations throughout development (Figure 2C).

We next assessed whether *FLT3*-TKD and *FLT3*-ITD influence subsequent hematopoietic commitment of hemogenic precursors. The emergence of primitive (CD45+CD34+) and total (CD45+) hematopoietic cells was analyzed throughout hEB development (Figure 2D-E). Blood cells did not emerge before day 10 of hEB development. Interestingly, both *FLT3*-TKD and *FLT3*-ITD enhanced (~3- to 4-fold) hematopoietic differentiation of hESCs. *FLT3*-TKD accelerated the emergence of CD45+ and CD45+CD34+ blood cells (Figure 2D-E). Importantly, *FLT3*-TKD and *FLT3*-ITD also increased the clonogenic potential of hematopoietic progenitors derived from hEBs. Hematopoiesis generated from *FLT3*-activated hESCs displayed a 1.5- to 2-fold increased clonogenic potential (CFUs) in semisolid cultures (Figure 2F).

FLT3 cooperates with MLL-AF4 to abrogate hematopoietic commitment of hESCs.

The contribution of *FLT3* to MLL-AF4–mediated hematopoiesis and leukemogenesis is controversial.[17,18,38] We first explored whether *FLT3*-activating mutations cooperate with MLL-AF4 in the hematopoietic fate of hESCs. EV- and MLL-AF4-hESCs were transduced with either *FLT3*-TKD or *FLT3*-ITD. More than 85% of the cells within the EV/*FLT3* mutation–expressing and MLL-AF4/*FLT3* mutation–expressing hESC cultures were transduced as measured by GFP expression (Figure 1B); PCR for MLL-AF4 (Figure 1C) and *FLT3*-TKD or *FLT3*-ITD (Figure 1D); and phosphosignaling analysis for AKT, ERK, and STAT5 (Figure 1E).

We investigated the effect of *FLT3*-TKD and *FLT3*-ITD in collaboration with MLL-AF4 on the emergence of hemogenic precursors throughout hEB development. We confirmed by PCR stable expression of ectopic *FLT3* mutations and MLL-AF4 on hEB differentiation (Figure 3A-B). Both *FLT3* mutations slightly impaired the specification into hemogenic precursors of MLL-AF4-hESCs (Figure 3C), whereas they completely blocked subsequent differentiation of MLL-AF4-hemogenic precursors into primitive (Figure 3D) and total blood cells (Figure 3E). *FLT3*-TKD and *FLT3*-ITD also abrogated the clonogenic potential of MLL-AF4–expressing hematopoietic derivatives (Figure 3F). These data indicate that MLL-AF4 inhibits the *FLT3*-TKD/ITD–mediated enhanced blood differentiation of hESCs.

The developmental impact of *FLT3*-TKD and *FLT3*-ITD in hematopoietic cell fate of hESCs may be the result of (i) direct regulation of hematopoietic specification of hESCs or (ii) effects on the proliferation or survival of the emerging hematopoietic cells. To address this, cell cycle distribution (Figure 4A) and apoptosis (Figure 4B) were analyzed within the hESC-derived hemogenic precursor population and within the CD45+ blood cells (Figure 4A-B). No differences in the proportion of cycling (Figure 4A) or apoptotic (Figure 4B) hESC-derived hemogenic precursors or blood cells were observed when *FLT3*-TKD or *FLT3*-ITD was expressed alone or together with MLL-AF4. This suggests that *FLT3*-activating mutations promote hESC hematopoietic specification rather than selective proliferation or survival of hESC-emerging hematopoietic derivatives.

Several groups have reported the presence of *FLT3*-TKD mutations in MLL-rearranged ALLs,[15,16,39] whereas others have shown that *FLT3* mutations in MLL-rearranged ALLs do not occur.[17,20,21,40,41] High expression of *FLT3* is a hallmark step in the pathogenesis of MLL-AF4+ ALL,[17] but it has not been resolved whether the constitutive activation of *FLT3* in MLL-AF4+ ALL is because of the presence of activating *FLT3* mutations or simply the result of an increased expression of *FLT3*. We thus investigated whether ectopic expression of *FLT3-WT* cooperates with MLL-AF4 in the hematopoietic fate of hESCs. MLL-AF4-hESCs were transduced with *FLT3-WT*, and > 90% of the cells within the MLL-AF4/*FLT3-WT*-hESC cultures were transduced as measured by GFP expression (Figure 5A); PCR for *FLT3* (Figure 5B); and phosphosignaling analysis for AKT, ERK, and STAT5 (Figure 5C). Ectopic expression of *FLT3-WT* barely impaired specification into hemogenic precursors of MLL-AF4-hESCs (Figure 5D), but it completely blocked differentiation of MLL-AF4-hemogenic precursors into primitive (Figure 5E) and total blood cells (Figure 5F). Additionally, *FLT3-WT* overexpression fully abrogated the clonogenic potential of MLL-AF4–expressing hESC-hematopoietic derivatives (Figure 5G). This finding indicates that *FLT3* activation, either through activating mutations or overexpression of the WT gene, abolishes hematopoietic differentiation of MLL-AF4-hESCs.

FLT3 activation does not cooperate with MLL-AF4 for in vitro transformation of hESC-derived hematopoietic cells.

We have reported previously that MLL-AF4 on its own is not sufficient for in vitro transformation of hESC-derived hematopoietic

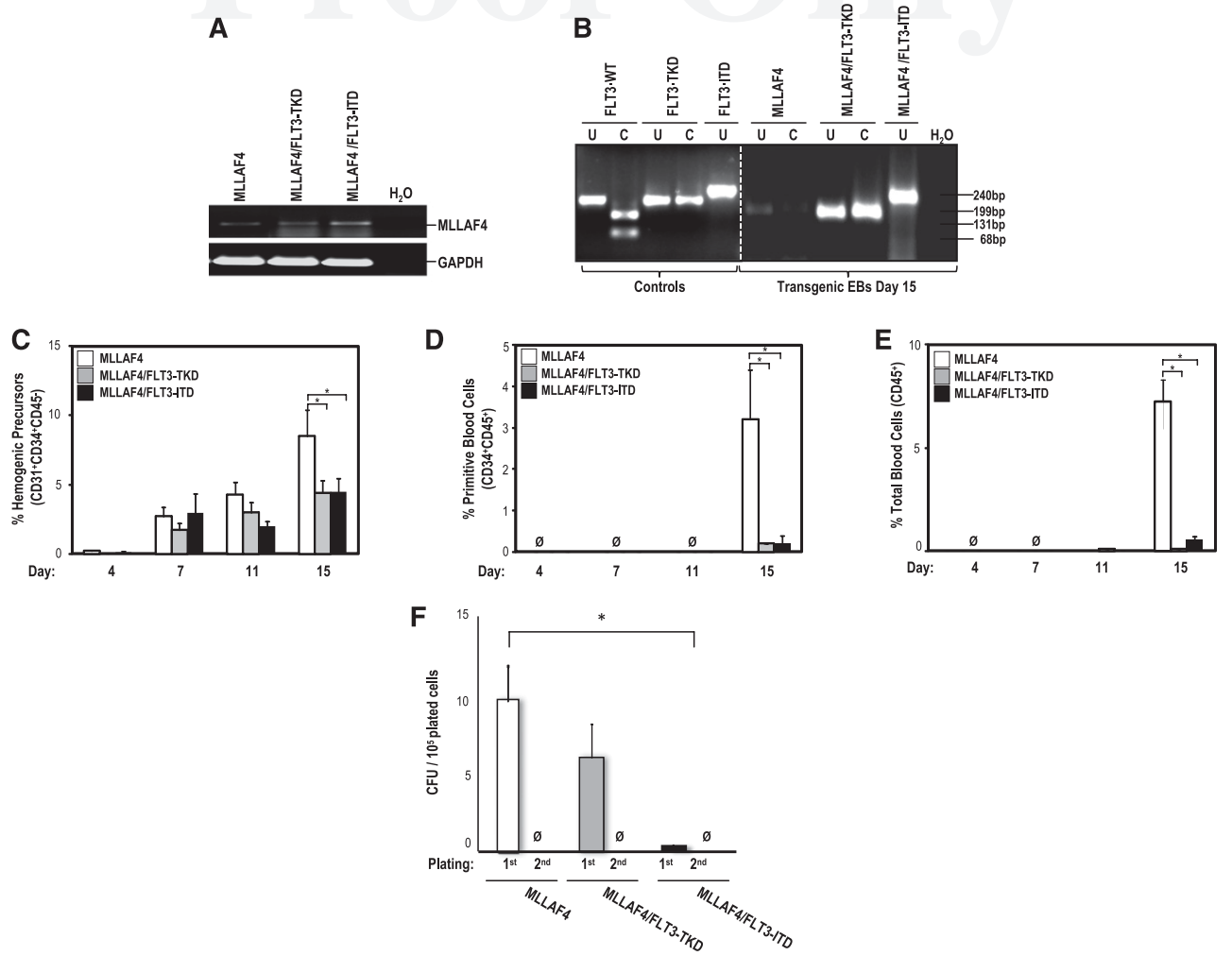


Figure 3. *FLT3*-activating mutations block hematopoietic differentiation from MLL-AF4-hESCs. (A) RT-PCR confirming stable expression of MLL-AF4 in transgenic day 15 hEBs. (B) PCR confirming the presence of either *FLT3*-TKD or *FLT3*-ITD mutations in transgenic day 15 hEBs. *FLT3*-activating mutations only impair late specification into hemogenic precursors of MLL-AF4–expressing hESCs (C) but completely block differentiation of MLL-AF4–expressing hESCs into primitive blood cells (D) and total blood cells (E). (F) CFU readout from d15 hEBs confirms a significant decrease in hematopoietic potential in hESCs coexpressing MLL-AF4 and *FLT3* mutations. Data are presented as mean±SEM for 9 independent experiments. MLL-AF4/*FLT3* mutation–expressing hESC-derived hematopoietic cells do not show stable in vitro replating efficiency in secondary CFU assays.

cells[14] and that secondary oncogenic hits may be required, making *FLT3* activation a candidate.[15-18,21,39,42] The data presented here reveal that expression of the MLL-AF4 fusion oncogene together with *FLT3* mutations does not confer either an in vitro proliferative or survival advantage to hESC-derived hemogenic precursors nor to CD45+ hematopoietic cells (Figure 4A-B). Functionally, *FLT3* activation alone (Figure 2F) or in combination with MLL-AF4 expression (Figure 3F, 5G) does not confer stable in vitro replating efficiency of hematopoietic cells in CFU assays, confirming that *FLT3* mutations are not secondary or cooperating hits for MLL-AF4 in the transformation of embryonic hematopoietic cells.

Transcriptional changes underlie the inhibition of hematopoiesis mediated by *FLT3*-TKD in MLL-AF4–expressing hESC-derived CD45+ hematopoietic cells.

To identify patterns of gene expression that could explain molecularly the developmental impact of *FLT3*-TKD in MLL-AF4–expressing hESC-derived CD45+ hematopoietic cells, we next performed a microarray analysis. A heatmap representation of global gene expression changes indicates that *FLT3*-TKD acts as a global transcriptional repressor when overexpressed in MLL-AF4–expressing hESC-derived CD45+ cells (Figure 6A). The genes differentially expressed in MLL-AF4 vs EV hESC-derived CD45+ cells and MLL-AF4/*FLT3*-TKD vs MLL-AF4 hESC–derived CD45+ cells were analyzed at the functional level using the IPA software. Analysis of the altered genes revealed that several gene functions (Figure 6B) and canonic pathways (Figure 6C) were altered in both MLL-AF4 vs EV and MLL-AF4/*FLT3*-TKD vs MLL-AF4 hESC–derived CD45+ cells. Among the altered gene functions, gene expression, cancer, embryonic development, hematologic system development and function, and hematopoiesis are the most significantly modulated cell functions on the expression of MLL-AF4 or MLL-AF4 together with *FLT3*-TKD (Figure 6B). Those genes differentially regulated in MLL-AF4 (vs EV) hESC–derived CD45+ cells, and in MLL-AF4/*FLT3*-TKD (vs MLL-AF4) hESC–derived CD45+ cells, which were classified by the IPA software as involved in hematopoietic system development and function, were analyzed in more detail (Figure 6D). MLL-AF4 expression enhances several hematopoietic functions (positive *z* score) mainly related to proliferation and differentiation of hematopoietic cells, while inhibiting (negative *z* score) biofunctions linked to cell movement, migration, and chemotaxis. Interestingly,

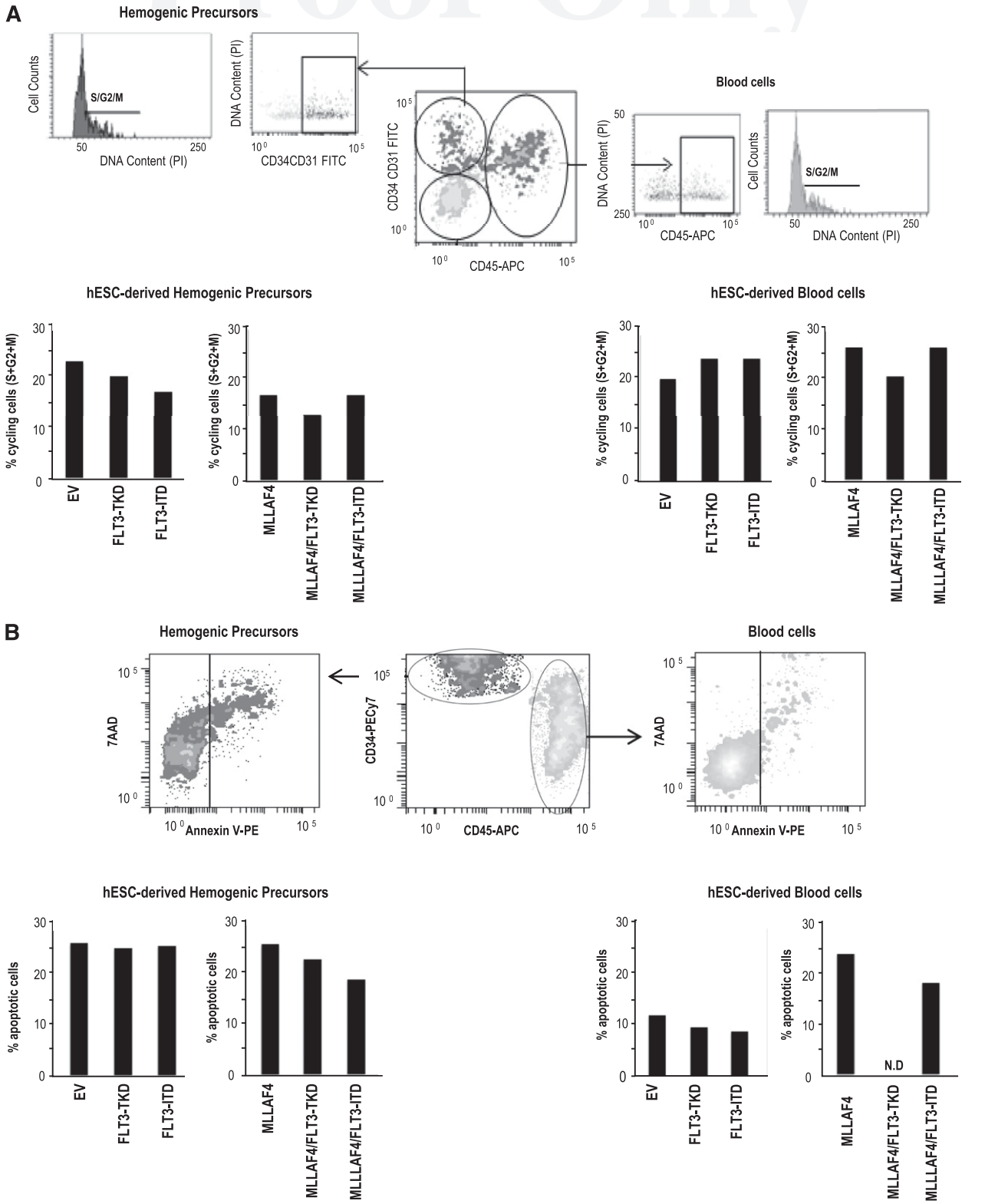


Figure 4. Cell cycle and apoptosis analysis reveal that the effect of *FLT3* mutations alone or in combination with MLL-AF4 on hESC hematopoietic specification is independent of proliferation or survival. (A) Top, representative flow cytometric analysis showing how cell cycle analysis was analyzed in hemogenic precursors and hematopoietic cells. Bottom, similar proportion of cycling (S+G2+M) hemogenic precursors (left) and hematopoietic cells (right) among the distinct transgenic hESCs. (B) Top, representative flow cytometric analysis showing how apoptosis was analyzed in hemogenic precursors and hematopoietic cells. Bottom, similar proportion of apoptotic (Annexin V+) hemogenic precursors (left) and hematopoietic cells (right) among the distinct transgenic hESCs. N.D: not determined because of the complete absence of CD45+ cells for analysis.

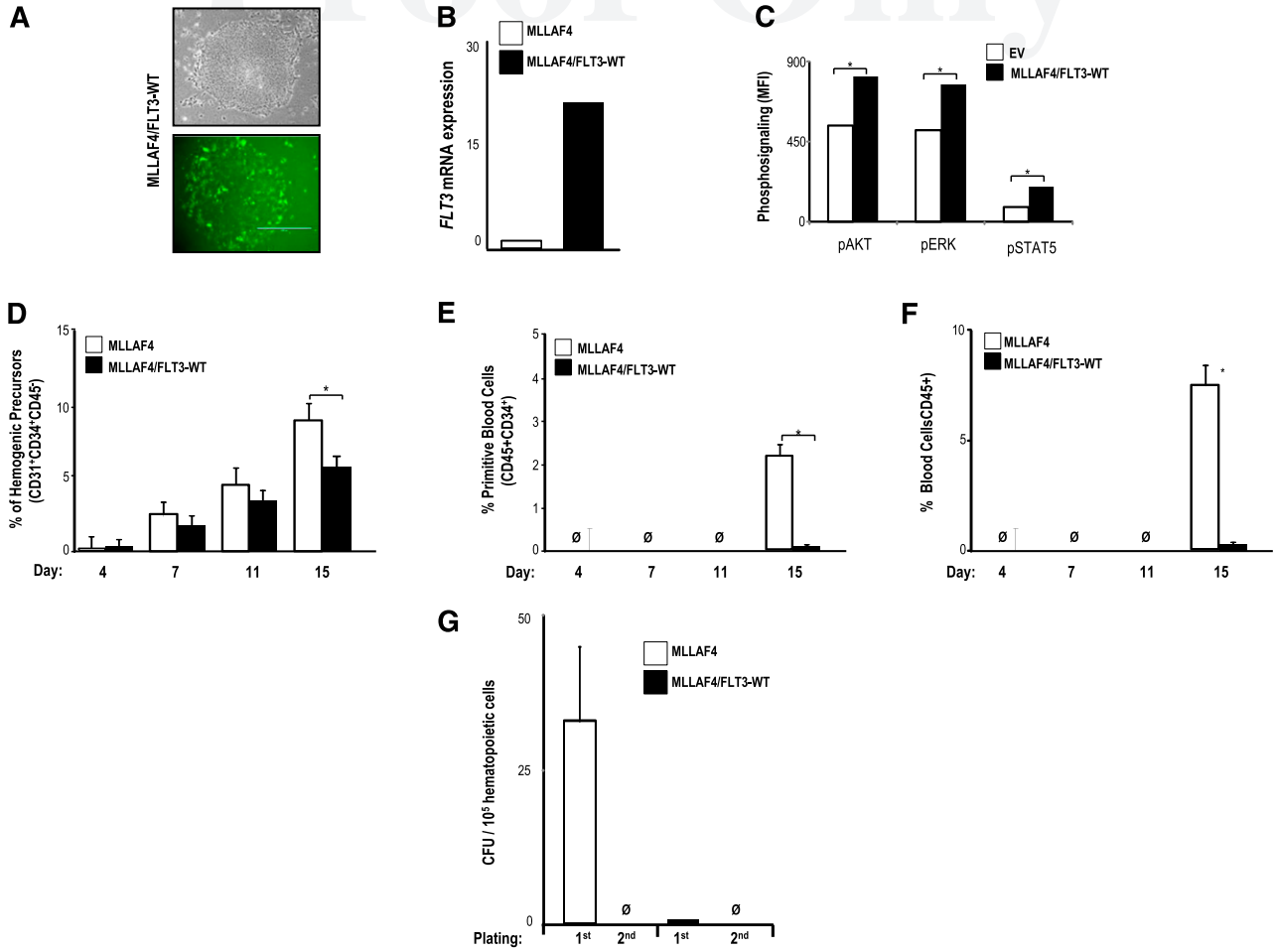


Figure 5. *FLT3* WT overexpression also abolishes hematopoietic differentiation from MLL-AF4-hESCs. (A) Phase-contrast morphology (top) and fluorescence microscopy (bottom) of colonies from MLL-AF4/*FLT3*-WT-hESCs. (B) Quantitative RT-PCR confirming efficient transduction and stable expression of *FLT3*-WT. (C) Phosphosignaling analysis of transduced hESCs, showing increased AKT, ERK, and STAT5 phosphorylation in cells transduced with *FLT3*-WT. *FLT3*-WT slightly impairs specification into hemogenic precursors of MLL-AF4–expressing hESCs (D), whereas it completely abolishes differentiation of MLL-AF4–expressing hESCs into primitive blood cells (E) and total blood cells (F). (G) CFU readout from day 15 hEBs confirms a significant decrease in hematopoietic potential in hESCs coexpressing MLL-AF4 and *FLT3*-WT. Data are presented as mean±SEM for 5 independent experiments.

ectopic expression of *FLT3*-TKD in MLL-AF4–expressing hESC-derived CD45+ cells has a strong impact on these biofunctions associated with a marked inhibition (negative *z* score) of biofunctions involved in the physiology and homeostasis of blood cell differentiation, and function (Figure 6D). Overall, this analysis indicates that transcriptional changes underlie the inhibition of hematopoiesis on *FLT3*-TKD expression in MLL-AF4–expressing hESC-derived CD45+ hematopoietic cells.

