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PII: S0304-3835(18)30317-3

DOI: 10.1016/j.canlet.2018.04.042

Reference: CAN 13882

To appear in: *Cancer Letters*

Received Date: 31 March 2018

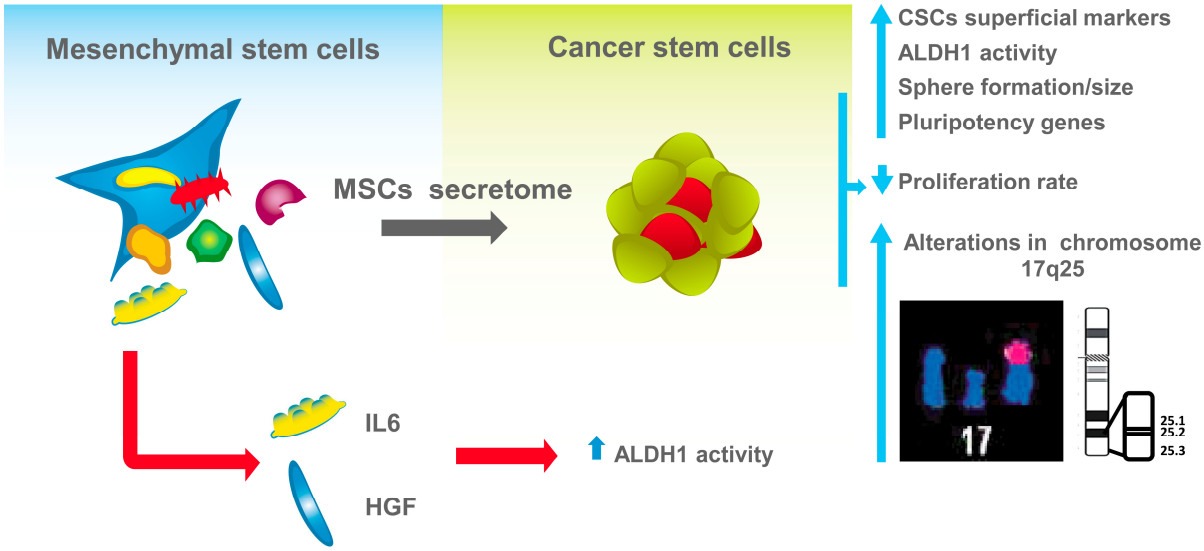
Revised Date: 27 April 2018

Accepted Date: 27 April 2018

Please cite this article as: G. Jiménez, M. Hackenberg, P. Catalina, H. Boulaiz, C. Griñán-Lisón, M.Á. García, M. Perán, E. López-Ruiz, A. Ramírez, C. Morata-Tarifa, E. Carrasco, M. Aguilera, J.A. Marchal, Mesenchymal stem cell's secretome promotes selective enrichment of cancer stem-like cells with specific cytogenetic profile, *Cancer Letters* (2018), doi: 10.1016/j.canlet.2018.04.042.

ABSTRACT

Cancer stem cells (CSCs) are responsible for tumour initiation, metastasis and cancer recurrence, however the involvement of microenvironment is crucial. Here, we have analyzed how human mesenchymal stem cells (MSCs)-derived conditioned medium (CM) affect colon and melanoma CSCs enrichment and maintenance. Our results strongly suggest that the secretome of CM-MSCs selects and maintains subpopulations with high expression of CSCs markers and ALDH1 activity, low proliferation rates with G1 phase arrest, and notably retain *in vivo* these properties. Cytogenetic analyses indicated that CM-cultured cells contain alterations in chromosome 17 (17q25). Subsequent SKY-FISH analyses suggested that genes located in 17q25 might be involved in stem-cell maintenance. The characterization of secreted proteins present in CM-MSCs revealed that four cytokines and seven growth factors are directy linked to the CSCs enrichment reported in this study. Further analyses revealed that the combination of just IL6 and HGF is enough to provide cancer cells with better stemness properties. In conclusion, this study demonstrates how specific chromosomal alterations present in CSCs subpopulations might represent an advantage for their *in vitro* maintenance and *in vivo* stemness properties.



Mesenchymal stem cell´s secretome promotes selective enrichment of cancer stem-like cells with specific cytogenetic profile

Running Title: Secretome of MSC improves cancer stem cell culture

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ABSTRACT

Cancer stem cells (CSCs) are responsible for tumour initiation, metastasis and cancer recurrence, however the involvement of microenvironment is crucial. Here, we have analyzed how human mesenchymal stem cells (MSCs)-derived conditioned medium (CM) affect colon and melanoma CSCs enrichment and maintenance. Our results strongly suggest that the secretome of CM-MSCs selects and maintains subpopulations with high expression of CSCs markers and ALDH1 activity, low proliferation rates with G1 phase arrest, and notably retain *in vivo* these properties. Cytogenetic analyses indicated that CM-cultured cells contain alterations in chromosome 17 (17q25). Subsequent SKY-FISH analyses suggested that genes located in 17q25 might be involved in stem-cell maintenance. The characterization of secreted proteins present in CM-MSCs revealed that four cytokines and seven growth factors are directy linked to the CSCs enrichment reported in this study. Further analyses revealed that the combination of just IL6 and HGF is enough to provide cancer cells with better stemness properties. In conclusion, this study demonstrates how specific chromosomal alterations present in CSCs subpopulations might represent an advantage for their *in vitro* maintenance and *in vivo* stemness properties.

Keywords: secretome, cancer stem-like cells, mesenchymal stem cells, cytogenetic, interleukin-6, hepatocyte growth factor

INTRODUCTION

Cancer is considered a genetic disease characterized by multiple genetic mutations, chromosomal/microsatellite instability, deregulation of transcriptional control and epigenetic changes [1]. The cancer stem cells (CSCs) model assumes that a small subpopulation of cells with stemness characteristics reside within the tumor and is responsible of tumor initiation, maintenance, progression and the development of secondary tumors. Main features of CSCs include: (i) indefinite self-replication, (ii) asymmetric cell division, (iii) the ability to maintain a quiescent state, (iv) resistance to cytotoxic agents, and (v) increased aldehyde dehydrogenase 1 enzymatic activity (ALDH1) [2]. In addition, CSCs are characterized by their high genomic instability [3], indeed, it has been shown that cancer cells can be converted into CSCs by increasing genomic instability. Consistently, tumor growth and metastasis can be inhibited by maintaining genomic stability on cancer cells [4, 5].

Traditional cancer therapies rely on surgical resection of the tumor followed by chemotherapy or radiotherapy to kill fast dividing cells, although quiescent CSCs could resist this therapy [2]. Therefore, to effectively treat cancer, novel approaches that target CSCs might be developed. However, CSCs only constitute a small subpopulation within the tumor mass which hinders their isolation and thus has hampered their molecular characterization. Moreover, the tumor niche plays a key role in the maintenance and support of CSCs populations [5]; however, upon *in vitro* culturing CSCs, the microenvironment is lost. Until now, CSCs culturing methods had exploited their specific abilities such as the formation of spheres in non-adherent substrates and their capability to grow in serum-free medium [6]; nevertheless, this methodology does not reproduce the tumor complex composition or the tumor microenrivonment. Notably,

the tumor microenvironment is composed of tumor cells and non-tumor cells such as mesenchymal stem cells (MSCs), fibroblasts, endothelial cells and various types of immune system cells [7]. The recruitment of MSCs to the tumor is an essential process that provides a microenvironment [7] with immunosuppressive properties [8], and with the capacity to promote angiogenesis [9], metastasis [10], and thus supporting cancer cell proportion and proliferation [11]. Previous studies have demonstrated that MSCs interact with tumor cells *via* secretion of signaling molecules that may stimulate cancer growth [7].

To further explore the role of MSCs on cancer development and CSCs, in this study we have analysed the role of MSCs in the generation of a CSCs niche (Fig. 1 and Supplementary Table S1). In doing that, we have discovered that conditioned medium (CM) obtained from MSCs enhances the enrichment and maintenance of colon and melanoma CSCs subpopulations when compared with conventional sphere forming media (SM).

MATERIALS AND METHODS

Cell lines

Human colon (HCT116) and melanoma (A375) cancer cell lines were obtained from American Type Culture Collection (ATCC), and cultured following ATCC recommendations. Moreover, three primary cell lines of human colon adenocarcinoma were obtained from the Biobanco del Sistema Sanitario Público de Andalucía. Two primary cell lines were expanded until low passage 2 (Patient 2, P2, and Patient 3, P3) and one primary cell line (Patient 1, P1) was expanded until passage 3 (low passage) and passage 6 (high passage) (More detailed characterization of primary cell lines is

shown in Supplementary Fig.S1). These cells were maintained in advanced DMEM/F12 (Gibco) supplemented with 10% FBS (Gibco) and 5% Penicillin/Streptomicin (Sigma-Aldrich).

Conditioned Medium

MSCs from lipoaspirate were seeded in 75 cm^2 culture flasks to 40% confluence in complete medium (DMEM, 10% FBS, 1% penicillin/streptomycin), at 24 hours were washed with phosphate-buffered saline (PBS) to remove any traces of medium or serum, and conventional sphere medium (SM) was added (DMEM-F12, 1% streptomycin-penicillin, 1 mg/mL hydrocortisone (Sigma-Aldrich), 4 ng/mL heparin (Sigma-Aldrich), 1X ITS (Gibco); 1X B27 (Gibco), 10 ng/mL EGF (Sigma-Aldrich), 10 ng/mL FGF (Sigma-Aldrich)). Every 48 hours the medium was collected and replaced by fresh medium, until the culture flasks reached 80-90% confluence. The collected medium was called conditioned medium (CM), passed through a filter of 0.22 μM and stored at -20 °C until its use.

Culture conditions

In order to distinguish the effect of different culture conditions in the selection and enrichment of CSCs, thirteen different culture conditions based on medium, attachment, transwell (T) and cell sorting were established (Fig. 1 and Supplementary Table S1). Cells were cultured in each condition during 12 days, and every 72 hours spheres were disaggregated. For this purpose, spheres were collected by centrifugation at 1500 rpm during 5 minutes, incubated 5 minutes at 37°C with trypsin-edta (Sigma-Aldrich) and inactivated with FBS. Then, cells were washed with PBS and seeded again in the same culture condition.

In vivo study

Detailed in Supplementary data.

Conventional and spectral karyotyping

Conventional and spectral karyotyping are detailed in Supplementary data.

Gene and microRNAs analysis of selected chromosome bands

We converted the names of the chromosome bands into the corresponding chromosomal coordinates of genome assembly hg19/GRCh37 by means of the 'cytoBand' table located at the UCSC Table Browser. Once obtained the chromosome coordinates of the 'hotspots' in bed format, we determined the genes (RefSeq genes) and microRNAs that were contained within these regions by means of the 'Intersect' tool implemented in Galaxy [12].