These transcriptional changes support that *FLT3*-TKD cooperates with MLL-AF4 to abrogate the hematopoietic commitment of hESCs. However, *FLT3*-TKD did not cooperate with MLL-AF4 to confer in vitro or in vivo transformation of hESCs/hESC-derived hematopoietic cells. Guenther et al[19] recently reported 159 MLL-AF4 direct target genes identified in leukemic samples, many of which are key for hematopoietic stem cell identity and self-renewal in human leukemia. We thus analyzed our gene expression data to determine whether these specific MLL-AF4 target genes are modulated in our cellular system. Ectopic expression of MLL-AF4 alone or together with *FLT3*-TKD induces transcriptional changes in subsets of this gene list in hESC-derived CD45+ cells (Figure 7A). Specifically, 75 and 106 MLL-AF4 target genes are transcriptionally modulated on expression of MLL-AF4 alone or MLL-AF4 along with *FLT3*-TKD, respectively (Figure 7B). These MLL-AF4 target genes differentially expressed in MLL-AF4 vs EV and MLL-AF4/*FLT3*-TKD vs MLL-AF4 hESC–derived CD45+ cells were analyzed using IPA. We found that MLL-AF4 target genes regulated by MLL-AF4 alone did not enhance biofunctions related to transformation. However, the subset of MLL-AF4 target genes regulated by expression of *FLT3*-TKD in MLL-AF4–expressing hESC-derived CD45+ hematopoietic cells is associated with an enhancement in biofunctions related to leukemia and cell transformation (positive *z* score) (Figure 7C-D).

It was reported that inhibition of DNA damage response (DDR) barriers accelerated MLL-rearrangement leukemogenesis.[43] Our GEP reveals that several pathways involved in DDR are affected by expression of MLL-AF4 alone or in conjunction with *FLT3*-TKD (Figure 6C). We found a global downregulation of many components associated with DDR signaling pathways such as DNA repair by homologous recombination, nonhomologous end-joining, or ATM signaling (supplemental Figure 2A-C). This finding supports the observed *FLT3*-mediated inhibition of hematopoiesis in MLL-AF4–expressing hESCs and indicates that although *FLT3*-TKD does not cooperate with MLL-AF4 to confer functional transformation of hESCs or hESC-derived cells, it induces

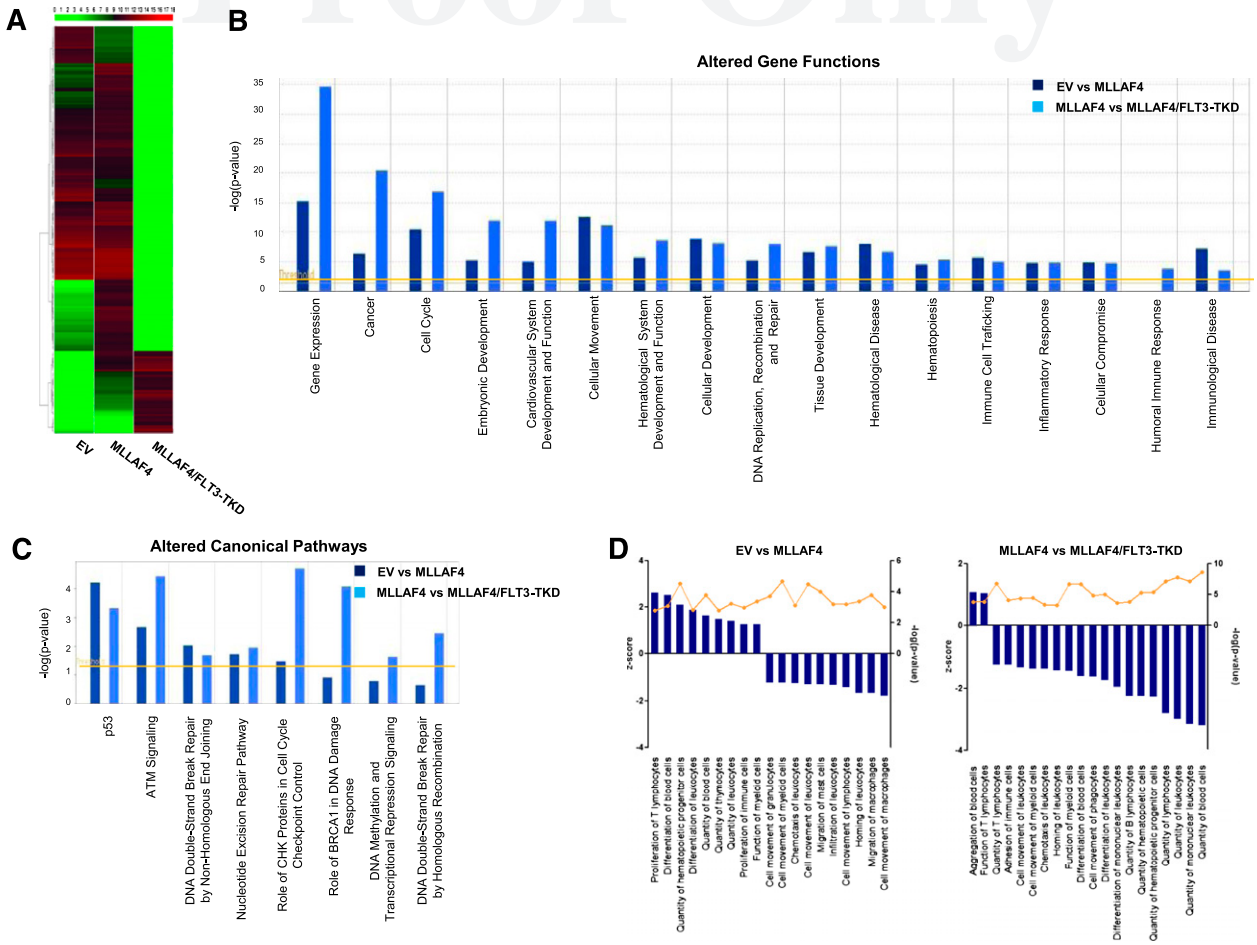


Figure 6. Gene expression profiling identifies gene functions and signaling pathways altered in MLL-AF4 and MLL-AF4/*FLT3*-TKD hESCs-derived CD45+ hematopoietic cells. (A) Heatmap diagram depicting the global gene expression profiling for EV-, MLL-AF4-, and MLL-AF4/*FLT3*-TKD hESC-CD45+ blood cells. The upper color bar codifies the gene expression in a log2 scale. Expression levels vary from highly expressed (red) to nonexpressed (green) genes. (B-D) After gene expression microarray analysis, the groups of genes differentially expressed (P value $< .01$; 2-fold regulation) in MLL-AF4 vs EV and MLL-AF4/*FLT3*-TKD vs MLL-AF4 hESC-CD45+ cells were compared, and the lists of gene functions and canonic pathways significantly altered were generated using the IPA software. IPA software–based data mining generated a list of significantly modulated gene functions (B) and canonic pathways (C) in MLL-AF4 vs EV hESC-CD45+ cells and MLL-AF4/*FLT3*-TKD vs MLL-AF4 hESC-CD45+ cells. (D) A more profound analysis was then performed for all of the genes classified by the IPA software as involved in hematopoietic system development and function/hematopoiesis. The IPA analysis–based z score is an estimation of the activation/inhibition status of a given category within an altered gene function.

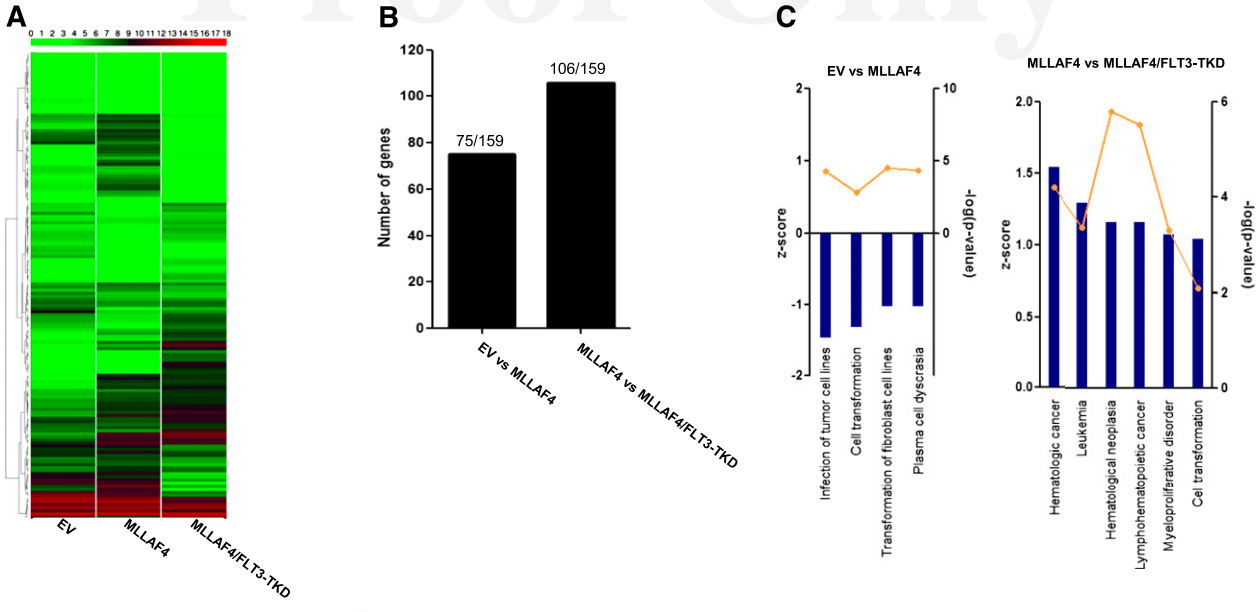
transcriptional modulation of MLL-AF4 target genes in MLL-AF4/*FLT3*-TKD hESCs–derived CD45+ hematopoietic cells.

Discussion

MLL-AF4+ ALL is a dismal leukemia that manifests in the first year of life.[6] MLL-AF4 is the initiating leukemogenic event with an in utero origin.[6] Our understanding about how MLL-AF4 deregulates early hematopoietic development is very limited, because despite recent advances in studies on mice[7,9] and human cellular systems,[13,14] current models do not recapitulate the disease phenotype or latency. Studies using cells from patients with MLL-AF4 are unable to address the developmental genesis of the hematopoietic system. Biphenotypic MLL-AF4+ infant ALL harbors and expresses fusion both in B cells and monocytes, suggesting that MLL-AF4 may arise in an ontogenically early hematopoietic stem cell.[3] We recently reported that MLL-AF4 affects the developmental fate of human CB-CD34+ hematopoietic stem cells and hESCs but was insufficient for leukemogenesis, suggesting that additional cooperating oncogenic hits are required for MLL-AF4–mediated leukemogenesis.[13,14]

Despite current controversy about the presence of *FLT3* gene mutations in MLL-AF4+ ALL,[15-17,20,21,39] GEP identified the *FLT3* gene consistently highly expressed in MLL-AF4+ ALL, indicating that *FLT3* activation may represent a candidate-cooperating lesion in MLL-AF4–mediated ALL. hESCs and hESC derivatives enable the study of early human development that cannot otherwise be addressed by patient sample analyses or mouse models. Hematopoietic differentiation of hESCs represents a strategy to study the onset of hematopoiesis, particularly the emergence of the earliest events leading to specification of the hematopoiesis.[11,12] We have thus explored the role of *FLT3* alone, or in cooperation with MLL-AF4, in the hematopoietic fate of hESCs and hESC-hemogenic precursors.

Activation of *FLT3* in hESCs promoted hematopoietic specification. Opposite to CD45+ blood commitment, *FLT3* mutations exerted neither blockage nor promotion of hESC specification into hemogenic precursors. These data suggest a dispensable role of *FLT3* in the specification of human hemogenic endothelium. In fact, GEP revealed that *FLT3* is expressed in hESC-derived CD45+ blood cells but not in CD45-CD31+ hemogenic precursors.



D

Category	Functions	Molecules
Cancer	Hematologic cancer	ADAM10, ARID4B, BCAT1, BCL2, ETV6, HOXA9, ITGA4, KDM6A, LMO2, MEIS1, PBX3, SATB1, SYK
	Leukemia	ADAM10, ARID4B, BCL2, ETV6, HOXA9, ITGA4, LMO2, MEIS1, SYK
	Hematological neoplasia	ADAM10, ARID4B, BCAT1, BCL2, BRD2, BTG1, ETV6, HMGA2, HOXA9, IKZF2, ITGA4, KDM6A, LMO2, MEIS1, PBX3, SATB1, SSBP2, SYK, TWIST1
	Lymphohematopoietic cancer	ADAM10,ARID4B,BCAT1,BCL2,BRD2,BTG1,ETV6,HMGA2,HOXA9,IKZF2,ITGA4,KDM6A,LMO2,MEIS1,PBX3,SATB1,SSBP2,SYK,TWIST1
	Myeloproliferative disorder	ARID4B, BCL2, ETV6, HMGA2, HOXA9, KLF3, MEIS1
	Cell transformation	AMD1, BCL2, CDK6, ETV6, HMGA2, HOXA9, MEIS1, PBX3

Figure 7. Comparative analysis of the MLL-AF4 target genes reported by Guenther et al[19] in MLL-AF4 and MLL-AF4/*FLT3*-TKD hESC-derived CD45+ hematopoietic cells. (A) Heatmap diagram depicting the expression of the MLL-AF4 target genes reported by Guenther et al[19] for EV-, MLL-AF4-, and MLL-AF4/*FLT3*-TKD hESC-CD45+ blood cells. The upper color bar codifies the gene expression in a log2 scale. Expression levels vary from highly expressed (red) to nonexpressed (green) genes. (B) Number of MLL-AF4 target genes (of the159 reported by Guenther et al[19]) found differentially expressed in MLL-AF4 vs EV hESC-CD45+ cells and MLL-AF4/*FLT3*-TKD vs MLL-AF4 hESC-CD45+. (C) The MLL-AF4 target genes reported by Guenther et al[19] differentially expressed in MLL-AF4 vs EV hESC-CD45+ cells (left panel) and MLL-AF4/*FLT3*-TKD vs MLL-AF4 hESC-CD45+ (right panel) were classified by IPA software. The *z* score is an estimation of the activation/inhibition status of a given category within an altered gene function. (D) List of MLL-AF4 target genes differentially expressed between MLL-AF4/*FLT3*-TKD and MLL-AF4 hESC-CD45+ cells.

FLT3-activating mutations affect myeloid vs lymphoid lineage restriction of adult HSPCs.[44] *FLT3* also plays a key role in controlling the homeostasis of B-cell development; it is highly expressed in hematopoietic stem cells and pro-B cells but becomes down-regulated at the pre-B-cell stage. This is in keeping with the accumulation of *FLT3* expression in patients with MLL-AF4+ who display a differentiation block at the pro-B-cell stage. In human embryonic hematopoiesis, cell cycle or apoptosis did not account for the enhanced hematopoiesis found on *FLT3* activation, indicating that *FLT3*-mediated effects underlie hESC specification rather than selective proliferation or survival of hESC-emerging hematopoietic derivatives. This mechanism suggests that, signaling downstream, the activated receptor may differ between embryonic and adult/definitive hematopoiesis. Although *FLT3*-TKD and *FLT3*-ITD mutations have been reported to signal through distinct mechanisms, their ectopic expression in hESCs induced similar phosphorylation of the signal-transducing effectors AKT, ERK, and STAT5. Activation of *FLT3* in hESCs was compatible with pluripotency, in line with previous findings supporting the importance of AKT and ERK in hESC maintenance.[45]

Intriguingly, *FLT3* activation in MLL-AF4-hESCs robustly abrogated hematopoietic commitment of hESCs but barely affected MLL-AF4-hESC specification into hemogenic precursors, indicating that MLL-AF4 cooperates with *FLT3* to abolish hematopoietic commitment of hESCs. Similarly, Furuichi et al[38] have reported that *FLT3* activation antagonizes with MLL rearrangements to inhibit proliferation of MLL-rearranged cell lines. Additionally, Sexauer et al[46] have recently shown that *FLT3*-ITD blocks terminal myeloid differentiation. In MLL-AF4-hESCs, activation of *FLT3* blocked hematopoietic commitment without affecting proliferation or survival of hESC-emerging hematopoietic derivatives. GEP analysis revealed that expression of *FLT3*-TKD in MLL-AF4–expressing hESC-derived CD45+ cells resulted in a genetic profile consistent with the inhibition of biofunctions involved in the physiology and homeostasis of blood cell differentiation and function, indicating that transcriptional changes underlie the inhibition of hematopoiesis on *FLT3*-TKD expression in MLL-AF4–expressing hESC-derived CD45+ hematopoietic cells. Similar to what occurs in primary MLL-AF4+ ALL, MLL-AF4–expressing hESC-derived CD45+ cells expressed high levels of MYC, HOXA9, SET, and RAN, which are proto-oncogenes activated by MLL-AF4 via aberrant H3K79 dimethylation and DOT1L recruitment.[47]

Coexpression of both MLL-AF4 and *FLT3* mutations did not immortalize hESCs or hESC-derived hematopoietic derivatives. hESCs and hESC hematopoietic cells coexpressing both MLL-AF4 and *FLT3* mutations did not display a proliferative and/or survival advantage and failed to confer replating efficiency in hematopoietic CFU assays. This finding suggests that either additional oncogenic hits[7] may be required for leukemogenesis, or that embryonic cells are not the appropriate cellular target for MLL-AF4–mediated

transformation. It cannot be ruled out that other embryonic precursors or fetal HSPCs represent better candidate target cells in which MLL-AF4 originates and/or exerts its oncogenic function. Yamaguchi et al[18] reported that MLL-AF4 together with *FLT3*-TKD conferred the mouse cell line 32Dc IL3-independent robust proliferative capacity, supporting the need for further (epi)-genetic oncogenic events.

Guenther et al[19] reported 159 MLL-AF4 direct target genes. Analysis of these 159 MLL-AF4 target genes in our GEP datasheet shows that 75 and 106 MLL-AF4 target genes are transcriptionally modulated on expression of MLL-AF4 alone or MLL-AF4 along with *FLT3*-TKD, respectively. These genes differentially expressed in MLL-AF4/*FLT3*-TKD hESC–derived blood cells were analyzed using IPA software, and it was found that expression of *FLT3*-TKD in MLL-AF4–expressing hESC-derived CD45+ cells regulates a subset of MLL-AF4 target genes involved in cell transformation and leukemia. Additionally, it has been reported that DDR is a rate-limiting event for acquisition of stem cell properties in MLL-mediated transformation, as experimental inhibition of the DDR barrier accelerated leukemia development.[43] Our GEP reveals a global downregulation of many components associated with DDR master signaling pathways. These GEP data indicate that although *FLT3*-TKD does not cooperate with MLL-AF4 to transform hESCs or hESC-derived hematopoietic cells, it induces transcriptional modulation of MLL-AF4 target genes in hESCs-derived CD45+ hematopoietic cells, partially resembling the scenario found by Guenther et al[19] by ChIP-Seq analysis.

Epigenetics represent a key player in MLL-AF4–driven transformation.[48,49] In contrast to many human cancers, which are commonly characterized by hypomethylation in nonpromoter regions, MLL-AF4+ ALL displays genome-wide hypermethylation at nonpromoter sequences.[48,49] Global hypomethylation is usually associated with genomic instability, allowing additionally acquired genetic hits to propel a premalignant clone into a fully transformed state. The global hypermethylation of MLL-AF4+ ALL might explain why additional genetic lesions have not been discovered in MLL-rearranged ALL.[40,41]

We provide a hESC model to deepen our understanding about the mechanisms underlying the developmental impact of the MLL-AF4 and *FLT3* signaling pathway during human early embryonic development. It also offers a unique in vitro system in which to test the ability of potential cooperating oncogenic events (reciprocal AF4-MLL that causes epigenetic remodeling and strong transcriptional enhancement)[50] or causal genotoxic compounds to induce leukemic transformation.

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Authorship

Contribution: C.B. and P.M. designed the research of this manuscript. C.B., V.A., R.M., O.N.-M., D.R.-M., V.R.-M., P.J.R., and M.J.A.-B. performed research and analyzed data. C.B. and P.M. wrote the manuscript. The manuscript has been seen and approved by all authors.