To shed light on the putative functions of the microRNAs contained within this region, we use miRPath [13]. This program accepts a set of microRNAs as input and i) determines the target genes either by predicting or retrieving them from a list of experimentally verified interactions, ii) calculates the number of target genes in every KEGG pathway comparing this number to the expectation. Those pathways that show a statistically significant higher number of target genes are reported. In order to study the genes located on 17q25, we analysed the protein interaction network for those genes with stem cell features by means of the string webserver (http://nar.oxfordjournals.org/content/early/2014/10/28/nar.gku1003.full). Only experimentally verified connections and those obtained by text-mining were considered.

Secretome analysis

Detailed in Supplementary data.

Statistical analysis

Statistical calculations were performed using SPSS 13.0 software for Windows (SPSS, Chicago, IL, USA). All graphed data represent the mean + /-SD from at least three experiments. Differences between treatments were tested using the two tailed Student´s T test. Assumptions of Student´s T test (homocedasticity and normality) were tested and assured by using transformed data sets [log(dependent variable value + 1)] when necessary. P-values < 0.05 (*,#) and P-values <0.01 (**,##) were considered statistically significant in all cases.

RESULTS

MSC-drerived Conditioned Medium induces sphere formation and the acquisition of a CSC-like phenotype on cancer cells.

We initially evaluated the effect of MSCs conditioned medium (CM) on the enrichment and culturing of CSCs subpopulations derived from colon (HCT116) and malignant melanoma (A375) cell lines, and both primary cell colon cancer cultures at low and high passages. The efficacy of the CM was compared with routinely used serum free sphere-forming medium (SM) and with inserted transwells (T) containing MSCs under different culture setting (Fig. 1B and Supplementary Table S1): i) monolayer culture ii) non-sorted (NS) cells growing under sphere-forming conditions; iii) ALDH1-sorted cells growing under sphere-forming conditions (S+, ALDH1 activity; S-, non ALDH1 activity). Additionally, monolayer adherent cell cultures supplemented with FBS were used as a control.

After 12 days in culture, cells were characterized by ALDH1 activity and the expression of specifics melanoma (CD44) and colon (CD44 and CD326) CSCs surface markers. Consistently, ALDH1 activity was significantly enhanced (**p<0.01) in HCT116 cells growing in cell suspension (NS) with CM when compared to cells grown on SM. Intriguingly, ALDH1 activity and CD44 expression were significantly enhanced (**p<0.01) in the subpopulation negative for ALDH1 activity (S-) growing in presence of CM (Fig. 2A). A significant increase in CD44 expression was found for A375 cells grown in CM for both NS and sorted S- subpopulations (Fig. 2B) when compared to cells cultured with SM. Moreover, ALDH1 activity of A375 cells was increased in the sorted ALDH1+ (S+) subpopulation grown with CM compared to cells grown with SM (Fig. 2B). Cell cultured in suspension (NS, S+ and S-) with T displayed values for CD44 and/or CD326 expression and/or ALDH1 activity which were intermediate between those for CM and SM. (Supplementary Fig. S2).

Notably, when we analyzed the primary colon cancer cell line P3 at a low passage, we detected a significant increase in the expression of the CSCs marker CD44 in the presence of CM, even in cells cultured in attaching conditions (Fig. 2C). Moreover, CM significantly increased CD44 expression and ALDH1 activity in NS populations with respect to cells cultured in the presence of SM. In addition, cells grown with CM showed significantly higher expression for CD44 and CD326 in S+ subpopulations, and higher ALDH1 activity in S- subpopulations in comparison to those grown in SM (Fig. 2C). Similar results were observed in the S- subpopulations from both HCT116 and A375 cell lines (Fig. 2A and B). In concordance with P3 cells, CM significantly augmented ALDH1 activity and/or CD44 and CD326 expression of primary cell cultures in patients 2 and 1 (P2 and P1) (Supplementary Fig. S3). Furthermore, P1 at

low passage, P2 and P3 cells cultured with T showed similar values that cells cultured with SM. On the other hand, P1 at a high passage cultured with T showed the lowest expression of stem cell markers and ALDH1 activity in comparison to SM or CM grown cells (supplementary Fig. S3). These data suggest that CM is better than SM to select cells with CSCs-like phenotypic properties.

Furthermore, these data were confirmed using the sphere formation assays, showing that CM favors the formation of larger spheres (Fig. 2). Futther, the number and the size of spheres were significantly higher in HCT116 and A375 established cells lines, as well as in the primary colon cancer cell lines when cells were grown in presence of CM compared to the conventional SM (Fig. 2).

CM induces low proliferation rates

CSCs are associated with reduced proliferative potential, thus, we compared the proliferation rate in the different growth conditions.

Proliferation assay demonstrated that cell suspension cultures of NS, S+ and S- with CM or T grow significantly slower (**$p<0.01$) than cells cultured with conventional SM (Fig. 3A and Supplementary Fig. S4A). An exception was observed in A375 S+ cells cultured with CM, which showed a similar behavior to cells grown in SM. In addition, cell cycle analyses of cells cultured with CM showed an accumulation of cells predominantly in G1 phase, with a concomitant restriction of cells on division (Supplementary Fig. S5). Moreover, apoptotic cells presents in each culture condition were quantified and results showed a higher apoptosis rate in cells cultured with T in

comparison with cells cultured with CM (#p-valor) or SM (*p-valor) (Supplementary Fig. S4B).