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Q:4 References

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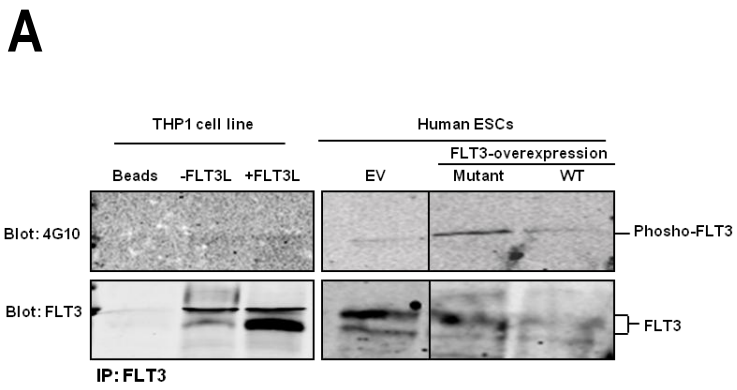
Q:5 Q:6 Q:7

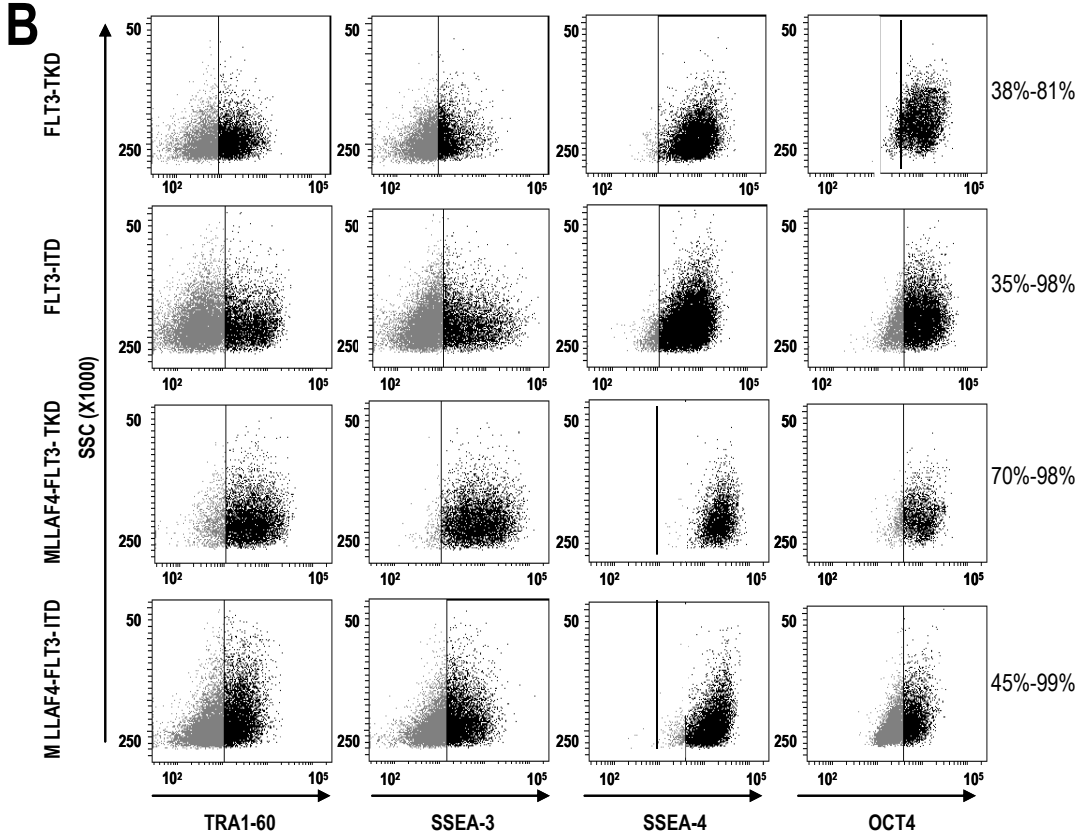
LEGEND TO SUPPLEMENTAL FIGURES

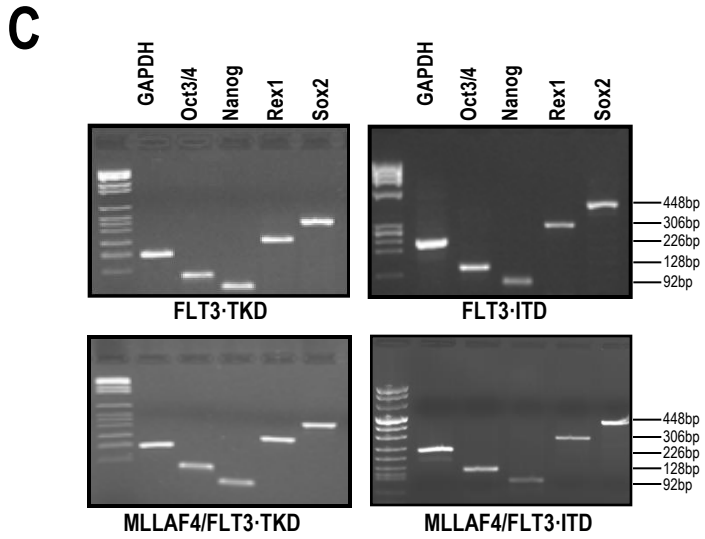
FIGURE S1. Enforced expression of FLT3 mutations alone or together with MLL-AF4 fusion gene is compatible with hESC pluripotency. (A) Activation of FLT3 in hESCs through ectopic expression of either FLT3 mutants or FLT3-WT induces FLT3 receptor phosphorylation. As a control, the MLL-rearranged THP1 cell line before and after treatment of FLT3 ligand was used. (B) Similar expression of the hESC markers TRA-1-60, SSEA-3, SSEA-4 and OCT4 in the indicated hESCs. (C) RT-PCR showing comparable mRNA expression levels of the pluripotency markers SOX2, NANOG, OCT3/4 and REX1 in the indicated hESCs. (D) Representative histological analysis of teratomas formed from transgenic hESCs depicting three germ layer developmental potential.

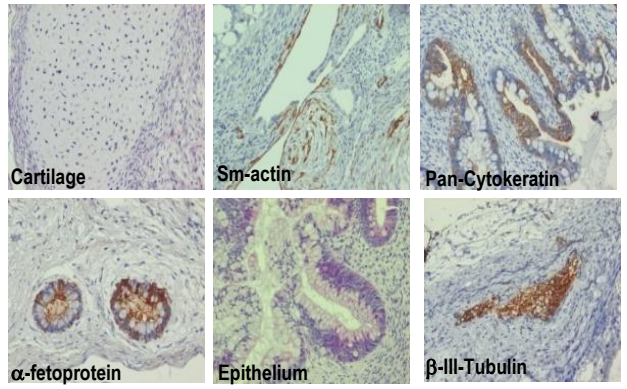
FIGURE S2. Schemes provided by IPA software specifically showing expression changes in genes involved in master signaling pathways associated with DNA damage repair. (A) DNA repair by Homologous Recombination, (B) DNA repair by Non-Homologous End Joining and (C) ATM signaling. For each signaling pathway, the cartoon on the left compares MLLAF4 *vs* EV hESC-derived CD45+ cells whereas the cartoon on the right compares MLLAF4/FLT3-TKD *vs* MLLAF4 hESC-derived CD45+ cells. Among the differentially expressed genes, the up-regulated ones are shown in red and the down-regulated ones in green. Open (no-colored) genes are not differentially expressed between the indicated conditions.

Bueno et al Figure 1S ↑





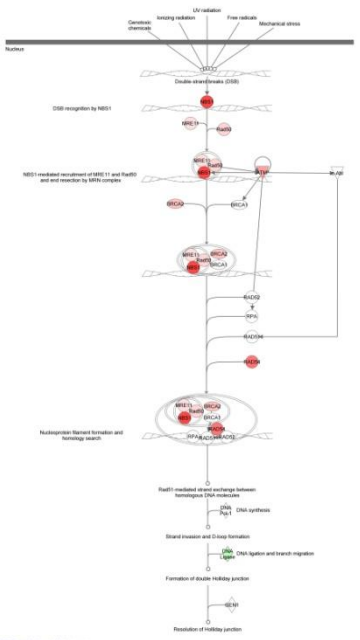




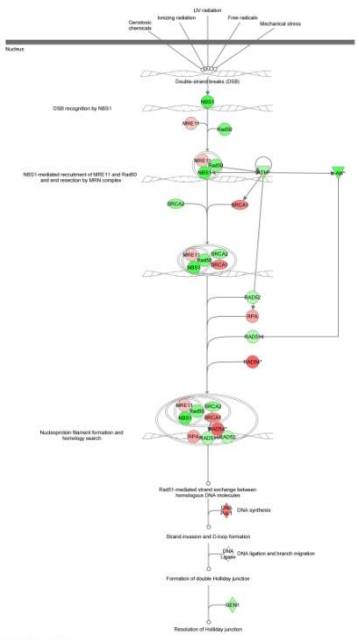
A

DNA repair by HR

EV vs MLLAF4



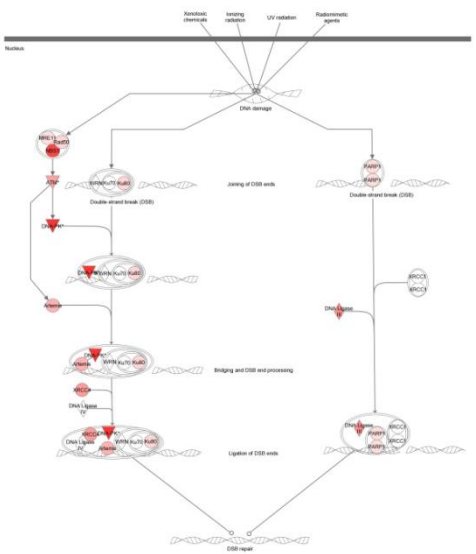
MLLAF4 vs MLLAF4/FLT3-TKD



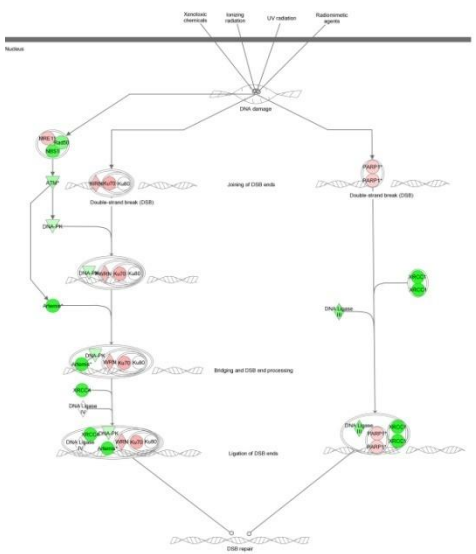
B

DNA repair by NHEJ

EV vs MLLAF4



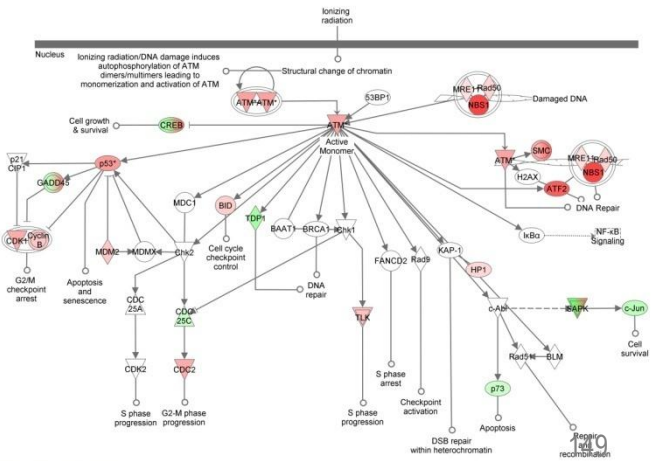
MLLAF4 vs MLLAF4/FLT3-TKD



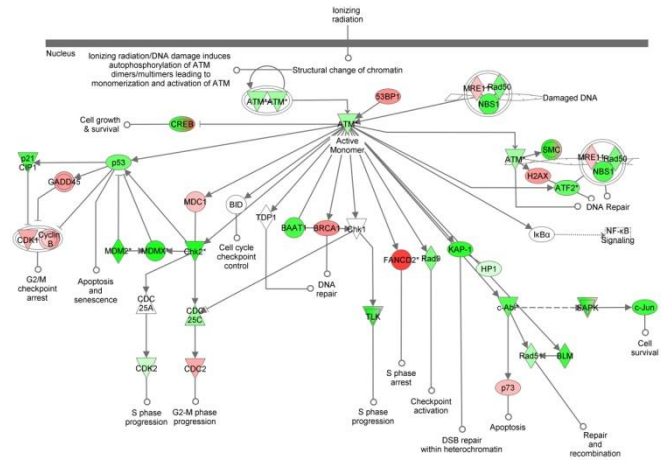
C

ATM signaling

EV vs MLLAF4



MLLAF4 vs MLLAF4/FLT3-TKD



Inside Blood

The ongoing conundrum of MLL-AF4 driven leukemogenesis.

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In this issue of *Blood*, Bueno and colleagues explored the developmental impact, as well as the transforming capacity of the MLL-AF4 fusion protein in combination with FLT3 activation in human embryonic stem cells.[1]

MLL-AF4+ pro-B acute lymphoblastic leukemia (ALL) in infants represents an aggressive, high-risk type of childhood leukemia arising from prenatally acquired pre-leukemic t(4;11) chromosomal translocations. However, despite various reported attempts, accurately mimicking MLL-AF4 driven leukemogenesis in mice remains a difficult task. Enforced expression of the MLL-AF4 fusion protein in cord blood derived human hematopoietic stem cells (HSCs) transplanted into immunodeficient mice either result in the development of malignancies that deviate from the pro-B ALL phenotype as observed in humans, or do not lead to neoplasia at all.[2] Obviously these discrepancies raise important questions: (I) Does MLL-AF4+ pro-B ALL in infants arise in CD34+ HSCs? (II) Are MLL-AF4 fusion proteins driving leukemogenesis on their own, or are cooperative genetic lesions required? (III) do MLL-AF4 fusion proteins exert sufficient transforming capacity? For instance, Bursen *et al.* recently showed that not MLL-AF4, but enforced expression of its reciprocal fusion protein AF4-MLL in murine hematopoietic stem/progenitor cells induced ALL in mice, without the requirement of MLL-AF4.[3] In contrast, Tamai *et al*. showed that enforced expression of MLL-AF4 in murine HSCs is sufficient to induce ALL, but demonstrated that the process of transformation is significantly accelerated by cooperative *K-Ras* mutations. Nonetheless, these experiments remain to be repeated in HSCs of human origin in order to appreciate the relevance of AF4-MLL and/or RAS activation in the development of MLL-AF4+ pro-B ALL. Moreover, the reciprocal *AF4-MLL* fusion transcript is present in the majority, but not all MLL-AF4+ ALL patients[3], and *RAS* mutations are found in ~25% of the cases.[5] Hence, distinct mechanisms of transformation, as well as the involvement of yet unknown genetic events, can not be ruled out.

Meanwhile, dr. Pablo Menendez and his co-workers have been elegantly addressing the question of the cell of origin from which MLL-AF4+ pro-B ALL may arise. Based on their earlier observations that bone marrow derived mesenchymal stem cells from MLL-AF4+ pro-B ALL patients harbor and express the *MLL-AF4* fusion gene[6], Menendez *et al*. reasoned that this type of leukemia may well arise in pre-hematopoietic mesodermal or hemangioblastic precursors sprouting from differentiating human embryonic stem cells (hESCs). To test this hypothesis, this research group recently created a cellular system to study early hemato-endothelial development in MLL-AF4 expressing hESCs. Interestingly, introducing MLL-AF4 expression in hESCs enhanced the specification of hemogenic precursors, but impaired further hematopoietic commitment in favor of and endothelial cell fate. Alas, MLL-AF4 expression alone appeared not sufficient to induce leukemia in hESC-derived hematopoietic cells.[7] In the present study Bueno *et al*.[1] explored the impact of Fms-like tyrosine receptor 3 (FLT3) activation on the hematopoietic fate of their MLL-AF4 expressing hESCs. Patients with MLL-AF4+ pro-B ALL frequently display constitutive FLT3 activation, usually as a result of high-level *FLT3* expression, or sporadically due to activating mutations within the tyrosine kinase domain.[8] Activated FLT3 positively affects several signal transduction pathways all of which favor cell survival and proliferation, and supposedly provides (pre-)leukemic cells with a growth advantage and possibly with enhanced transforming capacity. Hence, FLT3 activation may

well be an additional genetic event required for MLL-AF4 driven leukemogenesis. Interestingly, Bueno *et al*. show that in MLL-AF4 expressing hESCs, activated FLT3 is capable of abolishing hematopoietic differentiation, indeed suggesting a role for FLT3 activation in the development of MLL-AF4+ pro-B ALL. However, FLT3 activation did not seem sufficient to cooperate with MLL-AF4 in transforming hESC-derived hematopoietic cells.

Nonetheless, the presented MLL-AF4 expressing hESC model represents an intriguing experimental system that hopefully soon will also be used to explore the impact of *RAS* mutations, or perhaps converted into an AF4-MLL expressing hESC model. In the mean time it remains important to keep searching for alternative (epi)genetic hits that potentiate MLL-AF4 driven leukemogenesis, for example using whole genome sequencing approaches.

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9.- Ligand-independent FLT3 activation does not cooperate with MLL-AF4 to immortalize/transform cord blood CD34+ cells.

Montes R., Bursen A., Prelle C., Marschalek R., Chillón M.C., Romero-Moya D., Prieto C., Navarro-Montero O., Bueno C., Menendez P.

(Sometido a publicación, Leukemia 2013)

INTRODUCCIÓN. La LLA pro-B posee un pronóstico fatal y una latencia muy corta, manifestándose antes del primer año de vida. Esta leucemia se caracteriza por presentar la traslocación t(4;11), dando lugar al oncogén de fusión MLL-AF4. El origen de la traslocación es prenatal, y hasta la fecha se desconocen qué eventos/efectores colaboran con la expresión de MLL-AF4 en el desarrollo de la enfermedad. Los fenómenos que conducen a la activación constitutiva del receptor FLT3 es uno de los posibles eventos secundarios candidatos, dado que se ha demostrado que la LLA con reordenamiento de *MLL* presenta altos niveles de expresión de *FLT3*. Además, dado el origen prenatal, las HSPCs derivadas de SCU suponen un posible estadio ontogénico neonatal para el estudio y modelaje de la enfermedad.

OBJETIVO. Conocer el efecto de la activación del receptor FLT3, por mutación puntual o por sobrexpresión, por sí mismo o en colaboración con la expresión de MLL-AF4, sobre HSPCs CD34+ de SCU.

MATERIAL Y MÉTODOS. Las HSPCs CD34+ aisladas mediante procedimientos magnéticos a partir de SCU fueron trasducidas con vectores lentivirales que contenían MLL-AF4, FLT3-TKD, FLT3-WT y vector vacío (control). Las células trasducidas fueron separadas mediante FACS según la expresión de su gen reportero: GFP para MLL-AF4 o GFP-control; dTo para FLT3-TKD, FLT3-WT, o bien GFP+dTo+ para MLL-AF4/FLT3-TKD y MLL-AF4/FLT3-WT. Dicha expresión fue confirmada por RT-PCR, qPCR y RFLP-PCR. Las células trasducidas y separadas fueron trasplantadas intratibia en ratones inmunodeficientes subletalmente irradiados. Los ratones fueron sacrificados 14 semanas post-trasplante aislándose células de la MO de la tibia inyectada, de la MO de la tibia no inyectada (contralateral), del bazo y de la sangre periférica. En estos órganos se valoró por citometría la reconstitución hematopoyética humana, así como la diferenciación multilinaje de la misma. Así mismo, se analizarón tanto parámetros bioquímicos-hematológicos en sangre periférica, como la morfología e histología de bazo e hígado. En los estudios *in vitro*, las células CD34+ trasducidas se utilizaron para ensayos de clonogenicidad en metilcelulosa, y para ensayos de proliferación en cultivo líquido, así como para análisis de ciclo celular y apoptosis.

RESULTADOS. La activación de FLT3 potencia la reconstitución hematopoyética FLT3, sólo o en combinación con MLL-AF4, y no afecta la diferenciación multilínea ni la movilización. *In vitro*, la activación de FLT3 no aumentó la capacidad clonogénica de las HSPCs, ni cooperó con MLL-AF4 en inducir trasformación oncogénica, pero sí aumentó de forma transitoria la expansión en cultivo líquido de las CD34+ debido a un aumento de la proliferación y de la supervivencia.

CONCLUSIÓN. A pesar de que las leucemias con reordenamiento de *MLL* muestran una alta expresión del receptor FLT3, la activación de FLT3 (por mecanismos de sobrexpresión o por mutación puntual) en HSPCs CD34+ de SCU que expresan MLL-AF4, no induce leucemia *in vivo*, e *in vitro* no supone la adquisición de ventaja proliferativa ni inmortalización, por lo que posiblemente son otros eventos secundarios, como pueden ser expresión del producto recíproco AF4-MLL, la actividad de la metiltrasferasa DOT1L asociada a reordenamientos de *MLL*, mutaciones del oncogén K-ras, o bien modificaciones epigenéticas, los eventos secundarios que cooperan en el proceso leucémico mediado por MLL-AF4.

Ligand-independent FLT3 activation does not cooperate with MLL-AF4 to immortalize/transform cord blood CD34+ cells

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Running Title: FLT3-TKD and MLL-AF4 in CB-CD34+ cells

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KEY POINTS

-FLT3.TKD mutations or increased expression of germline *FLT3* do not cooperate with MLL-AF4 fusion protein to transform CB-CD34+ cells either *in vitro* or *in vivo*.

-FLT3 activation augments multilineage hematopoietic engraftment, but not *in vitro* clonogenic potential of CB-CD34+ cells, without skewing hematopoietic lineage commitment.

ABSTRACT

MLL-AF4 fusion is hallmark in high-risk infant pro-B-acute lymphoblastic leukemia (pro-B-ALL). Our limited understanding of MLL-AF4-mediated transformation reflects the absence of human models reproducing this leukemia. Hematopoietic stem/progenitor cells (HSPCs) constitute likely targets for transformation. We previously reported that MLL-AF4 enhanced hematopoietic engraftment and clonogenic potential in cord blood (CB)-derived CD34+ HSPCs but was not sufficient for leukemogenesis, suggesting that additional oncogenic lesions are required for MLL-AF4-mediated transformation. MLL-AF4+ pro-B-ALL display enormous levels of *FLT3*, and occasionally *FLT3* activating mutations, thus representing a candidate cooperating event in MLL-AF4+ pro-B-ALL. We have explored whether FLT3.TKD mutation or increased expression of FLT3.WT cooperates with MLL-AF4 to immortalize/transform CB-CD34+ HSPCs. In vivo, FLT3.TKD/FLT3.WT alone, or in combination with MLL-AF4, enhances hematopoietic repopulating function of CB-CD34+ HSPCs without impairing migration or hematopoietic differentiation. None of the animals transplanted with MLL-AF4+FLT3.TKD/WT-CD34+ HSPCs showed any sign of disease after 14 weeks. In vitro, enforced expression of FLT3.TKD/FLT3.WT conveys a transient overexpansion of MLL-AF4-expressing CD34+ HSPCs associated to higher proportion of cycling cells coupled to lower apoptotic levels, but does not augment clonogenic potential nor confer stable replating. Together, FLT3.TKD/FLT3.WT does not suffice to immortalize/transform MLL-AF4-expressing CB-CD34+ HSPCs, suggesting the need of alternative (epi)-genetic cooperating oncogenic lesions.

Key words: MLL-AF4, FLT3.TKD, FLT3.WT, CB-CD34+ HSPCs, leukemogenesis.

INTRODUCTION

The translocation t(4;11)(q21;q23) encodes the fusion protein MLL-AF4 which is the hallmark genetic abnormality associated to infant pro-B/monocyte acute lymphoblastic leukemia (ALL). This newborn pro-B ALL is characterized by its dismal prognosis and very brief latency, raising the question of how this disease evolves so quickly (Pui et al. 1991; Greaves 1996; Pui 2000; Meyer et al. 2009). Compelling evidence indicates that MLL-AF4 arises prenatally during embryonic/fetal hematopoiesis (Ford et al. 1993; Greaves et al. 2003; Greaves and Wiemels 2003; Bueno et al. 2011). To understand the developmental impact of MLL-AF4, we first need to elucidate which is the target cell for transformation, and the cooperating oncogenic lesions underlying MLL-AF4-mediated transformation (Eguchi et al. 2005; Bueno et al. 2011; Marschalek 2011).

MLL-AF4-induced leukemogenesis has been difficult to model and *bona fide* MLL-AF4 disease models do not exist (Chen et al. 2006; Metzler et al. 2006; Krivtsov et al. 2008; Bursen et al. 2010). Our understanding of MLL fusions comes from murine models which do not recapitulate the human disease faithfully, suggesting that these mouse models may be missing essential components of leukemogenesis during early human development. It could be argued that the lack of a MLL-AF4 model may be due to: (i) a cell in a wrong developmental stage was targeted in the mouse; (ii) the impact of other secondary hits has not been properly addressed; or (iii) MLL-AF4 exerts its transforming function preferentially in human cells, indicating that the MLL-AF4 function has to be addressed using ontogenically primitive human stem cells. Among these, neo-natal (cord blood [CB]-derived) CD34+ hematopoietic stem/progenitor cells (HSPCs) or pre-natal (fetal- or embryonic-derived) cells represent ontogenically early candidate target cells.

We have recently reported that the expression of MLL-AF4 has a functional impact in CB-CD34+ HSPCs (Montes et al. 2011) and human embryonic stem cell (hESC)-derived hematopoietic cells (Bueno et al. 2012). In CB-CD34+ HSPCs, MLL-AF4 enhanced hematopoietic engraftment and clonogenic potential but was not sufficient for leukemogenesis (Montes et al. 2011). In hESCs, MLL-AF4 altered the developmental cell fate, skewing the early hemato-endothelial specification of hESCs (Bueno et al. 2012), but was also unable to transform hESCs or hESC blood derivatives. These data make us to hypothesize that the inability to develop a MLL-AF4 model may not be due to the human cell context or cell type targeted, but rather that additional oncogenic lesions are required for leukemogenesis.

Gene expression profiling showed that *FLT3* is highly expressed in MLL-AF4+ pro-B-ALL. Moreover, it was shown that other *MLL*-rearranged leukemias display FLT3.TKD (Tyrosine Kinase Domain) mutations in up to 20% of the cases, suggesting they may represent candidate cooperating events (Armstrong et al. 2004; Taketani et al. 2004). Accordingly, Yamaguchi *et al* (Yamaguchi et al. 2009a) have reported that FLT3.TKD cooperates with MLL-AF4 to induce *in vitro* aggressive proliferation of the mouse cell line 32Dc. Several other groups suggest, however, that *FLT3* mutations are not common in MLL-AF4+ pro-B-ALL, and that an increased transcriptional expression of *FLT3* may act as secondary cooperating hit (Stam et al. 2007a; Stam et

al. 2007b; Guenther et al. 2008b; Chillon et al. 2012). The latter is supported by Guenther *et al* (Guenther et al. 2008b) who reported that *FLT3* is a direct transcriptional target of MLL-AF4.

We have very recently reported that *FLT3* activation cooperates with MLL-AF4 to abrogate the hematopoietic specification of hESCs (Bueno et al. 2013 (in press)). However, whether *FLT3* activating mutations or increased expression of germline *FLT3* cooperates with MLL-AF4 in post-natal CB-derived CD34+ HSPCs remains unknown. Here, we have explored whether FLT3.TKD or FLT3.WT in combination with MLL-AF4 fusion protein suffices to immortalize/transform CB-CD34+ HSPCs *in vitro* and/or *in vivo*.

MATERIAL AND METHODS

CB collection and CD34+ HSPC isolation

Fresh umbilical CB units from healthy newborns were obtained from The Andalusian Public Cord Blood Bank upon approval by our local Ethics and Biozahard Board Committee. CB samples were pooled to reduce variability among individual CB units. Mononuclear cells were isolated using Ficoll-Hypaque. After lysing the red cells (StemCell Technologies, Vancouver, Canada), CD34+ cells were purified by magnetic bead separation using the human CD34 MicroBead kit and the AutoMACS Pro separator (Miltenyi) as per manufacturer's instructions (Bueno et al. 2008; Bueno et al. 2009). Purity of the CD34+ fraction was assessed by flow cytometry using anti-CD34-PE (Beckton Dickinson) and only CD34+ fractions showing purity >95% were used (Bueno et al. 2008; Bueno et al. 2009). The CD34- fraction was irradiated (1500 cGy), and used as accessory cells for co-transplantation with transduced CD34+ HSPCs.

Plasmid Construction and Lentiviral transduction

The MLL-AF4 cDNA (MLL-exon10 fused to AF4-exon8) and the FLT3.TKD (D835 mutation) and FLT3.WT cDNAs (kindly provided by Prof. H. Serve, University of Münster, Germany) were subcloned into the pRRL-EF1α-PGK-GFP/dTo vector (Bueno et al. 2013 (in press)). The following lentivectors were used: pRRL-EF1α-PGK-GFP/dTo (empty vector; EV), pRRL-EF1α-MLL-AF4-PGK-GFP (MLL-AF4), pRRL-EF1α-FLT3.TKD-PGK-dTo (FLT3.TKD), and pRRL-EF1α-FLT3.WT-PGK-GFP (FLT3.WT) (**Fig 1A**). VSV-G-pseudotyped viral particles were generated on 293T cells by calcium-phosphate transfection and concentrated by ultracentrifugation as described (Montes et al. 2011; Real et al. 2012a). Human CD34+ HSPCs ($2x10^6$ cells) were infected overnight with concentrated viruses in the presence of Polybrene (1μg/mL; Sigma) and the following hematopoietic cytokines SCF (100ng/mL), FLT3L (100ng/mL) and IL-3 (10ng/mL) (Peprotech). The following day, the viral supernatant was removed and transduced CD34+ HSPCs were washed (Montes et al. 2011).

For *in vitro* and *in vivo* functional experiments, transduced cells were sorted (FACSAria, Becton Dickinson -BD) based on reporter expression: GFP+ for MLL-AF4-transduced CD34+ cells, dTo+ for FLT3.TKD/WT-transduced cells, and merged-yellow for MLL-AF4 and FLT3 co-transduced cells.

Mice transplantation

NOD/LtSz-scid IL-2Rγ$^{-/-}$ mice (NSG) (Bueno et al. 2010a; Navarro-Montero et al. 2012) were housed under sterile conditions. The Animal Care Committee of the University of Granada approved all mouse protocols. Mice at 8 to 12 weeks of age were sublethaly irradiated (2.50 cGy) 6-16 hours prior to transplantation. The mice were anesthetized with isoflurane inhalation and intra bone marrow transplantation (IBMT) was performed as described in detail (Levac et al. 2005; Bueno et al. 2010a). Seventy-two hours after infection, CD34+ cells were sorted based on reporter expression, and $3x10^4$ transduced/sorted CD34+ HSPCs (along with $5x10^4$ irradiated accessory cells) were transplanted in a volume of 30-40 µl. For pain relief, 1 mg buprenorphine was administered immediately after transplantation (Levac et al. 2005; Bueno et al. 2010a).

Analysis of engraftment

Mice were killed 14 weeks post-transplant. BM was flushed from injected tibia (IT) and from contralateral tibia, femur and iliac crests (CL). Spleen, liver and PB were also collected and analyzed for human chimerism. Mice health was monitored throughout the entire experiment. Cells from BM, liver, spleen and PB were stained with anti-HLA-ABC-FITC (BD) and anti-CD45-PerCP-Cy5 (BD) to analyze human chimerism by flow cytometry. All engrafted mice were assessed for multilineage analysis using anti-CD33-PE for myeloid cells, anti-CD19-APC for B-cells and anti-CD34-PE for immature hematopoietic cells (all from Miltenyi). Within the CD19+ cell subset, the proportion of CD34+ and CD10+ cells (anti-CD10-PE; Miltenyi) was analyzed to distinguish between pro-B and pre-B cells.

Analysis of PB hematological counts and histology of spleen and liver

Hematological parameters including absolute counts of white blood cells (WBC), platelets and hemoglobin (Hg) levels as well as the WBC differential composition were determined in PB the day of sacrificing the animals using the hematological analyzer Mythic 22CT (Orphée) (Montes et al. 2011). Spleen and liver were visualized macroscopically, measured and weighted. Half of tissue was used for flow cytometry and the other half was fixed in formalin, paraffin-embedded, cut into 4µM sections and stained with hematoxylin-eosin for histology analysis (Gutierrez-Aranda et al. 2010; Montes et al. 2011).

***In vitro* liquid culture, cell cycle and apoptosis analysis of sorted/transduced CD34+ cells**

For liquid culture, transduced CD34+ cells were sorted based on reporter expression. Purified GFP+ (MLL-AF4-expressing), dTo+ (FLT3.TKD/WT-expressing) or merged-yellow (MLL-AF4 and FLT3-co-expressing) CD34+ HSPCs were allowed to expand for a 90-day period. To determine the growth kinetics in each experimental group, cells were counted every 5 days (Montes et al. 2011; Romero-Moya et al. 2013). For cell cycle and apoptosis analyses, transduced/sorted CD34+ HSPCs were stained with 10 µM BrdU (Sigma) for 20 minutes and fixed in 70% ice-cold ethanol. Subsequently, cells were incubated with 2M HCl, washed with PBS

containing 0.1% BSA and 0.2% Tween20 and incubated with 2 μL of anti-BrdU-FITC (BD) for 30 mins. Cells were finally suspended in PI buffer containing 5 μg of PI and 100μg/mL of RNAase. Cell cycle distribution was analyzed on a FACSCanto-II cytometer using the FACSDiva software (BD). Cycling cells were identified as BrdU+ (Montes et al. 2011; Romero-Moya et al. 2013). The apoptotic status of transduced CD34+ cells was assessed using the Annexin-V apoptosis detection kit (BD) according to the manufacturer's instructions (Ji et al. 2008; Bueno et al. 2010b). The transduced apoptotic cells were detected by gating the AnnexinV+ fraction.

Clonogenic Assay

Primary human colony forming unit (CFU) assays were performed by plating 1000 sorted/transduced CD34+ HSPCs into methylcellulose H4434 (Stem Cell Technologies) containing human growth factors: 50ng/ml SCF, 10ng/ml GM-CSF, 10ng/ml IL3 and 3U/ml Erythropoietin. Colonies were counted and scored at day 14 of the CFU assay using standard morphological criteria (Montes et al. 2011). For secondary re-plating, all the CFU colonies from each experimental condition were harvested from the methylcellulose and a single cell suspension was achieved and re-plated as above.

RNA extraction, cDNA synthesis and *MLL-AF4* and *FLT3* gene expression

RNA extraction and reverse transcription was performed using the Europe against Cancer Group protocol(Chillon et al. 2012). cDNA was used for conventional (*MLL-AF4*) and quantitative (*FLT3*) PCR. MLL-AF4 expression was confirmed using the following primers (Fw:5'-CAGGTCCAGAGCAGAGCAAAC-3' and Rw:5'-GAGCACTTGGAGGTGCAGATG-3') and PCR conditions (95ºC-10 min followed by 40 cycles of 95ºC-15s and 60ºC-60s) (Chillon et al. 2012). In qPCR experiments, *FLT3* expression was normalized to the expression of β-actin. qPCR was performed using SYBRGreen PCR Master mix (Applied BioSystem) and the 7500 RT-PCR System.

***FLT3* gene mutation analysis.** The presence of FLT3-TKD mutation was assessed by restriction fragment length polymorphism PCR (RFLP-PCR), using primers and PCR conditions previously described in detail (Catalina et al. 2009; Chillon et al. 2012; Bueno et al. 2013 (in press)).

Statistical analysis

Data are expressed as mean±SEM. Statistical comparisons were performed using either paired or unpaired Student's T test, as corresponding. Statistical significance was defined as a *p-value*<0.05.

RESULTS

FLT3.TKD or FLT3.WT do not cooperate with MLL-AF4 to enhance multilineage hematopoietic engraftment *in vivo*

Purified human CD34+ HSPCs were transduced with: (i) an empty lentivector (EV) expressing either GFP or dTo as reporter, (ii) MLL-AF4/GFP-expressing lentivector (MLL-AF4), (iii) FLT3.TKD/dTo-expressing lentivector (TKD), or (iv) simultaneously co-transduced with both MLL-AF4 and FLT3.TKD (**Fig 1A**). Gene transfer efficiency ranged from 10% to 40% as determined by FACS and fluorescence microscopy 48h after infection (**Fig 1B**). To determine whether FLT3-TKD cooperates with MLL-AF4 in regulating HSPCs, EV-, MLL-AF4-, FLT3.TKD- and MLL-AF4+FLT3.TKD-transduced CD34+ HSPCs were sorted based on reporter expression, and 30.000 transduced cells were transplanted via IBMT into sublethally irradiated mice (n=44) (Levac et al. 2005; Bueno et al. 2010a). Animals were monitored twice a week and were killed after 14 weeks. None of the transplanted mice displayed any sign of disease after 14 weeks EV-CD34+ HSPCs generated a human graft in 60% of the mice with a low (2.5%±4.4%) engraftment level (**Fig 1C**). However, MLL-AF4-, FLT3.TKD- and MLL-AF4+FLT3.TKD-CD34+ HSPCs engrafted basically in 100% of the mice (**Fig 1C**), and the level of chimerism was significantly higher (6.6-, 14.3- and 9-fold higher for MLL-AF4-, FLT3.TKD- and MLL-AF4+FLT3.TKD-CD34+ HSPCs, respectively) compared to EV-CD34+ HSPCs transplanted NSG mice (**Fig 1C**). Enforced expression of FLT3.TKD provided the highest level of engraftment but when co-expressed with MLL-AF4 the level of human chimerism slightly decreased, indicating that FLT3.TKD and MLL-AF4 do not synergize to promote more robust engraftment potential (**Fig 1C**). Direct injection of cells into the tibia provides the opportunity to assess migration of transplanted cells *in vivo*. The migration ability was assessed by analyzing the level of chimerism in the injected tibiae, CL, spleen, liver and PB (**Fig 1D**). The ectopic expression of MLL-AF4 or FLT3.TKD alone, or in combination, did not influence the migration capacity of the transduced CD34+ HSPCs as demonstrated by the similar capacity to colonize other hematopoietic tissues in all the animals (**Fig 1D**). To ensure that the observed effects in engraftment and migration are linked to the ectopic expression of MLL-AF4 and/or FLT3.TKD, the expression of MLL-AF4 and the presence of FLT3.TKD mutation were confirmed by RT-PCR and RFLP-PCR, respectively, in BM cells from a cohort of engrafted mice (**Fig 1E**).

We next characterized by FACS the composition of the human graft (**Fig 2A**). Multilineage repopulation was consistently observed in all engrafted mice, regardless of the expression of MLL-AF4, FLT3.TKD or MLL-AF4+FLT3.TKD and the tissue analyzed (**Fig 2B**). Regardless of the gene over-expressed and the tissue analyzed, the graft was composed predominantly of CD45+CD19+ B-lymphoid cells (60%-85%), followed by CD45+CD33+ myeloid cells (5%-13%) and CD45+CD34+ immature cells (3%-19%) (**Fig 2A,B**). Because MLL-AF4 expression is associated with a pro-B ALL, we analyzed further the phenotype of the CD45+CD19+ B-cell population (Menendez et al. 2002; Menendez et al. 2004; Montes et al. 2011). As depicted in **Fig 2B** (right panels), the early B-cell markers CD34 and CD10 were expressed by ~3-8% and ~50-90%, respectively, of the B-cell graft indicative of a normal B-cell development coexisting both B-cell progenitors

(CD19+CD10+CD34+), pre-B cells (CD19+CD10+CD34-) and more mature B cells (CD19+CD10-CD34-). Leukemic blasts in MLL-AF4+ ALL are characterized by CD10-CD19+ pro-B phenotype whereas up to 90% of the CD19+ B-cells in our lentiviral-based MLL-AF4/FLT3.TKD xenograft model display a CD10+ pre-B phenotype, suggesting that enforced expression of FLT3.TKD alone, or in combination with MLL-AF4, enhances the hematopoietic repopulating function of CB-CD34+ HSPCs without impairing normal hematopoietic differentiation.

Several groups reported the presence of *FLT3*.TKD mutations in MLL-rearranged ALLs (Armstrong et al. 2004; Taketani et al. 2004; Emerenciano et al. 2008) whereas others showed that *FLT3* mutations in MLL-rearranged ALLs do not occur (Stam et al. 2007a; Stam et al. 2007b; Bardini et al. 2010; Bardini et al. 2011; Chillon et al. 2012). High expression of *FLT3* is a hallmark step in the pathogenesis of MLL-AF4+ ALL (Chillon et al. 2012), but it is not resolved whether the constitutive activation of *FLT3* in MLL-AF4+ ALL is due to the presence of activating *FLT3* mutations or simply to an increased expression of *FLT3*. We thus investigated whether ectopic over-expression of FLT3.WT cooperates with MLL-AF4 in regulating CB-CD34+ HSPCs. Identical to FLT3.TKD experiments, EV-, MLL-AF4-, FLT3.WT- and MLL-AF4+FLT3.WT-transduced CD34+ HSPCs were sorted based on reporter expression, and 30.000 transduced cells were transplanted via IBMT into sublethally irradiated mice (n=42) (Levac et al. 2005; Bueno et al. 2010a). FLT3.WT- and MLL-AF4+FLT3.WT-CD34+ HSPCs engrafted in 100% of the mice (**Fig 3A**), and the level of chimerism was significantly higher (12.5-fold higher) compared to EV-CD34+ HSPCs transplanted NSG mice (**Fig 3A**). Enforced expression of FLT3.WT alone, or in combination with MLL-AF4, exerted identical level of engraftment, indicating that FLT3.WT does not cooperate with MLL-AF4 to enhance engraftment potential (**Fig 3A**). Identical to FLT3.TKD, the ectopic expression of FLT3.WT alone, or in combination with MLL-AF4, did not influence the migration capacity of the transduced CD34+ HSPCs as demonstrated by the similar ability to colonize CL, spleen, liver and PB in all the animals (**Fig 3B**). Transgene expression was confirmed by RT-qPCR in BM cells from a cohort of engrafted mice (**Fig 1C**). Multilineage repopulation was consistently observed in all mice and tissues engrafted with either FLT3.WT- or MLL-AF4+FLT3.WT-CD34+ HSPCs (**Fig 3D**). Identical to previous results with FLT3.TKD mutation, the graft was composed predominantly of B-lymphoid cells (50%-80%), followed by myeloid cells (5%-12%) and CD34+ immature cells (2%-14%) (**Fig 3D**). CD34 and CD10 were expressed in ~3-15% and ~50-90% of the CD19+ B-cells, respectively, indicative of a normal B-cell development. This indicates that enforced expression of FLT3.WT alone, or in combination with MLL-AF4, enhances the hematopoietic repopulating function of CB-CD34+ HSPCs without impairing normal hematopoietic differentiation.

FLT3 activation does not suffice to initiate MLL-AF4-mediated leukemogenesis *in vivo*

We have previously reported that MLL-AF4 on its own is not sufficient to initiate leukemia in CB-CD34+ HSPCs (Montes et al. 2011). We now report that neither FLT3.TKD nor FLT3.WT cooperates with MLL-AF4 fusion to confer leukemia *in vivo*. Although independent ectopic expression of MLL-AF4 or FLT3.TKD/WT enhanced *in vivo* repopulating function, co-transduction of both MLL-AF4 and FLT3.TKD/WT did not have an additive effect in the level of human engraftment (**Fig 1C** and **3A**). None of the animals transplanted with MLL-

AF4+FLT3.TKD/WT-CD34+ HSPCs showed any sign of disease over a 14-week period, and co-expression of MLL-AF4 and FLT3.TKD/WT did not impair normal hematopoietic differentiation (**Fig 2B** and **Fig 3D**). Furthermore, hematological parameters determined at the time of animal killing showed no signs of disease (**Fig 4**). No differences were observed between experimental groups for total WBC, platelet counts and Hg levels (**Fig 4A**) or differential WBC counts (**Fig 4B**). Accordingly, neither splenomegaly nor hepatomegaly was observed in mice transplanted with MLL-AF4+FLT3.TKD/WT-CD34+ HSPCs (**Fig 4C**). Thus, FLT3 activation does not cooperate with MLL-AF4 to transform CD34+ HSPCs, suggesting the need of alternative (epi)-genetic cooperating hits (Bursen et al. 2010; Prelle et al. 2012).