CSCs subpopulations selected by CM retain *in vivo* CSC-like phenotypic properties

We next tested the *in vivo* functional characteristics of CSCs to develop a tumor. First, *in vivo* limiting dilution was performed to establish the tumorigenic ability of CSCs subpopulations selected by CM and SM. For this purpose, we injected subcutaneous serially diluted cells (1000, 100 and 10) that were grown previously in CM or SM. CM grown cells of both cell lines development more remarkable number of tumors, that grew significantly faster and with higher tumor volume than SM at all dilutions. Moreover, cells that have been selected by SM did not grow at the lowest dilution (10 cells) in both cell lines (Fig. 4A). Tumor formation showed that CM selects cells with strongly *in vivo* tumorigenic ability.

Second, cell subpopulations from HCT116 and A375 cell lines cultured in suspension with CM (NS CM, S+CM and S-CM) were compared to cells cultured in monolayer with complete medium as a control (CTL). Tumors generated 38 days after subcutaneous injections of 1×10^4 cells were sectioned in two pieces, and a piece was used for immunohistochemical analyses and the other was disaggregated by enzymatic digestion and primary cultures established.One week after establishing primary cell cultures, ALDH1 activity, CD44 and CD326 expression were analyzed (Fig. 4B). Results indicated that both cell lines cultured *in vitro* with CM, prior to inoculating mice, displayed elevated ALDH1 activity (**$p<0.01$) after the *in vivo* tumorigenic process, and in the case of HCT116 accompanied with an increase for CD326 and CD44

expression (**p<0.01), in comparison to CTLs. It is important to note that NS and S- cells cultured with CM displayed same fold increase expression of CSCs markers and ALDH1 activity that S+CM cells after the tumorigenic process.

Immunohistochemistry of sections obtained from tumors showed high protein expression of OCT4 and SOX2 in S+ CM and NS-CM tumors in both HCT116 and A375 cell lines (Fig. 4C). In S- CM tumors, OCT4 expression was lower than S+ CM and NS-CM tumors, but slightly higher compared with control tumors in which low or practically null expression was observed in both cell lines. Furthermore, low expression of SOX2 was observed in S-CM tumors from A375 cell line and even lower in control tumors of the same cell line, while in HCT116 cell line, SOX2 expression was practically inestimable in S- CM and control tumors (Fig. 4C).

Cells cultured with conditioned medium show alterations in chromosome 17 that are related with both pluripotency-associated genes and microRNAs

Genomic instability is considered a key endogenous mechanism for accumulation of chromosome mutations. Here, we studied the karyotype of cells cultured with CM during 12 days, with the aim to establish a correlation between CSCs subpopulation selected by CM and chromosome alterations. The initial chromosomal analysis of the HCT116 and A375 cells showed that both cell lines have structural and numerical abnormalities at baseline. The A375 cell line is a hypotriploid with a modal number of 62 chromosomes. There are 8 chromosomes markers that are commonly found in each cell, and normal N2, N6, and N22 are present at one copy per cell. The HCT116 cell line showed that stemline chromosome number (the most basic clone) is near diploid with the modal number at 45 (62%) and polyploids occurring at 6.8%. The markers 10q+ and t(?8p;18q) are present in all metaphases and t(9q;?16p-), in 80% of the cells

karyotyped. N16 is monosomic in the presence of the translocation, but disomic in the absence of t(9q;?16p-), N10 and N18 are monosomic and other chromosomes from those mentioned above are disomic. G-band observations revealed the presence of the Y chromosome, but not in all cells (more than 80% of cells lacked the Y in G-band karyotypes). Once cultured in the CM, we observed that new chromosomal alterations appeared in both cell lines, increasing their genetic instability. Thus, we found a hot spot on chromosome 17 involved in different translocation that corresponds to the band 17q25 involved in the alteration (Fig. 5 and Supplementary Table S2).

To study the possible molecular changes induced by CM we performed a functional *in-silico* analysis of the genes and microRNAs located in the 17q25 region. We found that the chromosome bands 17q25 were affected in all culture conditions with CM, with the exception of A375 S+ CM. This region has a total of 7 annotated microRNAs (hsa-mir-636, hsa-mir-4316, hsa-mir-657, hsa-mir-3065, hsa-mir-338, hsa-mir-1250, hsa-mir-3186) and 541 annotated transcripts from 249 genes (Fig. 6C). Among these 7 microRNAs, only 3 are described in the literature: hsa-mir-636; hsa-mir-657 and hsa-mir-338. Only for mir-338, both arms are contained within miRBase and therefore we queried miRPath with 4 known mature sequences. Important stem cell related pathways are among the most significant results like Wnt signaling pathway (Fig. 6A). Furthermore, the target genes seem to accumulate rather upstream of the Wnt pathway (genes that are targeted by any of the 4 sequences are displayed in yellow in Fig. 6B).

Moreover, the region 17q25 contains a total of 249 genes involved in i) Wnt/β-catenin signaling (*NOTUM, HN1, TIMP2, FOXKs, MCRIP1*), ii) EGF signaling pathway (*GRB2, TBC1D16*), iii) PI3K/AKT/mTOR (*RPTOR, SECTM1*), iv) epithelial-

mesenchymal transition (*METTL23, EXOC7, ITGB4, FAM195B*), v) stem cell activation and differentiation (*CBX4*) and vi) invasion, metastasis and cell migration (*ARHGDIA, EXOC7, SPHK1, CBX8, SNHG16, LLGL2*) among other.

By means of the String webserver we analysed the protein interaction network of these genes (Fig. 6D). We observed that many of them are strongly connected, linking together genes with important functions in stemness properties. For example, *GRB2* seems to be the connection between *EGFR* and several member of the RAS/RAF family (*HRAS, KRAS, NRAS and RAF1*). Three proteins from 17q25 interact with *HRAS* which is connected to *KLF4*, a transcription factor with outstanding functions in self-renewal. Finally, 4 genes *TIMP2, RPTOR, ITGB4, GRB2* are directly connected to *MYC*, another crucial gene for stem-cell features.