Enforced expression of FLT3.TKD/FLT3.WT does not augment *in vitro* clonogenic potential but promotes a transient *in vitro* enhanced proliferation of MLL-AF4-expressing CD34+ HSPCs

The hematopoietic progenitor function was examined by analyzing the CFU capacity of transduced CD34+ HSPCs. CD34+ HSPCs expressing MLL-AF4 alone, or in combination with FLT3.TKD/FLT3.WT, displayed higher clonogenic potential (62 and 64 CFU, respectively, per 1000 plated CD34+ cells) than CD34+ cells transduced with EV- (35 CFU/1000 plated CD34+ cells) or FLT3.TKD/FLT3.WT (37 CFU/1000 plated CD34+ cells) in the absence of MLL-AF4 (**Fig 5A** and **Fig 6A**). Scoring of primary CFUs revealed no significant differences in CFU types between experimental groups (**Fig 5A** and **Fig 6A**). Importantly, primary CFU cultures were re-plated and the CFU potential significantly diminished in the secondary read-outs, regardless of the experimental condition indicating that co-expression of MLL-AF4 with FLT3.TKD/FLT3.WT does not confer stable replating efficiency (**Fig 5A** and **Fig 6A**). Reporter expression was successfully retained in the CFU assays. In keeping with the *in vivo* data, these CFU results confirm that FLT3 activation does not cooperate with MLL-AF4 to boost clonogenic capacity nor to impair normal hematopoietic differentiation.

We next analyzed the *in vitro* homeostasis of EV-, MLL-AF4-, FLT3.TKD-, FLT3.WT-, MLL-AF4+FLT3.TKD- and MLL-AF4+FLT3.WT-transduced CD34+ cultures. CD34+ HSPCs expressing either MLL-AF4 or FLT3.TKD/WT grew much faster than EV-CD34+ cells up to day ~50 of *in vitro* culture (**Fig 5B** and **Fig 6B**). This transient enhanced expansion of CD34+ HSPCs was further extended up to day ~80 of *in vitro* cultures of CD34+ HSPCs co-expressing MLL-AF4 and FLT3.TKD/WT, indicating that FLT3 activation enhances expansion of MLL-AF4-expressing CD34+ HSPCs (**Fig 5B** and **Fig 6B**). Importantly, the enhanced cell expansion promoted by MLL-AF4 in combination with FLT3 activation was transient, and after ~80 days the growth kinetics of MLL-AF4+FLT3-CD34+ cultures was similar to EV-CD34+ cultures, confirming that FLT3 activation in MLL-AF4-expressing CD34+ does not promote *in vitro* immortalization which is in line with the clonogenic data (**Fig 5A** and **Fig 6A**). Apoptosis and cell cycle analysis of the different experimental groups reveal that MLL-AF4 and FLT3.TKD/WT on their own, or in combination, seem to protect the CD34+ cells from apoptosis (~20% vs 36% for EV-CD34+ cells; **Fig 5C** and **Fig 6C**) while inducing cell proliferation as determined by BrdU staining (~55% vs 30% for EV-CD34+ cells; **Fig 5D** and **Fig 6D**). This increased proportion of cycling cells coupled to lower levels of apoptosis explains, at least in part, the enhanced *in vitro*

transient proliferation, and the *in vivo* repopulating cell function of CD34+ HSPCs expressing MLL-AF4, FLT3.TKD/WT or both together.

DISCUSSION

Several studies have attempted to recapitulate human infant pro-B ALL harboring MLL-AF4 fusion using mouse HSPCs (Daser and Rabbitts 2005; Chen et al. 2006; Metzler et al. 2006; Krivtsov et al. 2008; Bursen et al. 2010), but unfortunately, the resulting *in vivo* leukemias do not faithfully reproduce the actual human disease and the resultant phenotype/latency differs significantly from that seen in infant ALL (Daser and Rabbitts 2005; Chen et al. 2006; Metzler et al. 2006; Bursen et al. 2010). Potential reasons for this are: (i) an inappropriate cell type was targeted in the mouse; (ii) murine systems lack appropriate etiological content for essential secondary mutations (Eguchi et al. 2005); (iii) the impact of secondary cooperating oncogenic lesions has not been properly addressed, and (iv) MLL-AF4 transforming ability is dependent on a human cell context. Biphenotypic MLL-AF4+ infant ALL harbors and expresses the fusion both in B-cell and monocytic lineages, suggesting that MLL-AF4 may arise in a HSC, so that MLL-AF4 function has to be addressed using ontogenically primitive human stem cells. Among these, neo-natal (CB-CD34+ HSPCs) or pre-natal (fetal- or embryonic-derived) cells represent ontogenically early candidate target cells. Furthermore, the recent success in modeling the initiation and progression of acute leukemia mediated by the ectopic expression of MLL-ENL and MLL-AF9 in CB-derived CD34+ HSPCs (Barabe et al. 2007) provides a precedent proof-of-principle for examining the transforming function of MLL-AF4 in CB-derived CD34+ HSPCs.

We have previously reported that the expression of MLL-AF4 on its own has a functional impact in CB-CD34+ HSPCs(Montes et al. 2011) and hESC-hematopoietic cells (Bueno et al. 2012). In CB-CD34+ HSPCs, MLL-AF4 enhanced hematopoietic engraftment and clonogenic potential (Montes et al. 2011). In hESCs, MLL-AF4 altered the developmental cell fate, skewing the early hemato-endothelial specification of hESCs (Bueno et al. 2012). However, in either scenario MLL-AF4 was not sufficient on its own to immortalize/transform hESC-hematopoietic cells or CB-CD34+ HSPCs. This suggests that the inability to develop a MLL-AF4 model is not due to the human cell context, but rather that additional oncogenic lesions are required for leukemogenesis.

Gene expression profiling showed that *FLT3* is highly expressed in MLL-AF4+ pro-B-ALL. Moreover, it was shown that other MLL-rearranged leukemias display FLT3.TKD mutations in up to 20% of the cases, suggesting they may represent candidate cooperating events (Armstrong et al. 2004; Taketani et al. 2004; Chillon et al. 2012). Accordingly, Yamaguchi *et al* (Yamaguchi et al. 2009a) have reported that FLT3.TKD cooperates with MLL-AF4 to induce *in vitro* aggressive proliferation of the mouse cell line 32Dc. Several other groups suggest, however, that *FLT3* mutations are not common in MLL-AF4+ pro-B-ALL, and that an increased transcriptional expression of *FLT3* may act as secondary cooperating hit (Stam et al. 2007a; Stam et al. 2007b; Guenther et al. 2008b; Chillon et al. 2012).

We have very recently reported that *FLT3* activation does not cooperate with MLL-AF4 to immortalize/transform hESC-derived hematopoietic cells, suggesting the need of either alternative secondary oncogenic hits or a different target cell for transformation. Whether FLT3 activating mutations or increased expression of germline FLT3 cooperates with MLL-AF4 in post-natal CB-derived CD34+ HSPCs remains unknown. Here, we have explored whether FLT3.TKD or FLT3.WT in combination with MLL-AF4 fusion protein suffices to immortalize/transform CB-CD34+ HSPCs *in vitro* and/or *in vivo*. *In vivo*, FLT3.TKD/FLT3.WT alone, or in combination with MLL-AF4, enhanced hematopoietic reconstitution function of CB-CD34+ HSPCs but without impairing hematopoietic differentiation, and none of the animals transplanted with MLL-AF4+FLT3.TKD/WT-CD34+ HSPCs showed any sign of disease after 14 weeks. *In vitro*, enforced expression of FLT3.TKD/FLT3.WT conveys a transient overexpansion of MLL-AF4-expressing CD34+ HSPCs associated to higher proportion of cycling cells coupled to lower apoptotic levels, but does not augment clonogenic potential nor confer stable replating. Together, FLT3.TKD/FLT3.WT does not suffice to immortalize/transform MLL-AF4-expressing CB-CD34+ HSPCs. This suggests that either alternative (epi)-genetic cooperating oncogenic lesions different that FLT3 activation (Bursen et al. 2010) may be required for leukemogenesis, or that other target cell different from hESC-hematopoietic cells or CB-derived CD34+ cells (for example, fetal liver-derived CD34+ HSPCs) might represent a more appropriate cellular target for MLL-AF4-mediated transformation.

Bursen *et al* (Bursen et al. 2010) recently reported that the AF4-MLL fusion protein is capable of inducing ALL in mice without requirement of MLL-AF4. In addition, very recent work (Prelle et al. 2012; Driessen et al. 2013) demonstrated that RAS mutations are present in 14-36% of MLL-AF4 patients, and that RAS mutations in MLL-AF4 infant ALL is an independent predictor for a poor outcome. These studies indicate that AF4-MLL and RAS mutations may represent alternative genetic lesions contributing to MLL-AF4-driven leukemogenesis which have still not been addressed in a human stem cell context. Importantly, epigenetics represent a key player for MLL-AF4-driven transformation. In fact, leukemic transformation by the MLL fusions requires the H3K79 methyltransferase Dot1L (Krivtsov et al. 2008; Deshpande et al. 2013). Therefore, further work should determine the expression of Dot1L in potential target cells for transformation by MLL-AF4. Also, biochemical studies and ChIP-Seq studies should be conducted upon ectopic over-expression of MLL-AF4 to elucidate whether exogenous MLL-AF4 is capable of recruiting Dot1L histone methyltransferase and induce H3K79 methylation and subsequent transcriptional activation of MLL-AF4 target genes. Finally, in contrast to many human cancers which are commonly characterized by hypomethylation in non-promoter regions, MLL-AF4+ ALL displays genome-wide hypermethylation at non-promoter sequences (Stumpel et al. 2009; Stumpel et al. 2012b). Global hypomethylation is usually associated with genomic instability, allowing additionally acquired genetic hits to propel a pre-malignant clone into a fully transformed state. The global hypermethylation of MLL-AF4+ ALL might explain why additional genetic lesions have not been discovered in MLL-rearranged ALL (Bardini et al. 2010; Bardini et al. 2011).

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AUTHOR CONTRIBUTIONS

Designed research (R.Mo, C.B, P.M); performed research & analyzed data (R.Mo, C.B, A.B, R.Ma, C.C, D.R-M, O.N-M, C.P); wrote the paper (R.Mo, C.B, P.M). The manuscript has been seen and approved by all authors. The authors have no conflicts of interest to disclose.

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FIGURE LEGENDS

FIGURE 1. Hematopoietic engraftment of CB-CD34+ HSPCs expressing MLL-AF4, FLT3.TKD or a combination of both MLL-AF4 and FLT3.TKD. (A) Schematic representation of the lentiviral vectors used. The empty lentivector carries either GFP or dTo as fluorescence reporter. MLL-AF4 is expressed along with GFP as reporter. FLT3-TKD/FLT3-WT is expressed together with dTo as reporter. **(B)** Representative phase contrast and fluorescence microscopy photos of CD34+ HSPCs transduced with FLT3.TKD (dTo+), MLL-AF4 (GFP+) or co-transduced with both MLL-AF4 and FLT3.TKD (merged yellow). **(C)** Levels of long-term (14 weeks) hematopoietic chimerism of NSG mice (n=44) after IBMT of 30.000 transduced (sorted based on GFP/dTomato) EV-, MLL-AF4-, FLT3.TKD- or MLL-AF4+FLT3.TKD-expressing CD34+ cells. Engraftment was determined as % of hCD45+ >0.1% in the injected tibia. Each dot represents an individual mouse, and the horizontal line indicates the mean of each experimental cohort. **(D)** Level of human chimerism in the distinct hematopoietic tissues analyzed. (**E**) RT-PCR (top panel) and RFLP-PCR (bottom panel) confirming stable expression of MLL-AF4 mRNA, and the presence of FLT3.TKD mutation in mice killed 14 weeks after transplantation. The TKD mutation abrogates an EcoRV restriction site.

FIGURE 2. Flow cytometric analysis confirming multilineage engraftment. (A) Representative flow cytometry. The human graft is identified as the CD45+ fraction. The CD45+ human graft comprises B-lymphoid cells (CD19+), myeloid cells (CD33+) and immature cells (CD34+). About 5% and 80% of the CD19+ B-lymphoid population co-express CD34 and CD10, respectively, indicative of a normal B cell differentiation. **(B)** Multilineage and multiorgan human chimerism in the IT, CL, spleen, liver and PB demonstrating migration of human cells from the IT. No differences in the graft composition between EV-, MLLAF4-, FLT3.TKD- and MLLAF4+FLT3.TKD-expressing CD34+ HSPCs were found (n=44).

FIGURE 3. Multilineage hematopoietic engraftment of CB-CD34+ HSPCs expressing MLL-AF4, FLT3.WT or a combination of both MLL-AF4 and FLT3.WT. (A) Levels of long-term (14 weeks) hematopoietic chimerism of NSG mice (n=46) after IBMT of 30.000 transduced (sorted based on GFP/dTo) EV-, MLL-AF4-,

FLT3.WT- or MLL-AF4+FLT3.WT-expressing CD34+ cells. Engraftment was determined as % of hCD45+ >0.1% in the injected tibia. Each dot represents an individual mouse, and the horizontal line indicates the mean of each experimental cohort. **(B)** Level of human chimerism in the distinct hematopoietic tissues analyzed. **(C)** Representative RT-qPCR confirming stable expression of FLT3.WT in mice killed 14 weeks after transplantation. **(D)** Multilineage and multiorgan human chimerism in the IT, CL, spleen, liver and PB demonstrating migration of human cells from the IT. No differences in the graft composition between EV-, MLLAF4-, FLT3.WT- and MLLAF4+FLT3.WT-expressing CD34+ HSPCs were found (n=46).

FIGURE 4. Analysis of hematological parameters in PB and examination of the liver and spleen reveal no signs of leukemia in mice reconstituted with EV-, MLL-AF4-, FLT3.WT-, MLL-AF4+FLT3.WT-, FLT3.TKD-, or MLL-AF4+FLT3.TKD-expressing CD34+ HSPCs. (A) Top panels show absolute counts of WBC, platelets and Hg levels in the indicated mice groups (n=71). The horizontal line indicates the median of each experimental group. **(B)** Differential composition of lymphocytes, monocytes and neutrophils in the PB of the indicated mice groups (n=71). **(C)** Weight, representative macroscopic images and H&E staining of liver (left panels) and spleen (right panels) showing lack of splenomegalia and hepatomegalia in mice transplanted with 30.000 sorted/transduced CD34+ HSPCs expressing the indicated genes.

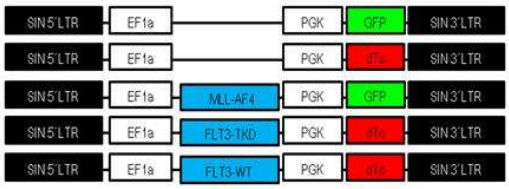
FIGURE 5. FLT3.TKD does not augment *in vitro* clonogenic potential but conveys a transient *in vitro* expansion of MLL-AF4-expressing CD34+ HSPCs. (A) Primary and secondary CFU plating showing a higher CFU potential mediated by MLL-AF4 expression (n=4). The CFU types were not different among the indicated conditions. Inset: representative fluorescence-microscopy of CFU colonies transduced with a lentivector expressing dTomato (EV or FLT3.TKD), GFP (MLL-AF4) or merged-yellow (MLL-AF4+FLT3.TKD). **(B)** *In vitro* cell growth over a 90 days period of purified EV-, MLL-AF4-, FLT3.TKD- and MLL-AF4+FLT3.TKD-expressing CD34+ HSPCs (n=6). Left insets: representative fluorescence images confirming reporter expression (GFP+, dTo+, merged-yellow) of FACS-purified transduced CD34+ cells. Right insets: representative RT-PCR (top panel) and RFLP-PCR (bottom panel) confirming stable expression of MLL-AF4 mRNA, and the presence of FLT3.TKD mutation **(C)** Apoptosis levels in CD34+ cells transduced as indicated. The inset displays a representative flow cytometry panel of 7-ADD *vs* Annexin V analysis. **(D)** Proliferation level measured as % of BrdU+ cells (S-phase) within the CD34+ fraction transduced as indicated. The inset displays a representative flow cytometry panel of BrdU *vs* PI analysis.

FIGURE 6. FLT3.WT does not augment *in vitro* clonogenic potential but induces a transient *in vitro* expansion of MLL-AF4-expressing CD34+ HSPCs. (A) Primary and secondary CFU plating showing a higher CFU potential mediated by MLL-AF4 expression (n=4). The CFU types were not different among the indicated conditions. Inset: representative fluorescence-microscopy of CFU colonies transduced with a lentivector expressing dTo (EV or FLT3.WT), GFP (MLL-AF4) or merged-yellow (MLL-AF4+FLT3.WT). **(B)** *In vitro* cell expansion over a 90 days period of purified EV-, MLL-AF4-, FLT3.WT- and MLL-AF4+FLT3.WT-expressing CD34+ HSPCs (n=6). Left insets: representative fluorescence images confirming reporter

expression (GFP+, dTo+, merged-yellow) of FACS-purified transduced CD34+ cells. Right panels: representative RT-PCR and RT-qPCR confirming stable expression of MLL-AF4 and FLT3.WT, respectively. **(C)** Apoptosis levels in CD34+ cells transduced with the indicated genes. **(D)** Proliferation level measured as % of BrdU+ cells within the CD34+ fraction transduced with the indicated genes.

Montes *et al.* Figure 1

A



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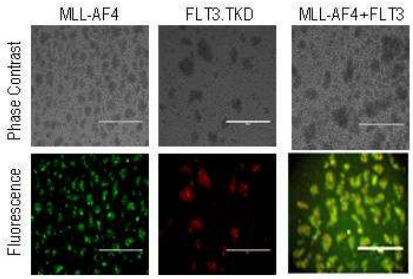

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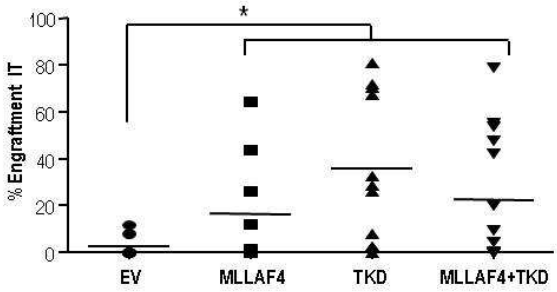


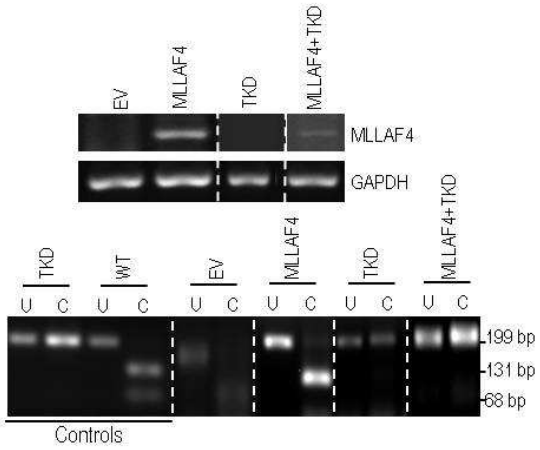
Nº of engrafted mice: 6/10 (60%) 9/9 (100%) 11/11 (100%) 13/14 (93%)

D

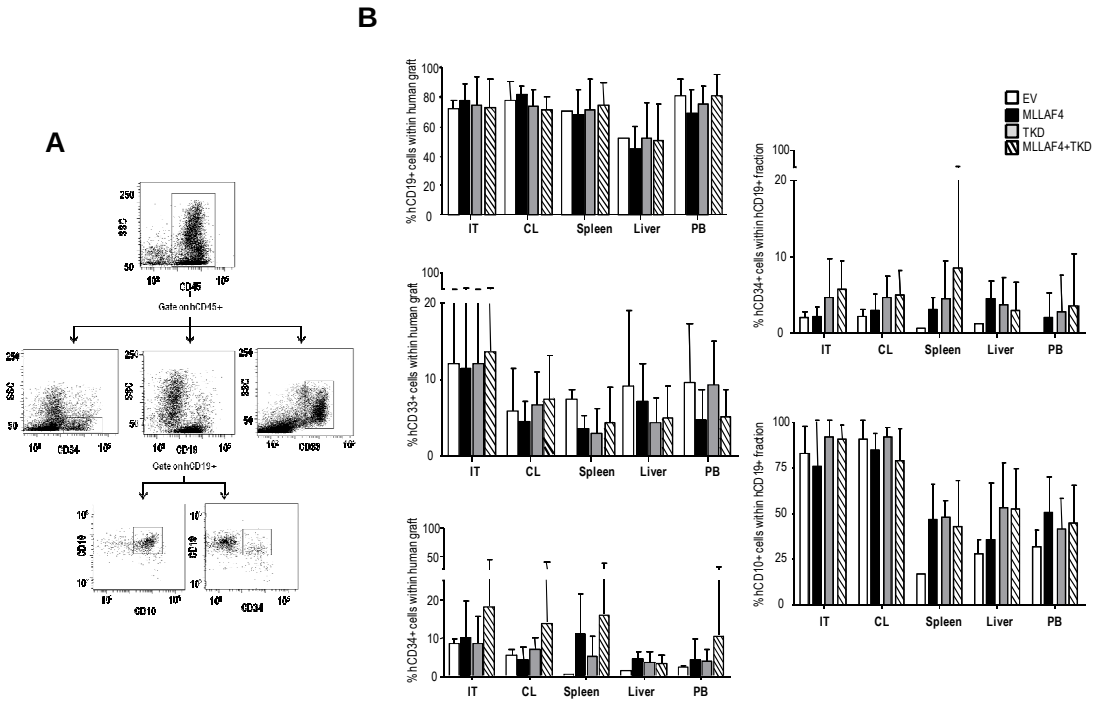
% of hematopoietc engraftment

	IT	CL	Spleen	Liver	PB
EV	2.5 ± 4.4	0.4 ± 0.9	0.3 ± 0.6	0.6 ± 0.7	0.1 ± 0.1
MLLAF4	16.5 ± 23.6	4.6 ± 7.4	2.7 ± 2.6	1.7 ± 1.8	0.8 ± 1.1
TKD	35.7 ± 31.6	15.1 ± 12.9	8.3 ± 8.5	4.6 ± 4.7	3.5 ± 4.2
MLLAF4+TKD	22.5 ± 27.4	4.2 ± 7.1	2.2 ± 2.5	1.3 ± 1.5	0.8 ± 1.3