Concentrated conditioned medium improves its CSCs enrichment properties.

Once it was established that CM possessed best properties for CSCs enrichment and culture, it was speculated that the beneficial characteristics could be optimized by concentrating it. For this purpose, CM was concentrated (CCM) 10-fold using ultrafiltration units, and different dilutions (25%, 50%, 75% and 100%) of this 10-fold CCM were used to grown HCT116 cell line in suspension.

We determined ALDH1 activity and specific colon CSCs-related cell surface markers. Results showed that all dilutions lead to an increase in ALDH1 activity and at least one colon CSCs marker, being the 10-fold concentration (100%) the best that induced highest increments in ALDH1 activity (1.8-fold, $*p<0.05$), in CD44 and CD326 expression (4.3 and 9.6-fold respectively, $**p<0.01$) compared to SM culture. Moreover, the expression of these markers only was significantly higher in 10-fold

concentrated CM respect to unconcentrated CM (ALDH1= $^{\#}p<0.05$, CD44 and CD326= $^{\#\#}p<0.01$) (Fig. 7A).

These data suggest that CCM was most efficient in maintaining stemness properties.

Secretome analysis of conditioned medium from MSCs cultures showed that combination of HGF and IL6 is a promising new alternative for CSCs selection and culture.

In order to characterize the secreted proteins associated with the improvement in CSCs culture, a multiplex-assay was performed in CM and in the supernatants from HCT116, Patient 1 and Patient 3 (Tables S3-S5) grown in each condition. Secretome analysis revealed high levels (upper of 5000 pg/ml) of EGF, FGF, HGF, IL6 and IL8/CXCL8. Furthermore, the presence of GMCSF, MSCF, VEGF-A, PIGF-1, IL12 and IL23 was also detected. All showed differential concentration in CM obtained from MSCs and supernatant of tumor cells cultured with CM (Supplementary Fig. S6 and Supplementary Tables S3-S5).

To select which of these cytokines and/or growth factors could improve the stemness enrichment properties of the SM, we analyzed ALDH1 activity in HCT116 colonospheres growth in SM supplemented with those factors individually or in combinations. As it can be appreciated in Fig. 7B, seven factors (FGF, GMCSF, HGF, VEGF, PIGF1, IL-6, IL8 and IL-23) significantly increased ALDH1 activity when compared with colonospheres cultured in conventional SM. Nevertheless, neither of factors added individually to SM enhanced ALDH1 activity of HCT116 spheres when compared with cells grown in CM. Additionally, we tested 17 combinations of

cytokines and/or growth factors and proved that 11 of these combinations significantly incremented ALDH1 activity (ranging from 1,1 to 3,22) in HCT116 spheres (Fig. 7C). When cells were cultured in SM enriched with the combination of all factors tested, ALDH1 activity experimented an increment (3,22 folds) comparable to results obtained with spheres growth in CM (2,84 folds). Interestingly, the combination of IL6 and HGF increased ALDH1 activity (2,25 folds), suggesting that the only addition of those two factors improves the SM properties, enhancing the acquisition of CSC-like properties.

DISCUSSION

Over the last years, it became clear that tumor development and its behavior is not determined by tumor cells alone, but the participation of microenvironment is crucial during tumorigenesis. In particular, the role of MSCs in supporting tumor development has been widely debated [7, 8]. In our study, we demonstrate that MSCs-derived secretome possess the ability to select and maintain *in vitro* and *in vivo* CSCs subpopulations with a specific phenotypic, genetic and cytogenetic profile.

First, we proved that CM obtained from adipose derived stem cells facilitates the enrichment and maintenance of CSC phenotype more efficiently than conventional SM medium as showed the higher expression of CD44 and CD326 surface markers, ALDH1 activity, and the ability to form spheres [15, 16]. Also, it has been demonstrated that CSCs show slow cycling and G1 phase arrest, which are related with a quiescent state [16]. Cells cultured with CM showed slow cycling characterized by a lower proliferation ratio through induction of G1 phase arrest. These results are in concordance with previous studies demonstrating that the secretome of MSCs is able to keep tumor cells arrested in G0/G1 [17, 18,].

In our study, even non stemmnes ALDH1 negative subpopulations (S-) acquired CSC-like phenotype after culture with CM. In agreement with our results, evidence has been provided that dedifferentiation of non-tumorigenic tumour cells towards CSCs can occur [19]. This conversion is supported by several factor such as inflammatory mediators and microenvironmental changes, and involves the reactivation of one or more pluripotency genes [20].

All together, these data reveal that CM allows the enrichment and maintenance of CSC-like phenotype for a long time in culture. In a previous study, we proved that enriched subpopulations with CSC-like properties show higher tumorigenic and metastatic *in vivo* potential [21]. In agreement with these results, cells grown in CM were more tumorigenic than those grown in SM as demonstrated limiting dilution assay and retain *in vivo* the overexpression of ALDH1, CD44 and CD326, which were previously described as CSCs markers with higher tumorigenic potential and prognosis markers in several cancers [22, 23]. Further, immunohistochemistry analysis of tumors showed high protein expression of SOX2 and OCT4 in tumors developed by CM cultured cells. It has been shown that pluripotency genes are critical for self-renewal, tumorigenecity and tumor progression [24].

Cancer develops in conjunction with genomic instability and multiple genetic mutations, but how these events prompt CSCs´s develop is still unclear [25]. In our study, cytogenetic assays carried out for both colon and melanoma cancer cell lines displayed that CSCs subpopulations enriched with CM presented alterations that were not present in the attached control ones. Specifically, an alteration in chromosome 17 (17q25) was common for both CSCs CM-selected tumor cell lines. Curiously, this area

constitutes a hot spot for chromosomal alterations in pluripotent stem cells [26, 27, 28]. Furthermore, this region locates also the *BIRC5* gene [28] that codes for survivin, a member of inhibitor apoptosis family. *Survivin* is highly expressed in most human tumours, but is completely absent in terminally differentiated cells [29] and it has been shown that the disruption of *survivin* induction pathways leads to increase in apoptosis and decrease in tumour growth. Moreover, it has been demonstrated that human embryonic stem cells (ESCs) with trisomy 17 generate more aggressive teratomas than diploid ESCs [30].

In addition, microRNA and gene profile assays carried out by chromosomal bands analyzes showed that CM selects chromosomal mutations that seems to affect pathways involved in CSCs phenotype. Among the 10 KEGG pathways that are most significantly enriched in target genes of the 4 analyzed microRNAs sequences, there are many relevant signaling pathways such as Wnt and PI3K-Akt. Especially the Wnt/β-catenin pathway is known to play an important role in self renewal of stem-cells [31] and whose de-regulation is associated with putative CSCs [32]. Interestingly, the target genes do not seem to be distributed randomly over the pathway, but i) apparently they do accumulate rather upstream in the pathway and ii) some important negative regulators of Wnt signaling are targets. For example, decreased expression of *CXXC4* has been shown to activate Wnt in renal cell carcinoma [33]. Furthermore, *NKD1* is a known Wnt antagonist able to suppress canonical Wnt signaling [34]. The analysis of the genes located within 17q25 showed that CM also induced the selection of cells with a modulated expression of *GRB2* and *TBC1D16*, two important genes involved in EGF signaling pathway that regulates colon and melanoma CSCs proliferation and apoptosis [35, 36, 37]. Besides, changes in genes involved in epithelial-mesenchymal transition,

invasion, metastasis and cell migration, and stem cell activation and differentiation pathways were detected. All these genes are related to the CSCs phenotype [38, 39]. Many of these genes interact at a protein level with key genes for stemness properties like *MYC* and *KLF4*. Hence, some of the genes located in 17q25 seem to be crucial in several stem-cell related pathways by interacting at a protein level with important genes. Finally, the secretome was analyzed in CM obtained form MSCs since it is known that these stromal cells secrete cytokines and/or growth factors that favors tumor growth rate, angiogenesis and metastasis [10, 11]. As result, we show that the addition of both IL-6 and HGF increases the stemness enrichment potential of conventional SM, implying an effective and cost-less procedure to obtain enriched CSCs subpopulations from tumor cell lines. The tumorigenic capacity of these two factors have been extensively described. For instance, IL-6 has been shown to induce non-cancer stem cells to express markers of CSCs, increase the ability to form *in vivo* tumors [40], and participates in carcinogenesis, metastasis through epithelial-mesenchymal transition, chemoresistance, epigenetic regulation and miRs regulation in cancer [41, 42]. Recently, we have demonstrated that the interaction between stromal cells and cancer cells increases the secretion of pro-inflammatory cytokines, including IL-6, and generates tumor-driving loops that promote CSCs expansion and metastatic progression [43]. Additionally, HGF overexpression encourage tumor growth, invasion and metastasis [44] in a pattern related with the Wnt pathway [45]. Currently, several therapeutic strategies are being tested in patients targeting the HGF pathway, such as humanized antibodies anti-HGF or against its receptor tyrosine kinase, cMET. Phase I to Phase III clinical trials in solid tumours have shown few adverse effects and dose-limiting toxicities in comparison to small-molecule inhibitors. Also, both improved objective response rate and median progression-free survival have been reported [46].

In summary, our results evidence that growth factors and cytokines secreted by MSCs can induce the enrichment of tumor subpopulations with CSCs-like phenotype, behavior and properties. Moreover, we show for the first time that MCSs-secretome selects cells subpopulations with specific cytogenetic profile, mainly an alteration on chromosome 17 (17q25), which might be already present in the original culture and selected for due to the CM and seems to be crucial in several stemness related pathways. Further studies are necessaries to stablish the importance of this chromosome alteration in CSCs and its translation to patients. Finally, this study provide a novel cytokine combination composed by IL6 and HGF that allows the culture of CSCs subpopulations during long time periods, improving the conventional methodology for enrichment and CSCs culture. The novel cell culture medium described in this work can be applied in different sectors, (i) from basic science for drug screening and CSCs biomarkers, (ii) to clinical applications such as *in vitro* diagnostic medical device that allows the culture of CSCs from the patients for subsequent analysis and to detect possible recurrence or diagnostic markers, and iii) for implement a personalized medicine. This in turn is the crucial first step for biological and clinical studies with the goal to establish new strategies against CSCs survival.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Economy and Competitiveness, Instituto de Salud Carlos III (FEDER funds, projects nº. PIE16/00045 and DTS17/00087), from the Ministerio de Economía y Competitividad (MINECO, FEDER funds, grant number MAT2015-62644.C2.2.R), from the Fundación Pública Andaluza Progreso y Salud, Consejería de Salud Junta de Andalucía in collaboration with JANNSSEN CILAG, S.A. (project numbers PI-0533-2014), and from the Chair

"Doctors Galera-Requena in cancer stem cell research". The authors also gratefully thank Jaime Lazuén and Gustavo Ortiz from the C.I.C. (University of Granada) for excellent technical assistance with flow cytometry and FACS studies. Also, we want to acknowledgement to Dr. Jose Luis García-Pérez for his contribution to the final manuscript.