E

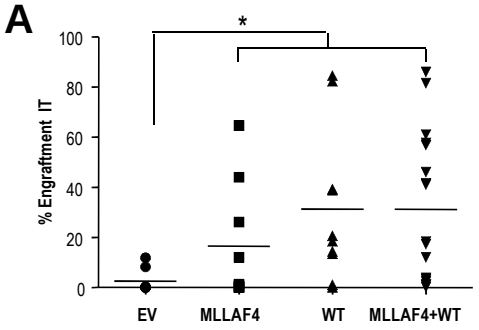


Montes *et al.* Figure 2



Montes *et al.* Figure 3

A

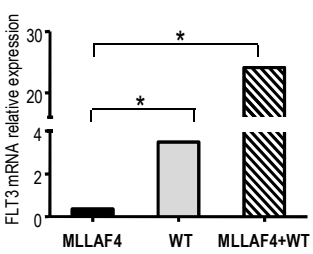


Nº engrafted mice: 6/10 (60%) 9/9 (100%) 10/10 (100%) 17/17 (100%)

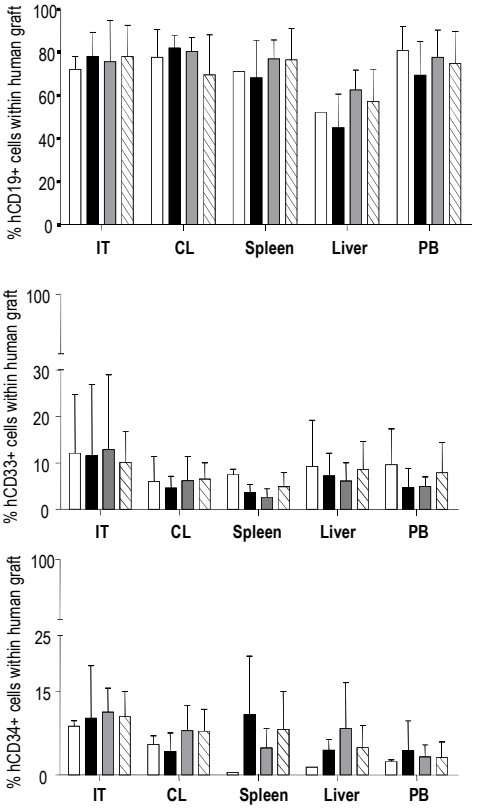
B

% of hematopoietc engraftment										
	IT		CL		Spleen		Liver		PB	
EV	2.5	4.4	0.4	0.9	0.3	0.6	0.6	0.7	0.1	0.1
MLLAF4	16.5	23.6	4.6	7.4	2.7	2.6	1.7	1.8	0.8	1.1
WT	31.3	30.4	13.5	9.5	8.3	8.5	8.6	7.9	5	4.5
MLLAF4 + WT	31.2	29.6	10.8	14.9	3.7	3.6	2.9	2.5	1.2	1

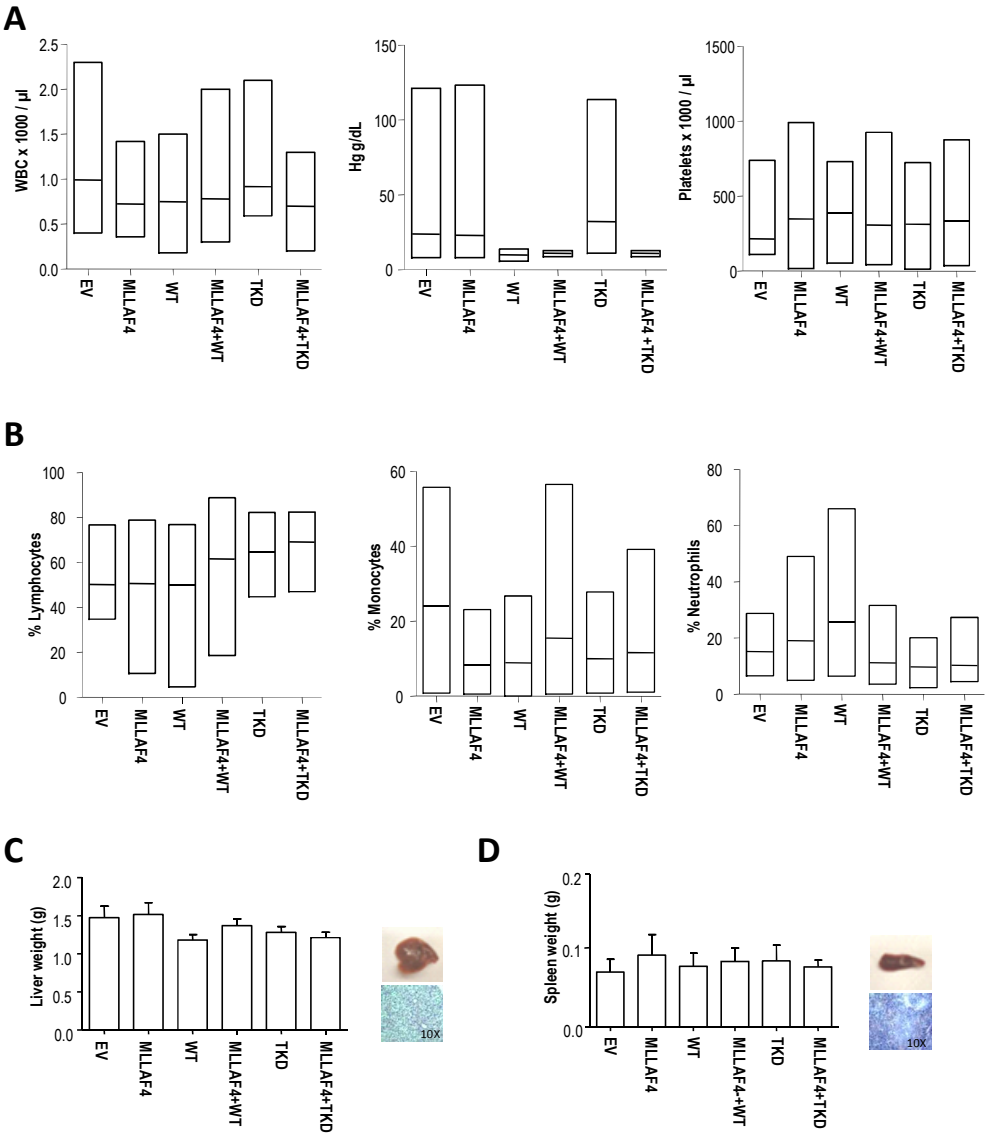
C



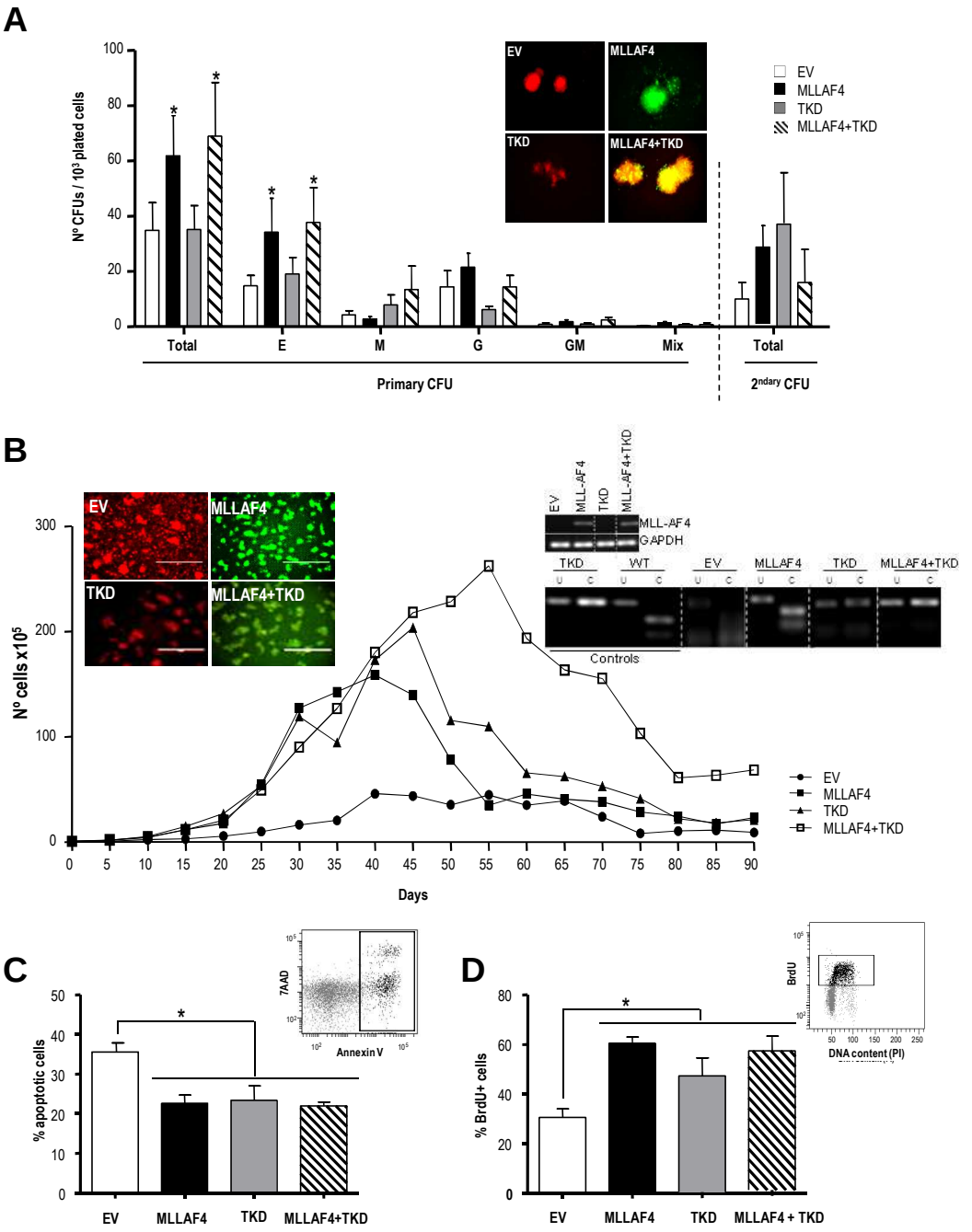
D



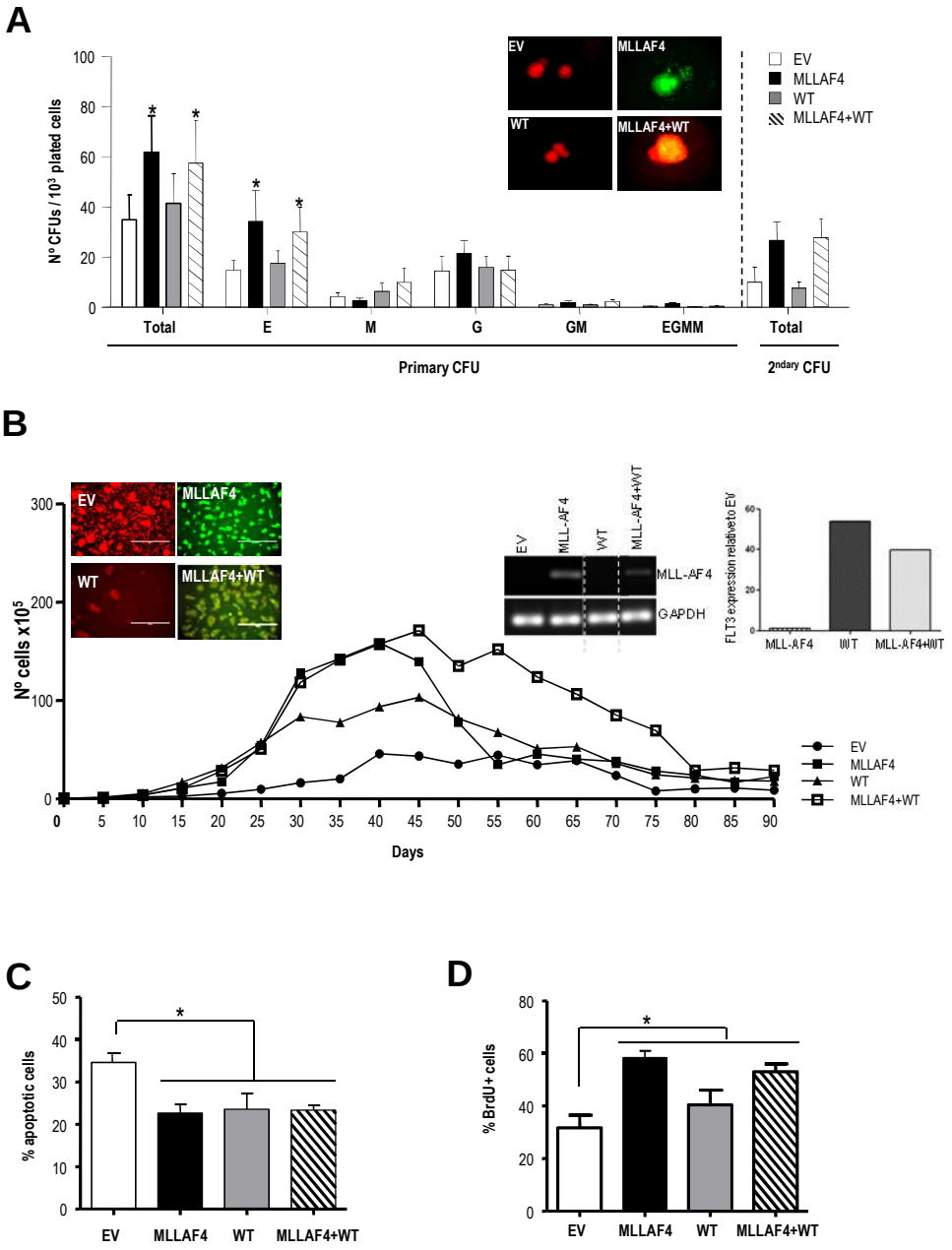
Montes *et al.* Figure 4



Montes *et al.* Figure 5



Montes *et al.* Figure 6



DISCUSIÓN

La LLA infantil con reordenamiento t(4;11) MLL-AF4+ constituye un subtipo de leucemia especialmente agresivo que se desarrolla con frecuencia antes del primer año de vida. Esta leucemia está representada casi en su totalidad por la LLA pro-B/monocítica con fenotipo $CD34^{+/-}CD19^{+}CD10^{-}NG2^{+/-}CD15^{+/-}CD65^{+/-}$. Esta LLA presenta una latencia muy corta (apareciendo antes del primer año de vida), además de una supervivencia libre de enfermedad y una supervivencia global a los 5 años inferior al 20% (Pui and Evans 2006) lo que la convierte en una de las neoplasias infantiles con peor pronóstico. En los últimos años, la aplicación a estos pacientes del protocolo INTERFANT-99, ha conseguido aumentar la tasa de supervivencia a aproximadamente 40% (Pieters et al. 2007), resultado que, aunque esperanzador, sigue siendo tremendamente adverso para más de la mitad de los niños diagnosticados con esta enfermedad.

Con respecto a la etiología de la LLA pro B, la traslocación t(4;11), que involucra a los genes *MLL* y *AF4*, es el evento oncogénico iniciador y característico y se detecta aproximadamente en el 100% de los casos. El origen prenatal de la traslocación se ha demostrado por estudios de clonogenicidad en gemelos monocigóticos, en los que ambos presentan la traslocación con el mismo punto de ruptura (Ford et al. 1993; Gale et al. 1997), y por estudios retrospectivos en los que la misma traslocación detectada al diagnóstico en el paciente estaba presente en las muestras recogidas en el momento del nacimiento (en la conocida como "prueba del talón" para detección de metabolopatías) (Greaves 2002). Los estudios en gemelos monocigóticos implican que han compartido una célula donde ocurrió la t(4;11) o bien que la célula donde se produce la traslocación se originó en un embrión/feto y migró por circulación trasplacental al otro feto, donde anidó y originó el mismo clon tumoral (anastomiosis). Sobre el origen de la ruptura de *MLL*, existen estudios que demuestran el efecto de componentes genotóxicos capaces de romper *MLL* en el feto pero no en la madre. De este modo, los reordenamientos de *MLL* pudieran ser el resultado de la exposición trasplacental durante el desarrollo embrionario a sustancias que alteran la función de la enzima topoisomerasa II (encargada de reparar rupturas en el DNA), altamente expresada durante el desarrollo fetal (Zandvliet et al. 1996; Blanco et al. 2004; Moneypenny et al. 2006; Barjesteh van Waalwijk van Doorn-Khosrovani et al. 2007; Libura et al. 2008). El etopósido, un inhibidor de la topoisomerasa II, es uno de los posibles agentes genotóxicos, utilizado en protocolos de quimioterapia y que se ha postulado como posible responsable del 5-15% de las leucemias agudas secundarias a tratamiento (Felix et al. 1998; Felix 2001; Libura et al. 2005; Felix et al. 2006). Desafortunadamente, aún hay pacientes con leucemias *MLL* reordenado que reciben etopósido como citostático, lo cual parece contradecir nuestro conocimiento actual sobre la etiología de las leucemias MLL. También se ha sugerido que dietas ricas en bioflavonoides, inhibidores también de la topoisomerasa II, pudieran causar rupturas del gen *MLL* dando lugar al gen de fusión MLL-AF4 (Strick et al. 2000; Spector et al. 2005). Recientemente, Bueno *y cols*. han mostrado que tanto las hESCs como las CD34+ de SCU son igual de sensibles al etopósido en términos de ruptura de *MLL* (Bueno et al. 2009). Los reordenamientos de *MLL* no confirieron ventaja proliferativa en ninguno de los dos casos, pero indujeron una mayor inestabilidad en las hESCs tras exposición continuada a dosis bajas de etopósido. Estos datos sugieren que la célula diana en LLA pro-B podría ser

una célula de origen embrionario o una célula hematopoyética temprana en desarrollo, lo que explicaría la presencia del gen de fusión en el momento del nacimiento.

La corta latencia de la enfermedad LLA pro-B (aparece en los primeros meses de vida) y su asociación a la traslocación t(4;11), sugieren que dicha anomalía génica pueda ser la única responsable del desarrollo de la enfermedad. Frente a esta hipótesis, el llamado "modelo de dos *hits*" para las leucemias infantiles propone la acción de otro o varios eventos secundarios que causarían su efecto sobre un clon pre-leucémico asintomático (portador de la traslocación) y provocaría el desarrollo de la enfermedad. Esto explicaría porque niños con la misma traslocación tienen latencias diferentes.

Disponer de modelos biológicos que permitan ampliar nuestro conocimiento sobre la etiología de la LLA pro-B MLL-AF4+ permitiría conocer los mecanismos por los que MLL-AF4 ejerce su acción oncogénica y diseñar estrategias terapéuticas más eficaces frente a la enfermedad. Sin embargo, las aproximaciones conseguidas en ratón hasta la fecha no han logrado reproducir ni el fenotipo ni la latencia observados en el humano (Chen et al. 2006; Metzler et al. 2006; Krivtsov et al. 2008). Los motivos por los que no se ha logrado reproducir la enfermedad pueden ser que: (i) en estos modelos murinos la célula diana en la que se expresa MLL-AF4 no se encuentre en el estadio ontogénico/jerárquico adecuado; (ii) exista un componente etiológico importante para el inicio de la enfermedad ausente en los modelos de ratón; (iii) sean necesarias lesiones oncogénicas secundarias a MLL-AF4 y que cooperen con la traslocación, o; (iv) la habilidad trasformante de MLL-AF4 depende exclusivamente de un contexto celular humano.

El origen prenatal de la enfermedad indica que la célula donde se produce el daño genético que origina la traslocación se encuentra en un estadio ontogénico y jerárquico muy temprano. Por otro lado, el fenotipo mixto de esta LLA (pro-B y monocítica) sugiere que MLL-AF4 tiene origen en una célula troncal inmadura o bien una HSPC que aún es capaz de diferenciarse a linaje linfoide y mieloide. Es por esto que tanto las hESCs como las HSCs de SCU (estado fetal/neonatal) constituyen potenciales dianas celulares donde MLL-AF4 pueda aparecer y tener su efecto pre-leucémico. El uso de estas células para el estudio de la LLA pro-B MLL-AF4+ supone una novedad, ya que hasta la fecha los modelos de estudio de la enfermedad han estado basados únicamente en ratón.

Discusión sobre aspectos técnicos.

Con respecto a las hESCs, desde que se derivaron por primera vez en 1998 (Thomson et al. 1998) a partir de las células de la masa celular interna del blastocisto, han sido constantes los esfuerzos por mejorar tanto las condiciones de derivación como de mantenimiento de dichas células manteniendo las características pluripotentes de las mismas a la vez que intentando minimizar el uso de componentes xenogénicos. Así, aunque inicialmente para su derivación y mantenimiento se utilizaban *feeders* de origen murino como MEFs o fibroblastos STO (Park et al. 2004), posteriormente se ha venido implementando el

uso de *feeders* de origen humano como HFFs y, más recientemente, hMSCs. Concretamente, las hMSCs incrementan la eficiencia de derivación de hESCs a partir de embriones congelados respecto a HFFs (Cortes et al. 2009). Además, en sistemas libres de *feeders*, en los que se utiliza medio condicionado por dichas células, el medio condicionado por hMSCs (hMSC-CM) favorece la diferenciación hematopoyética de las hESCs (Ramos-Mejia et al. 2012a), por lo que su utilización supone *a priori* una herramienta de utilidad para el estudio del desarrollo hematopoyético embrionario en humano. Por ello, hemos considerado necesario estandarizar las condiciones de cultivo de hESCs en hMSC-CM. Respecto al mantenimiento de la pluripotencia en hESCs mantenidas en medio condicionado por MEFs (MEF-CM), se ha propuesto un modelo de regulación paracrina (Bendall et al. 2007). Según este modelo, las hESCs mantenidas en MEF-CM se diferencian espontáneamente a células de morfología similar a fibroblasto (fibroblastoides) que secretan IGF-II y TGF-β en respuesta a la adición al medio de cultivo de bFGF. Dadas las ventajas mencionadas anteriormente respecto a la utilización de hMSC-CM en el mantenimiento de hESCs, nosotros nos planteamos si IGF-II y TGF-β ejercen en este sistema el papel que el descrito en el caso MEF-CM en cuanto al mantenimiento de homeostasis y pluripotencia de hESCs. HFFs y hMSCs presentan receptores para bFGF y secretan TGF-β en respuesta a bFGF al medio de cultivo. Con respecto a IGF-II, mientras que los HFFs secretan IGF-II al medio en respuesta a bFGF, las hMSCs no secretan IGF-II en respuesta a bFGF. Sin embargo, aunque el HFF-CM y el hMSC-CM tengan composiciones diferentes respecto a IGF-II, las hESCs cultivadas con ambos medios condicionados, son mantenidas de manera estable manteniendo la morfología, euploidía, expresión de marcadores de superficie y factores de trascripción asociados a pluripotencia. Además, hESCs mantenidas en cualquiera de estos medios mostraron idéntico potencial de diferenciación multilínea *in vivo* e *in vitro*. Estos datos señalan diferencias en la composición de HFF-CM y hMSC-CM en cuanto a los factores TGF-β e IGF-II, y sugieren que las *feeders* humanas podrían mantener el crecimiento indiferenciado de las hESCs por medio de la producción de IGF-II por parte de las células fibroblastoides diferenciadas a partir de las hESCs, al igual que ocurre con las *feeders* murinas (Bendall et al. 2007). Sin embargo, tampoco se detectó secreción de IGF-II por parte de las células fibroblastoides diferenciadas a partir de hESCs antes de ser mantenidas en hMSC-CM. Por tanto, nuestros datos indican que, a diferencia de lo observado con MEF-CM, IGF-II podría ser dispensable para el mantenimiento de hESCs en medio concidionado por *feeders* de origen humano. Es posible que las *feeders* de origen humano produzcan factores no caracterizados para el mantenimiento de las hESCs (Eiselleova et al. 2008). Estos metabolitos son aún desconocidos y serían necesarias técnicas de proteómica y metabolómica para profundizar en la naturaleza de los factores paracrinos y autocrinos existentes en el medio condicionado. Así mismo, estos estudios de proteómica/metabolómica deberían estar seguidos de estudios funcionales de validación.

La SCU supone una fuente accesible de HSPCs. Aunque las HSPCs frescas pueden ser utilizadas en transplante alogénico, los actuales protocolos terapéuticos y la disponibilidad donante-receptor,

demandan la utilización de un número elevado de células progenitoras por lo que es necesario recurrir a HSPCs previamente criopreservadas, habiéndose demostrado además que esta estrategia no se asocia con disminución en la efectividad de trasplante ni mayor predisposición a la aparición de enfermedad injerto contra huésped (Stockschlader et al. 1997; Berz et al. 2007). Los procesos de criopreservación y de descongelación son determinantes para todas las células, pero particularmente críticos en el caso de células primarias. El protocolo de criopreservación más utilizado para este tipo de muestras incluye la adición de DMSO (dimetilsulfóxido) al 10% *v/v* y una velocidad de congelación de -1ºC por minuto. En cuanto a los métodos actuales de expansión *ex vivo* de HSPCs, éstos conllevan una pérdida en la capacidad de diferenciación multilínea debido a que las células CD34+ en cultivo se diferencian a células más maduras. Por otra parte, los protocolos de congelación-descongelación provocan muerte celular y apoptosis inducida directamente por la criopreservación, así como una pérdida de HSPCs CD34+ esenciales para la reconstitución hematopoyética. Por ello se están realizando múltiples esfuerzos a nivel mundial para expandir HSPCs de SCU sin que ésto conlleve una pérdida de la capacidad multipotente de las HSPCs. Recientemente se ha demostrado que Y-27632, un potente inhibidor de la kinasa asocida a p160-Rho (ROCK), contribuye a la recuperación y descongelación de blastocistos (Cortes et al. 2009), hESCs (Li et al. 2009; Gauthaman et al. 2010; Pakzad et al. 2010), iPSCs (Claassen et al. 2009) y hMSCs (Heng 2009). Sin embargo, se desconocen los efectos de Y27632 sobre la supervivencia y mantenimento de HSPCs CD34+ derivadas de SCU, por lo que nosotros hemos testado el efecto de este inhibidor de ROCK en HSPCs de SCU tanto frescas como criopreservadas con el fin de conocer si favorece la supervivencia en los procesos de congelación-descongelación. Además, hemos querido evaluar si el inhibidor Y-27632 permite expandir HSPCs CD34+ en cultivo sin que éstas pierdan potencial de diferenciación multilínea. A diferencia de lo observado con blastocistos, hESCs, iPSCs y hMSC, el inhibidor Y-27632 tiene un efecto perjudicial tanto en la expansión en cultivo de células HSPCs CD34+ frescas como criopreservadas, independientemente de su adición al medio de cultivo o al medio de criopreservación. Estos datos desaconsejan la aplicación de Y-27632 en ningún procedimiento clínico con el fin de mejorar las tasas de supervivencia tras criopreservación así como la expansión de las células CD34+ manteniendo sus características indiferenciadas. La proteína Rho y ROCK juegan un papel muy importante en proliferación celular, apoptosis, progresión en el ciclo celular, migración, citoesqueleto de actina, polaridad celular e interacciones célula-célula (Hall 1994; Krawetz et al. 2009). Estas funciones de Rho explicarían el efecto beneficioso de Y-27632 en inhibir la apoptosis cuando hESCs, iPSCs y hMSCs son disociadas y separadas de plástico en cultivo durante el mantenimiento, criopreservación y descongelación de las mismas. El papel inhibidor de la apoptosis de Y-27632 se basa en la dependencia de Rho respecto a receptores asociados a proteína G y matriz extracelular (Hall 1994; Koyanagi et al. 2008), por lo que ejercería su acción sobre tipos celulares que crecen en adhesión. Sin embargo, las HSPCs mantienen crecimiento en suspensión, por lo que las vías de acción del inhibidor de ROCK, relacionadas con la interacción célula-célula (Krawetz et al. 2009), no serían efectivas en estos progenitores hematopoyéticos.

El ensayo *in vivo* de reconstitución hematopoyética a largo plazo es el único que evalúa con certeza el potencial multipotente de las HSCs (Dick 1989; Dick et al. 1991; Cavazzana-Calvo et al. 2011). En la actualidad existen diversas cepas de ratones inmunodeficientes que son utilizadas en los ensayos de reconstitución hematopoyética. La elección de la cepa a utilizar depende de las características propias de la misma. Debido a nuestro objetivo biológico encaminado a conocer el papel de MLL-AF4 y activación del receptor FLT3 *in vivo*, hemos creído conveniente verificar qué cepa de ratones inmunodeficientes es la más apropiada para estudios *in vivo* a largo plazo (12-18 semanas). Desde el descubrimiento de la mutación *scid* y el desarrollo posterior de la cepa de ratones inmunodeficientes NOD/SCID, ésta ha sido ampliamente utilizada por la comunidad científica (Bosma et al. 1983; Dick et al. 1997). Posteriormente se desarrollaron dos cepas NOD/SCID, la cepa NOD/SCID β2m$^{-/-}$ y la cepa NOD/SCID IL-2Rγ$^{-/-}$, que añaden nuevos defectos inmunes a la cepa NOD/SCID. Los ratones NOD/SCID β2m$^{-/-}$ no presentan expresión de la proteína β2-microglobulina, cadena ligera de los antígenos de histocompatibilidad HLA ABC (MHC I), por lo que la función de las células NK está comprometida. Como contrapunto, los ratones NOD/SCID β2m$^{-/-}$ desarrollan linfomas rápidamente (Christianson et al. 1997; Shultz et al. 2007). La cepa NOD/SCID IL-2Rγ$^{-/-}$ presenta deficiencias en la cadena gamma del receptor de IL-2, lo que bloquea la señalización de múltiples receptores de citocinas (Ishikawa et al. 2005; Shultz et al. 2005; Shultz et al. 2007). Además, al no desarrollar linfomas tímicos, permite estudios a más largo plazo. Por otro lado, la irradiación mieloablativa de los ratones receptores es una práctica habitual que permite eliminar barreras de histocompatibilidad y aumentar el espacio disponible en la MO lo que favorece el éxito del xenotrasplante (Garcia-Castro et al. 2008; Waskow et al. 2009). Sin embargo, la irradiación también destruye el microambiente de la MO y existen evidencias de que este microambiente ejerce una importante función en la efectiva reconstitución hematopoyética por parte de los HSPCs. Los ritmos biológicos circadianos tienen un efecto en la movilización, proliferación, diferenciación y capacidad de reconstitución hematopoyética de los HSPCs. Esta acción se realiza mediante la regulación que el sistema nervioso simpático ejerce sobre los HSPCs a través del estroma celular y otros factores que forman parte del microambiente de la MO (Mendez-Ferrer et al. 2009), que son suprimidos por el procedimiento de irradiación mieloablativa. Nosotros comparamos la capacidad de reconstitución hematopoyética de las cepas NOD/SCID, NOD/SCID β2m$^{-/-}$ y NOD/SCID IL-2Rγ$^{-/-}$, en condiciones de no irradiación mieloablativa utilizando la técnica de trasplante intratibia de HSPCs de SCU. La cepa NOD/SCID IL-2Rγ$^{-/-}$ generó la mayor cantidad de ratones con repoblación hematopoyética (90%), a la vez que ofreció un mayor nivel de dicha reconstitución y mayor movilización a otros órganos hematopoyéticos, mientras que las cepas NOD/SCID y NOD/SCID β2m$^{-/-}$ apenas mostraron injerto humano. Además, la reconstitución hematopoyética en los ratones NOD/SCID IL-2Rγ$^{-/-}$ fue multilínea componiéndose mayormente el injerto de células linfoides B, seguido de células mieloides y progenitores inmaduros. Así, la combinación de la robusta cepa NOD/SCID IL-2Rγ$^{-/-}$ con la sensibilidad del trasplante intratibia de HSPCs proporciona una herramienta metodológica efectiva en los ensayos de reconstitución hematopoyética, incluso sin acondicionamiento previo del animal, por lo que, para la realización de nuestros estudios nos decantamos

por la utilización de la cepa NOD/SCID IL-2Rγ$^{-/-}$ y del trasplante intratibia de progenitores hematopoyéticos.

Discusión sobre aspectos biológicos.

La expresión de NG2 (molécula reconocida por el anticuerpo monoclonal 7.1 (Smith et al. 1996)) se ha venido asociando a las leucemias con *MLL* reordenado (Behm et al. 1996). Esta asociación ha motivado que, desde hace años, NG2 se haya incorporado a los paneles diagnósticos usados para el inmunofenotipaje de leucemias mieloblásticas agudas pediátricas y adultas (Smith et al. 1996), (Behm et al. 1996; Hilden et al. 1997; Wuchter et al. 2000; Schwartz et al. 2003; Zangrando et al. 2008). Concretamente, la expresión de NG2 se ha relacionado de manera específica con dos tipos de traslocaciones de *MLL*, t(4;11) (q21;q23) y t(9;11) (p13;q23), que codifican para los genes de fusión MLL-AF4 y MLL-AF9, respectivamente, pero no a otros tipos de reordenamientos de *MLL*. Sin embargo, la asociación de NG2 con los reordenamientos de *MLL* ha venido siendo motivo de controversia clínica, ya que hay pacientes con *MLL* reordenado que no expresan NG2, mientras que exiten leucemias agudas y de células dendríticas con *MLL germline*, con positividad para NG2 (Bueno et al. 2004). Respecto a esta controversia, nosotros nos planteamos profundizar en la asociación biológica entre la expresión de NG2 y los reordenamientos de *MLL*, analizando si NG2 depende de la traslocación de *MLL* con algún gen concreto, o bien si la expresión de NG2 en leucemias sin reordenamiento de MLL se debe a la existencia de una población de HSPCs CD34+ que co-expresan NG2, dónde se podría iniciar el proceso leucémico. Nuestros resultados indican heterogeneidad en la expresión de NG2 respecto a la presencia de *MLL* reordenado, ya que líneas celulares sin reordenamiento de *MLL* fueron tanto NG2+ (REH y 293T) como NG2- (KG1a), mientras que líneas celulares con reordenamiento de *MLL* fueron tanto NG2+ (MV4;11 (MLL-AF4) como NG2- (THP-1 (MLL-AF9) y RS4;11 (MLL-AF4)), por lo que la expresión de NG2 no parece asociada a reordenamientos de *MLL*, ni a un gen concreto traslocado con *MLL*. Estos datos sugieren que la expresión de NG2 podría ser dependiente del origen celular donde la alteración leucémica tiene lugar, bien un progenitor hematopoyético (HPC) comprometido a linaje o bien un progenitor hematopoyético más inmaduro (HSC). La expresión de NG2 fue confirmada en los HPCs CD34+ de muestras de MO, SCU y sangre periférica movilizada proveniente de donantes sanos. Por otro lado, debido al origen prenatal de las traslocaciones MLL-AF4, analizamos la expresión de NG2 en hESCs, como potencial diana celular de la traslocación, confirmándose la expresión de NG2 en una fracción de las hESCs. Por tanto, la expresión de NG2 en progenitores CD34+, precursores pDC y hESCs, sugiere que NG2 en leucemia aparecería únicamente en los pacientes donde el proceso leucémico se inicia, independientemente del tipo de leucemia. Sin embargo, son necesarios más estudios clínicos, citogenéticos e inmunofenotípicos que puedan aclarar el significado de la expresión de NG2 en

leucemias humanas. Estos estudios requieren la inclusión prospectiva de un elevado número de casos clínicos.

Pese a la necesidad de disponer de modelos *in vivo* que desarrollen la enfermedad LLA pro-B del lactante MLL-AF4+, actualmente no se dispone de ninguno que reproduzca la enfermedad de manera fidedigna. Las aproximaciones que se han realizado en ratón hasta la fecha dan lugar a una latencia mayor y a un fenotipo discordante al observado en humano (Chen et al. 2006; Krivtsov et al. 2008; Meyer et al. 2009). Es posible que estas aproximaciones no hayan podido reproducir la enfermedad debido a: (i) que la célula diana utilizada en estos estudios murinos no se encuentre en el estadio ontogénico/jerárquico adecuado, (ii) que MLL-AF4 tenga un efecto deletéreo cuando se expresa con promotores retrovirales que produzcan niveles de la proteína de fusión no fisiológicos y posiblemente con efectos negativos, (iii) que en estos modelos murinos carezcan de factores etiológicos o eventos secundarios necesarios en el proceso de leucemogénesis. Ante estos antecedentes, en nuestro estudio utilizamos vectores lentivirales para inducir la expresión ectópica de MLL-AF4 en hESCs (estadio prenatal) y HSPCs de SCU (estadio neonatal).

En HSPCs CD34+ de SCU, la expresión de MLL-AF4 tiene un impacto fenotípico y funcional. *In vivo*, la expresión de MLL-AF4 es capaz de aumentar la efectividad de la repoblación hematopoyética de las células CD34+ trasplantadas intratibia en ratón, aumentando tanto el nivel de dicha repoblación como la movilización a otros órganos hematopoyéticos. Esta repoblación fue multilínea, sin bloqueo de la diferenciación, ni sesgo hacia ningún estadio hematopoyético. Estos resultados contrastan con trabajos previos en modelos murinos en los que la activación de MLL-AF4 en precursores linfocitarios induce el desarrollo o selección de células B (Metzler et al. 2006), posiblemente debido a que las células dianas utilizadas en ambos trabajos se encuentran en estadios de desarrollo diferentes. Estos datos muestran además que los estudios murinos pueden no ser extrapolables al humano, lo que hace necesario utilizar en estudios de leucemogénesis en células humanas (Menendez et al. 2005; Menendez et al. 2006; Bueno et al. 2009). Los animales fueron sacrificados entre las semanas 12 y 16 post-trasplante, periodo suficiente para mimetizar la corta latencia de la LLA pro-B MLL-AF4+ infantil. Durante toda su monitorización y en el momento del sacrificio, los animales no mostraban signos de enfermedad. Esta ausencia de enfermedad fue corroborada tanto por los parámetros hematológicos-bioquímicos, como por la histología de bazo e hígado. *In vitro*, la expresión de MLL-AF4 aumentó la capacidad clonogénica de los HSPCs CD34+, aunque sin otorgar ventaja a ningún tipo de precursor concreto. Sin embargo esta capacidad clonogénica disminuyó en los ensayos de clonogenicidad secundarios, indicando que MLL-AF4 no es capaz de inmortalizar dichos precursores. Los ensayos de proliferación mostraron que MLL-AF4 aumentaba la tasa proliferativa de las células CD34+. Este efecto se acompaña de una mayor proporción de células en fase de síntesis y menor proporción de apoptosis, y se corroboró por la mayor expresión en las células MLL-AF4 de genes anti-apoptóticos y menor expresión de genes pro-apoptóticos. Estos datos contrastan con el potencial oncogénico de otras fusiones *MLL* como MLL-ENL y MLL-AF9 (Barabe et al.

2007). El grupo de John Dick ha demostrado que estas fusiones son oncogénicas por sí mismas cuando se expresan ectópicamente en HSPCs de SCU. Esto indica que distintas fusiones MLL parecen tener diferente potencial oncogénico.

Al igual que lo observado con HSPCs, la expresión ectópica de MLL-AF4 no fue suficiente para inducir trasformación *in vivo* o *in vitro* ni de las hESCs ni de las células hematopoyéticas diferenciadas de las mismas. *In vivo*, MLL-AF4 no proporcionó a las hESCs potencial de repoblación hematopoyética en ratones inmunodeficientes. *In vitro*, las células que expresan MLL-AF4 no muestran ninguna ventaja proliferativa ni de supervivencia y disminuye la aparición de colonias hematopoyéticas en ensayos de clonogenicidad seriados. Sin embargo, MLL-AF4 favorece la especificación endotelial de las hESCs. Dado que el precursor hemogénico obtenido de la diferenciación de hESCs es el responsable del desarrollo endotelial y hematopoyético (Park et al. 2004; Vodyanik et al. 2005; Wang et al. 2005b; Vodyanik et al. 2006), estos datos indican que la expresión de MLL-AF4 desvía el compromiso del precursor hemogénico hacia endotelio.

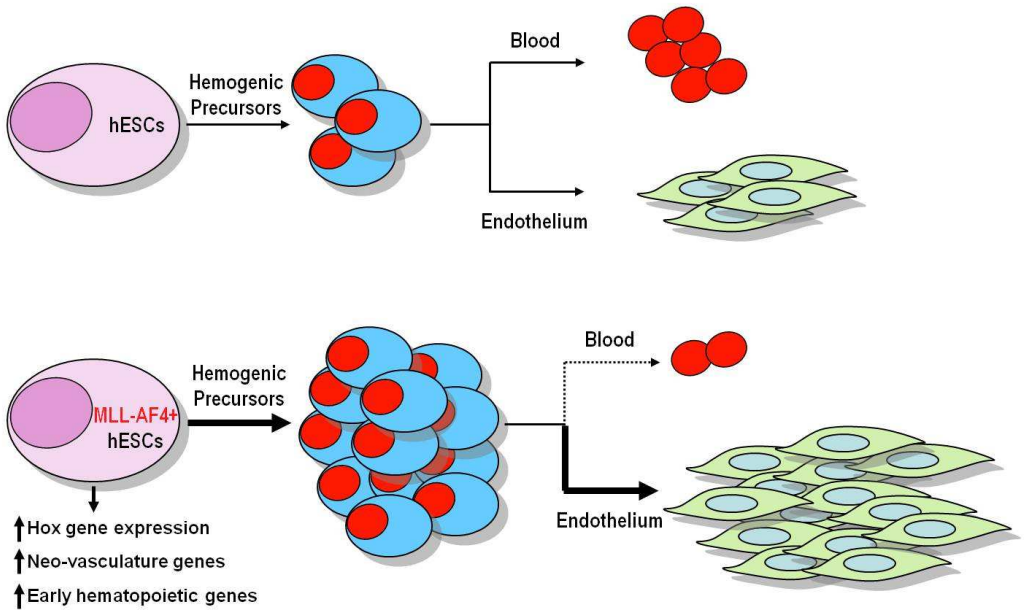


Figura 16. Figura ilustrativa del impacto de la expresión de MLL-AF4 en hESCs. La expresión de MLL-AF4 en hESCs aumenta la expresión de genes *Hox*, genes relacionados con la formación de neo-vasculatura, y genes de hematopoyesis temprana. A pesar de que aumenta la producción de precursores hemogénicos, la diferenciación hematopoyética a partir de los mismos se vé comprometida, a la vez que se favorece la especificación endotelial.

La especificación hacia endotelio de hESCs MLL-AF4+ favorece la maduración endotelial posterior, mostrando una marcada expresión de genes clave en funciones de neovasculatura. El oncogén de fusión leucémico BCR-ABL, al igual que otras alteraciones génicas asociadas a linfomas, se ha encontrado en células endoteliales de pacientes con leucemia mieloide crónica y linfoma de células B, respectivamente, sugiriendo que las células endoteliales podrían ser parte del clon neoplásico (Streubel et al. 2004; Fang et

al. 2005), y que es el precursor hemogénico (en lugar de los HSPCs) la célula diana del primer evento oncogénico, que podría ocurrir durante las fases de desarrollo embrionario, o bien a partir de hemangioblastos existentes en adultos. Además, recientemente se ha demostrado mediante la diferenciación hacia mesendodermo (Brachyury+, MixL1+) de hESCs, la existencia de un precursor común a MSCs y células endoteliales (Vodyanik et al. 2010). La existencia de dicho precursor junto la expresión de MLL-AF4 mostrada en blastos leucémicos y MSC en la MO de pacientes infantiles MLL-AF4+ (Menendez et al. 2009), sugieren que la fusión MLL-AF4 se origina y tiene un impacto sobre desarrollo temprano de mesodermo pre-hematopoyético o precursores hemangioblásticos. Es conocido que las traslocaciones de *MLL* regulan de forma positiva la expresión de genes *Hox* (Stam et al. 2005; Bach et al. 2010; Stam et al. 2010). Esto ocurre mediante la activación mediada por la marca H3K79me2 en los genes diana de MLL-AF4 (Guenther et al. 2008). Stam *y cols.* (Stam et al. 2005; Stam et al. 2010) han mostrado la existencia de dos subgrupos respecto a los pacientes de LLA infantil MLL-AF4+, teniendo los pacientes que pieden la expresión de genes *HoxA* un riesgo más elevado de recaida. En contraste, Trentin *y cols*. (Trentin et al. 2009) recientemente han cuestionado la función que la activación de genes *Hox* tiene sobre la LLA infantil MLL-AF4+, determinando estos autores que los pacientes pueden subdividirse en alta y baja expresión de genes *Hox*, sin demostrarse relevancia clínico-biológica.