CONFLICT OF INTEREST

None of the authors have a conflict of interest to declare.

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704 **FIGURE LEGENDS**

705 **Fig. 1. Schematic representation of the steps followed to obtain the conditioned**
706 **medium and workflow of culture conditions.** (A) Conditioned medium (CM) was
707 obtained from MSCs expanded with conventional sphere medium (SM). (B) Workflow
708 of culture conditions: (B1) attached cells cultured with complete medium
709 (HCT116/A375), SM, transwell (T) or CM. (B2) Non-sorter cells cultured in suspension
710 with SM, T, or CM. (B3) ALDH1-sorter cells (positive S^+/negative S^- subpopulations)
711 cultured in suspension with SM, T, or CM.

712

713 **Fig. 2. Phenotypic properties and tumoro sphere-forming ability of colon and**
714 **melanoma CSCs.** Fold increase of ALDH1 activity and CD44 and CD326 expression
715 of cells cultured in monolayer (attached cells) or in suspension with sphere medium
716 (SM) or conditionated medium (CM) relative to attached cells supplemented with serum
717 medium (value 1). Representative images and quantification of number and size of
718 tumorospheres formed in HCT116 cell line during culture with SM or CM (A), A375
719 cells (B) and primary cells of human colon adenocarcinoma at low passage (Patient 3)
720 (C). Statistical significance indicated *($p < 0.05$) or **($p < 0.01$) when cells cultured in
721 suspension with SM were compared with cells cultured with CM, and when cells
722 cultured in monolayer with CM were compared with conventional medium. Scale bar:
723 100µm.

724

725 **Fig. 3. Proliferation assay in CSCs enriched subpopulations.** Proliferation rates of
726 no-sorter (NS), sorter positive (S+) and sorter negative (S-) cells growing in suspension
727 culture with SM or CM. Statistical significance indicated *($p < 0.05$) or **($p < 0.01$).

728

Fig. 4. *In vivo* analysis of tumorigenic capacity of colon and melanoma CSCs selected by CM. (A) Tumor development for *in vivo* limiting dilution assay. Mean tumor volume over time following injection of 1000, 100 and 10 cells that were previously cultured with SM and CM, (graphed as mean ± SD (n = 6)), number of tumor generated by each condition, and representative images of tumors. (B) Fold increase of ALDH1 activity and CD44 and CD326 expression in primary cell culture of isolated tumors generated by HCT116 and A375 cell lines. Values were normalized with tumors induced by control cells cultured in monolayer with complete medium (CTL). Statistical significance indicated **($p < 0.01$). (C) Representative panel of immunohistochemistry for OCT4 and SOX2 expression. Expression of both proteins in paraffin sections of xenograft tumors developed in NSG mice 38 days after tumor cells injection of A375 and HCT116 cell lines and their respective CSCs enriched subpopulations (NS CM, S+CM and S-CM) (n=6). Sections (n=6) were counterstained with hematoxylin (blue nuclei). Original magnification: 20x. (D) Histogram showing quantification of positive OCT4 or SOX2 staining results. Statistical significance indicated *($p < 0.05$) or **($p < 0.01$).

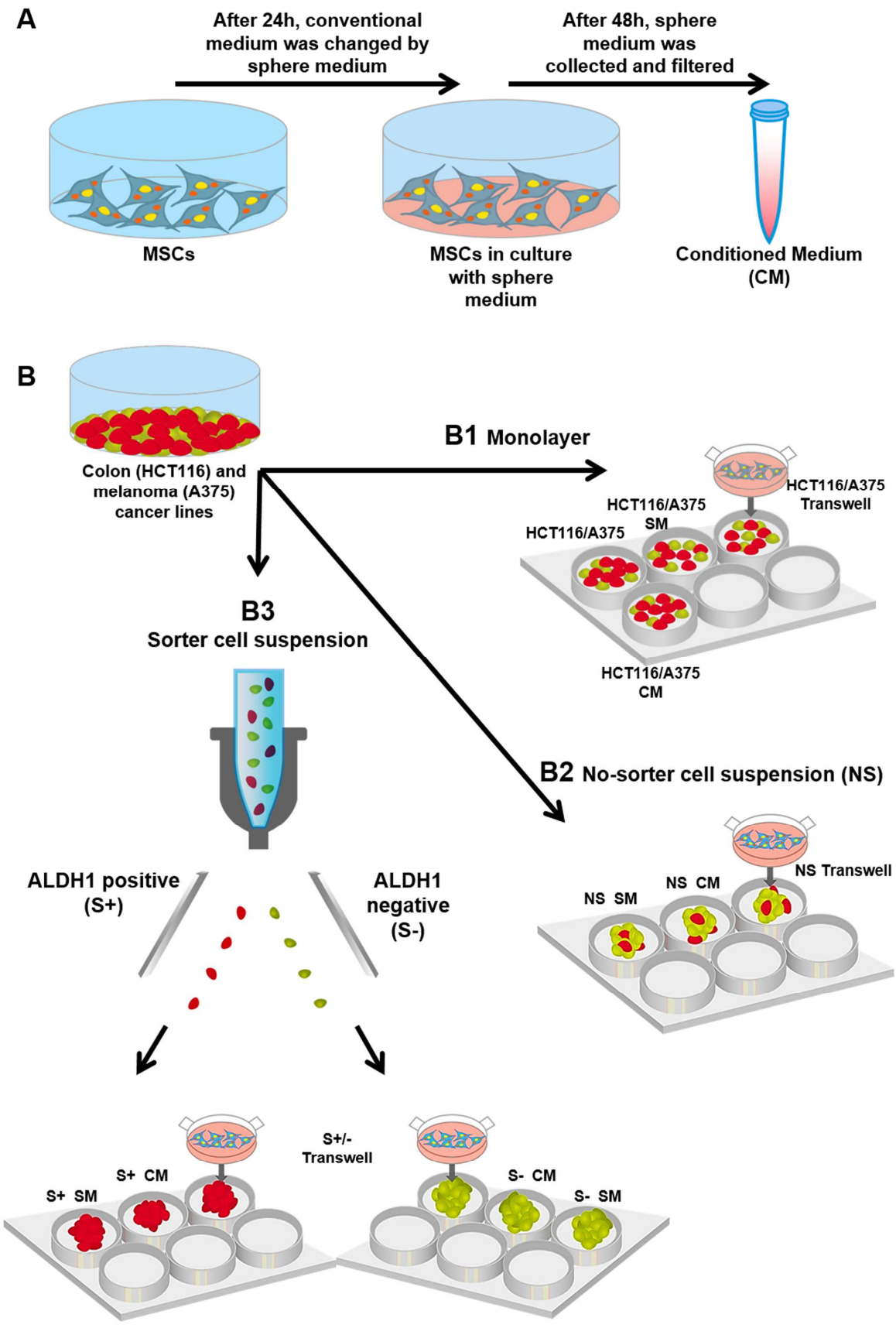
how many tumors did they analyze and how many tumor sections did they stain.