Por tanto, y a pesar de tener un impacto funcional y fenotópico en HSPCs de SCU y hESCs, la expresión ectópica de MLL-AF4 no es suficiente para desarrollar el proceso de trasformación leucémica. Una de las posibles causas es la posible necesidad de eventos oncogénicos secundarios a la expresión de MLL-AF4. En este sentido, alteraciones en la función del receptor FLT3 son potenciales candidatos a colaborar con MLL-AF4 ya que el receptor FLT3 está altamente expresado en LLAs con reordenamiento de *MLL* (Armstrong et al. 2003). Así también, Ono *y cols*. (Ono et al. 2005) y Yamaguchi *y cols*. (Yamaguchi et al. 2009) han mostrado que mutaciones activadoras de *FLT3*, cooperan con MLL-ENL y MLL-AF4, respectivamente, en inducir leucemia en modelos murinos. Aunque múltiples estudios han mostrado que mutaciones activadoras de *FLT3* son comunes en LMA (mutación FLT3-ITD) (Nakao et al. 1996; Yamamoto et al. 2001; Gilliland and Griffin 2002), en el caso de LLA existe controversia al respecto. Mientras que varios grupos muestran la presencia de mutaciones puntuales en el dominio TKD de FLT3 en el 3-20% de las LLAs con *MLL* reordenado, otros autores (Stam et al. 2007a; Stam et al. 2007b; Bardini et al. 2010; Bardini et al. 2011) defienden que no ocurren mutaciones en dichas leucemias. Respecto a esta controversia, *FLT3* es consistentemente sobre-expresado en LLA con reordenamientos de MLL debido a la presencia de mutaciones activadoras o a un incremento de la expresión transcripcional con el fin de proporcionar a los blastos ventaja proliferativa y de supervivencia. Esto ha sido recientemente sugerido por Guenter *y cols*., mostrando que *FLT3* podría ser una diana directa de MLL-AF4 (Guenther et al. 2008). Nuestro grupo, en colaboración con varios hospitales de España, especialmente el H.C. de Salamanca y H. Dr. Negrín de Las Palmas, ha mostrado en pacientes con LLA, la ausencia de mutaciones ITD y TKD del receptor FLT3. Sin embargo, los subgrupos con MLL-AF4+ y

TEL-AML1+ presentaron niveles de expresión más altos de *FLT3*. Además, comprobamos que dentro de los pacientes MLL-F4+, la expresión de *FLT3* tiene una importancia pronóstica dado que los pacientes con mayor nivel de expresión presentan una evolución significativamente peor de la enfermedad, siendo la expresión de *FLT3* un factor pronóstico independiente en un análisis estadístico multivariante y asociándose el mayor nivel de expresión de *FLT3* con unos valores de supervivencia total (0% *vs* 71%;p<0.002) y de supervivencia libre de enfermedad (0% vs 43%;p=0.03), extremadamente bajos. Por tanto, y según nuestros datos, *FLT3* podría ser una diana terapéutica en LLA MLL-AF4+, enfatizando el valor de futuros estudios y ensayos clínicos que utlizen inhibidores de FLT3 en LLAs adultas e infantiles con reordenamiento MLL-AF4 (Armstrong et al. 2003). De hecho, nos sentimos orgullosos de que nuestros resultados hayan ayudado a un paciente de 10 meses del Hospital Virgen de la Arrixaca (Murcia) con niveles muy altos de *FLT3*, que fue tratado con inhibidor de FLT3 (sorafenib) fuera de protocolo (uso compasivo), alcanzando la remisión completa y pudiendo ser candidato a trasplante de progenitores hematopoyéticos alogénico. Aunque este es un único caso, ejemplariza la necesidad de fomentar la investigación traslacional, en la que haya una mayor conexión entre investigación básica y clínica.

La activación constitutiva de *FLT3* promueve en hESCs la diferenciación hematopoyética, sin bloqueo de la especificación hacia precursores hemogénicos. Este resultado aporta nuevos datos a nuestro conocimiento de la aorta-gonada-mesonephros, sugiriendo que *FLT3* podría ser dispensable en la especificación del endotelio hemogénico. Sin embargo, la activación de *FLT3* coopera con la expresión de MLL-AF4 en hESCs para inhibir la diferenciación hematopoyética sin afectar ni a la proliferación ni a la supervivencia de las células derivadas de hESCs. La co-expresión de MLL-AF4 y la activación de *FLT3* no indujo inmortalización ni de hESCs ni de las células hematopoyéticas derivadas de las mismas, e inhibió el potencial clonogénico de dichas células. En HSPCs CD34+ de SCU, la activación constitutiva de *FLT3* mediante mutaciones activadoras o mediante aumento de expresión, por sí solo o en cooperación con MLL-AF4, potencia la reconstitución hematopoyética *in vivo*, pero fue incapaz de inducir trasformación tras 14 semanas post-trasplante. *In vitro*, la co-expresión de MLL-AF4 con la activación constitutiva de *FLT3* proporciona una ventaja proliferativa transitoria pero fue incapaz de inducir inmortalización. Estos datos indican que la activación de *FLT3* no coopera con MLL-AF4 para iniciar el proceso oncogénico. Debido a los altos niveles de expresión de *FLT3* en los pacientes, es posible que la expresión alta de *FLT3* tenga un papel en el mantenimiento pero no en la iniciación ("*triggering*") de la LLA.

Estos datos sugieren que otras lesiones oncogénicas (epi)-genéticas diferentes a *FLT3* podrían ser requeridas para el desarrollo de enfermedad. Recientemente se ha mostrado que la presencia de ambas proteínas recíprocas de la fusión de *MLL* (MLL-AF4 y AF4-MLL) o AF4-MLL por sí solo es capaz de trasformar progenitores murinos Lin-/Sca1+ (Bursen et al. 2010), sugiriendo que ambas proteínas contribuyen o tienen efectos sinérgicos sobre el fenotipo leucémico. Sin embargo, Kumar *y cols*. han

mostrado en líneas celulares de leucemia MLL-AF4+, que la proteína MLL-AF4 es crítica para la proliferación celular leucémica y supervivencia, mientras que la inhibición de AF4-MLL no tiene efecto sobre las células por lo que podría ser dispensable (Kumar et al. 2011). También se ha mostrado que en una fracción de las LLA pro-B, el producto recíproco AF4-MLL no se expresa (Kowarz et al. 2007). Esto ha llevado a discusiones académicas sobre si AF4-MLL es necesario para iniciar *vs* mantener el proceso leucémico. Por otro lado, el papel que las mutaciones de *RAS* tienen en las LLAs MLL-AF4+ merece ser estudiado. De hecho, recientes trabajos (Prelle et al. 2012; Driessen et al. 2013) han mostrado que las mutaciones de *RAS* están presentes en el 14-36% de los pacientes con traslocación MLL-AF4, y que la presencia de estas mutaciones es un factor de mal pronóstico en las LLAs MLL-AF4+ infantiles. Estos estudios indican que AF4-MLL y las mutaciones de *RAS* podrían representar lesiones génicas alternativas que contribuyen al proceso de iniciación de leucemia mediado por MLL-AF4 el cuál aún no ha sido reproducido en un contexto celular inmaduro en humanos. Estos estudios son parte de los objetivos que a día de hoy se persiguen en nuestro grupo de investigación. Por otro lado, se sabe que la trasformación leucémica mediada por las fusiones de *MLL* con *AF4*, *AF9 AF10* y *ENL* requieren de la metiltrasferasa DOT1L, con la que interactúan directa o indirectamente (Hess 2004; Krivtsov et al. 2008; Deshpande et al. 2013), de modo que las proteínas de fusión mantienen el reconocimiento específico de los elementos diana de MLL, a la vez que adquieren la habilidad de reclutar a DOT1L hacia estas localizaciones, induciendo la metilación aberrante H3K79 (Okada et al. 2005; Monroe et al. 2010). La metilación ectópica H3K79, resultante del reclutamiento de DOT1L por parte de las fusiones de *MLL*, potencia la expresión de genes como *HoxA9* y *Meis1* (Guenther et al. 2008; Krivtsov et al. 2008; Nguyen et al. 2011). DOT1L no está alterado en la enfermedad *per se*, sino que es su actividad enzimática en un lugar no habitual la consecuencia directa de la traslocación cromosómica, que afecta a los pacientes con reordenamiento de *MLL*. Es posible que la expresión de DOT1L sea el evento trasformante que coopere con MLL-AF4. De hecho, datos de nuestro laboratorio sugieren que las hESCs no expresan DOT1L, lo que indica que quizás su ausencia basal impide que el MLL-AF4 ectópico pueda reclutar DOT1L y mediar la metilación H3K79, un firma distinguible de las leucemias con *MLL* reordenado. Así, la inhibición de DOT1L podría proporcionar la base farmacológica del tratamiento terapéutico de la enfermedad, lo que ha llevado al desarrollo de moléculas con actividad inhibitoria de DOT1L (Daigle et al. 2011; Yu et al. 2013). Una de estas moléculas (EPZ-5676) está siendo utilizada en un ensayo clínico humano en fase I (Epizyme), lo que supone la primera vez que un inhibidor de metiltrasferasa es utilizado en una fase clínica en oncología. La realización de estudios bioquímicos y de *ChIP-Sequencing* junto con la expresión de MLL-AF4 exógeno, podrían revelar si MLL-AF4 es capaz de reclutar DOT1L e inducir la metilación aberrante H3K79 y la subsecuente activación trascripcional de los genes diana de MLL-AF4.

No podemos obviar en futuros estudios alteraciones meramente epigenéticas. El cáncer se asocia, en general, a una hipermetilación en secuencias promotoras pero a una hipometilación global del genoma. Esta hipometilación global del genoma es parcialmente responsable de la mayor inestabilidad genética en

cáncer (Calvanese et al. 2008). Sin embargo, recientemente el grupo de Rob Pieters y Ronald Stam (Stumpel et al. 2013) ha puesto de manifiesto que esta LLA pro-B MLL-AF4+ es una entidad particular ya que cursa con una hipermetilación global del genoma, al contrario que la mayoría de los tumores. Esto se asocia con una mayor estabilidad genética, explicando, al menos en parte, porque Bardini *y cols*. (Bardini et al. 2010) y otros estudios (Greaves et al. sometido) no han encontrado alteraciones génicas recurrentes. Esta supuesta estabilidad genómica no concuerda con la agresividad y latencia de la LLA, lo que nos hace continuamente replantearnos si esta entidad es un "*single-hit cancer*" o "*multiple-hit cancer*". Estudios futuros de metilación del DNA e histonas deberían aportar información muy relevante respecto a esta cuestión.

CONCLUSIONES Y PERSPECTIVAS FUTURAS

CONCLUSIONES

Respecto a la primera parte metodológica:

1.- El IGF-II es dispensable en el mantenimieno de hESCs con medio condicionado por *feeders* humanos (HFFs y hMSCs), mientras que es necesario cuando las hESCs se mantienen en *feeders* murinos (MEFs).

2.- El inhibidor de la kinasa de Rho, Y-27632, tiene un efecto negativo al utilizarse durante los protocolos de criopreservación, descongelación y expansión de las células CD34+ derivadas de SCU. Este efecto adverso desaconseja la introducción de Y-27632 en protocolos clínicos o de investigación relativos a criopreservación y mantenimiento de dichas células.

3.- El modelo de xenotrasplante usando la cepa de ratón inmunodeficiente NOD/SCID IL-2Rγ$^{-/-}$ proporciona una mayor proporción de repoblación hematopoyética multilínea y favorece la migración de las células CD34+ sin acondicionamiento previo, a diferencia de lo observado en las cepas NOD/SCID y NOD/SCIDβ2m$^{-/-}$.

Respecto al estudio de la relación genotipo-fenotipo entre los reordenamientos de *MLL* y la expresión del marcador NG2.

1.- Nuestros datos en líneas celulares, SCU y MO normal apoyan datos clínicos que indican la existencia de leucemias agudas con reordenamiento *MLL* pero sin expresión de NG2 y viceversa, leucemias NG2+ sin reordenamiento *MLL*. La presencia de un porcentaje de células CD34+ NG2+ en SCU y MO normal sugiere que la expresión de NG2 en leucemias puede estar limitada a aquellos casos donde el proceso leucémico se originó en una célula NG2+.

Respecto al estudio del impacto funcional del gén de fusión MLL-AF4 en células troncales progenitoras neonatales y embrionarias.

1.- La expresión ectópica de MLL-AF4 en HSPCs no es suficiente para trasformar *in vivo*, o inmortalizar *in vitro* HSPCs derivadas de SCU. La expresión ectópica de MLL-AF4 en HSPCs CD34+ derivadas de SCU aumenta la capacidad de reconstitución hematopoyética de dichos progenitores *in vivo*, su eficiencia y la movilización a otros órganos hematopoyéticos, siendo multilínea dicha reconstitución. *In vitro*, la expresión ectópica de MLL-AF4 aumenta el potencial clonogénico de las HSPC CD34+, y aumenta la tasa proliferativa.

2.- En hESCs, la expresión de MLL-AF4 no es suficiente para inducir trasformación oncogénica. Sin embargo, MLL-AF4 potencia la especificación hacia precursores hemogénicos pero inhibe la diferenciación hematopoyética de dichos precursores hacia hematopoyesis en favor de la diferenciación endotelial de los mismos.

Respecto al estudio de la activación constitutiva del receptor FLT3 y su posible papel en el proceso leucemogénico de MLL-AF4.

1.- Las LLAs no presenta mutaciones ITD y TKD del receptor FLT3. Sin embargo, los subgrupos con MLL-AF4+ y TEL-AML1+ presentan niveles de expresión más altos de FLT3. Dentro de los pacientes MLL-AF4+, la expresión de *FLT3* tiene una importancia pronóstica dado que los pacientes con mayor nivel de expresión presentan una evolución (supervivencia global y libre de enfermedad) significativamente peor de la enfermedad.

2.- La activación constitutiva del receptor FLT3 mediante mutación (ITD o TKD) o sobre-expresión no coopera con MLL-AF4 para inmortalizar/trasformar las células hematopoyéticas derivadas de las hESCs. La activación constitutiva de FLT3 potencia la diferenciación hematopoyética de las hESCs, mientras que la co-expresión de MLL-AF4/FLT3 inhibe la especificación hematopoyética. Esta inhibición está regulada por cambios trascripcionales mediados por la activación de FLT3 en las hESCs que expresan MLL-AF4.

3.- En células CD34+ derivadas de SCU, la activación constitutiva independiente de ligando del receptor FLT3 mediante la mutación en TKD o mediante sobre-expresión no coopera con MLL-AF4 en el desarrollo del proceso leucémico. *In vivo*, la co-expresión de MLL-AF4 junto con la activación de FLT3 aumenta la reconstitución hematopoyética. *In vitro*, la co-expresión de MLL-AF4 junto con la activación de FLT3 disminuye la capacidad clonogénica, aunque es capaz de aumentar la capacidad proliferativa de manera transitoria.

PERSPECTIVAS FUTURAS.

Debido a que ni la expresión de MLL-AF4 por sí sola o junto con la activación constitutiva de FLT3, son suficientes para inducir trasformación oncogénica en células troncales prenatales (hESCs) y células troncales hematopoyéticas neonatales (HSPCs de SCU), nuestro grupo de investigación se plantea realizar en el futuro los siguientes estudios:

1.- Utilizar células de hígado fetal/médula ósea humana como potencial diana celular sobre la que MLL-AF4 pudiera causar efecto oncogénico.

2.- Estudiar el efecto de la expresión ectópica de la metiltrasferasa DOT1L junto con la expresión de MLL-AF4, del producto recíproco de la traslocación t(4;11) (AF4-MLL), y de mutaciones activadoras de k-Ras en hESCs y células CD34+ de SCU.

3.- Reprogramar blastos provenientes de leucemias con la traslocación *MLL* para obtener iPSCs de dichas leucemias, y estudiar en las mismas los mecanismos celulares iniciales de la trasformación oncogénica, así como estudiar la activación específica a estadio pro-B de la fusión MLL-AF4.

4.- Realizar estudios de secuenciación masiva para identificar mutaciones somáticas recurrentes en esta leucemia.

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GLOSARIO DE ABREVIATURAS

Abreviatura	Inglés	Castellano
ABC	ATP-binding cassette	Casete de unión a ATP
AF4	Gene fused with *MLL* in chromosome 4 region q11	Gen traslocado con *MLL* localizado en cromosoma 4 región q11
AGM	Aorta-Gonada-Mesonephros	Aorta-Gónada-Mesonephros
ATP	Adenosine triphosphate	Adenosín trifosfato
BFU-E	Burst-forming Unit-Erythroid	Unidad formadora de colonias eritroides
BCR	Breakpoint Cluster Region	Región donde se acumulan roturas génicas
bFGF	basic Fibroblast Growth Factor	Factor de crecimiento de fibroblasto básico
CD	Cluster of Diferentiation	Marcador de diferenciación
CFU-E	Colony-forming Unit- Erythroid	Unidad formadora de colonias eritroides
ChIP	Chromatin Immunoprecipitation	Inmunoprecipitación de cromatina
CM	Conditioned Media	Medio condicionado
CSC	Cancer Stem Cell	Célula troncal tumoral
DOT1L	Disruptor of Telomeric silencing-1 like	Interruptor de silenciamiento telomérico-1
DNA	Deoxyribonucleic acid	Ácido desoxiribonucleico
EFS	Event-free survival	Supervivencia libre de enfermedad
ELISA	Enzyme-Linked ImmunoSorvent Assay	Inmunoensayo de detección de moléculas solubles
EPO	Erythropoietin	Eritropoyetina
FAB	French-American-British	Franco-Americano-Británico
FLT3	Fms-like Growth Factor 3	Factor de crecimiento tipo FMS 3
G-CSF	Granulocytic-Colony Stimulating Factor	Factor de estimulante de colonias granulocíticas.
GM-CSF	Granulocytic-Monocytic Stimulating Factor	Factor de estimulante de colonias granulo-monociticas
hESC	human Embrionic Stem Cell	Célula troncal embrionaria humana
HFF	Human foreskin fibroblast	Fibroblasto de prepucio humano
HFF-CM	Human foreskin fibroblast-Conditioned	Medio condicionado por fibroblastos

	Media	de prepucio humano
HLA	Human Leukocyte Antigen	Antígeno leucocitario humano
hMSC	Human Mesenchymal Stem Cell	Células troncales mesenquimales humanas
hMSC-CM	Human Mesenchymal Stem Cell-Conditioned Media	Medio condicionado por células troncales mesenquimales humanas
HPC	Hematopoietic Progenitor Cell	Célula progenitora hematopoyética
HSC	Hematopoietic Stem Cell	Célula troncal hematopoyética
HSPC	Hematopoietic Stem/Progenitor Cell	Célula troncal/progenitora hematopoyética
I-FISH	Immune-Fluorescence *In Situ* Hybridisation	Inmune-Hibridización *in situ* fluorescente
IGF-II	Insulin-like Growth Factor-II	Factor de crecimiento tipo insulínico-II
IL	Interleukine	Interleucina
iPSC	Induced Pluripotent Stem Cell	Célula troncal pluripotente inducida
ITD	Internal Tandem Duplication	Duplicación interna en tándem
LIC	Leukemia Initiating Cell	Célula iniciadora de leucemia
M-CSF	Monocytic-Colony Stimulating Factor	Factor de estimulante de colonias monocíticas
MEF	Mouse Embryonic Fibroblast	Fibroblasto embrionario murino
MEF-CM	Mouse Embryonic Fibroblast-Conditioned Media	Medio condicionado por fibroblastos embrionarios murinos
MLL	Mixed Lineage Leukemia	Leucemia de linaje mixto
Mll	Murine Mixed Lineage Leukemia	Leucemia de linaje mixto murino
MRD	Minimal Residual Disease	Enfermedad mínima residual
MSC	Mesenchymal Stem Cell	Célula troncal mesenquimal
NG2	Nerve/glial antigen-2	Antígeno neuro/glial-2
NOD	Non-Obese Diabetic	Diabético no-obeso
PCR	Polymerase Chain Reaction	Reacción en cadena de la polimerasa
pDC	Plasmacytoid dendritic cells	Células dendríticas plasmocitoides
RFLP	Restriction fragment lengh polymorphism-mediated	Polimorfismo de longitud de fragmentos de restricción
RNA	Ribonucleic acid	Ácido ribonucleico
ROCK	P160-Rho-associated coiled-coil kinase	Kinasa asociada a p160-Rho
RT-PCR	Reverse transcription-Polymerase Chain Reaction	Trascripción reversa-Reacción en cadena de la polimerasa

SCF	Stem Cell Factor	Factor de crecimiento de células troncales
SCID	Severe-Combined Immunodeficiency	Inmunodeficiencia severa combinada.
SCU	Umbilical cord blood	Sangre de cordon umbilical
SET	Suppresor of variegation, Enhancer of zeste, Trithorax.	Dominio epigenético presente en *MLL*
SNP	Single nucleotide polymorphism	Polimorfismo de nucleótido simple
SNV	Single nucleotide variation	Variación de nucleótido simple
TGF-β	Tumor Growth Factor beta	Factor de crecimiento tumoral beta
TPO	Trombopoietin	Trombopoyetina
qPCR	Quantitative PCR	PCR cuantitativa