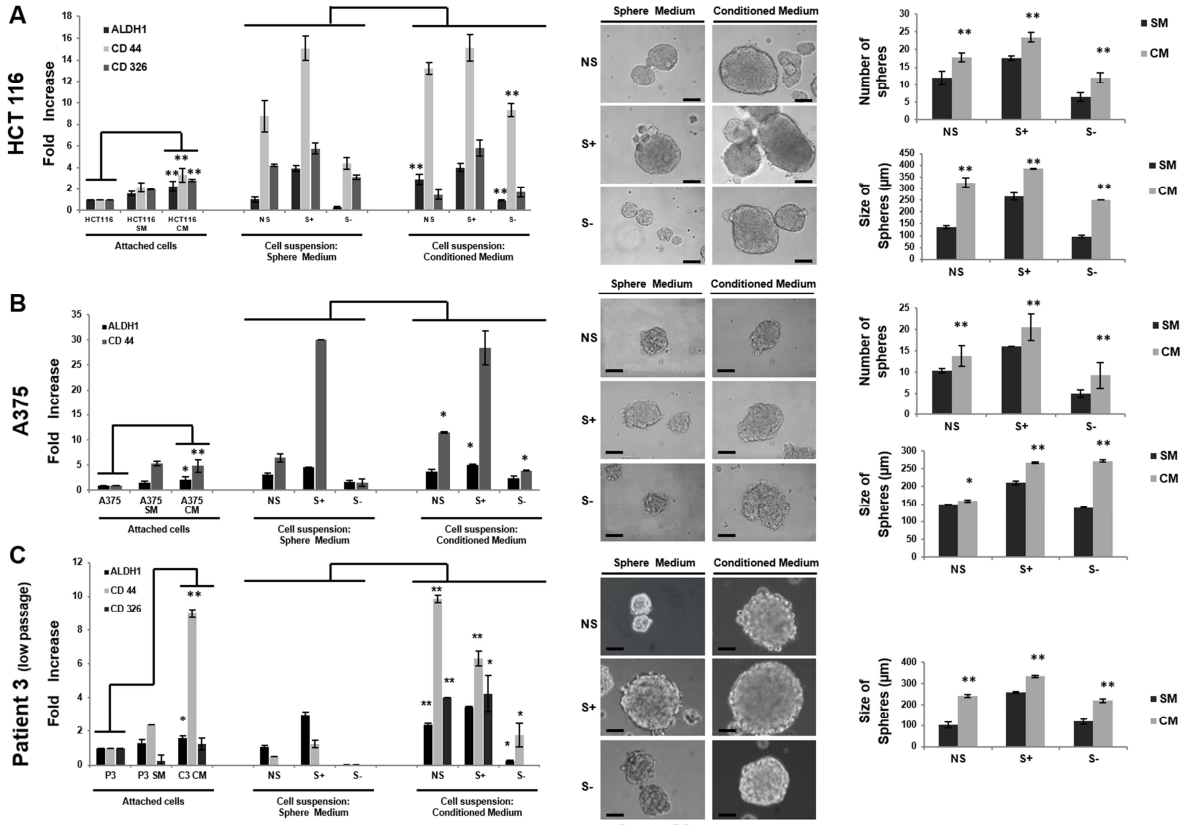
Fig. 5. G-banding stemline and Spectral karyotyping. (A) G-banding stemline of HCT116 cell line shows stemline chromosome number is near diploid with the modal number at 45 (62%) and polyploids occurring at 6.8%. The A375 cell line is a hypotriploid with a modal number of 62 chromosomes. There are 8 chromosomes markers that are commonly found in each cell, and normal N2, N6, and N22 are present at one copy per cell. That new chromosomal alterations appeared, increasing the genetic instability in both cell lines as the cell culture advances in different culture conditions.

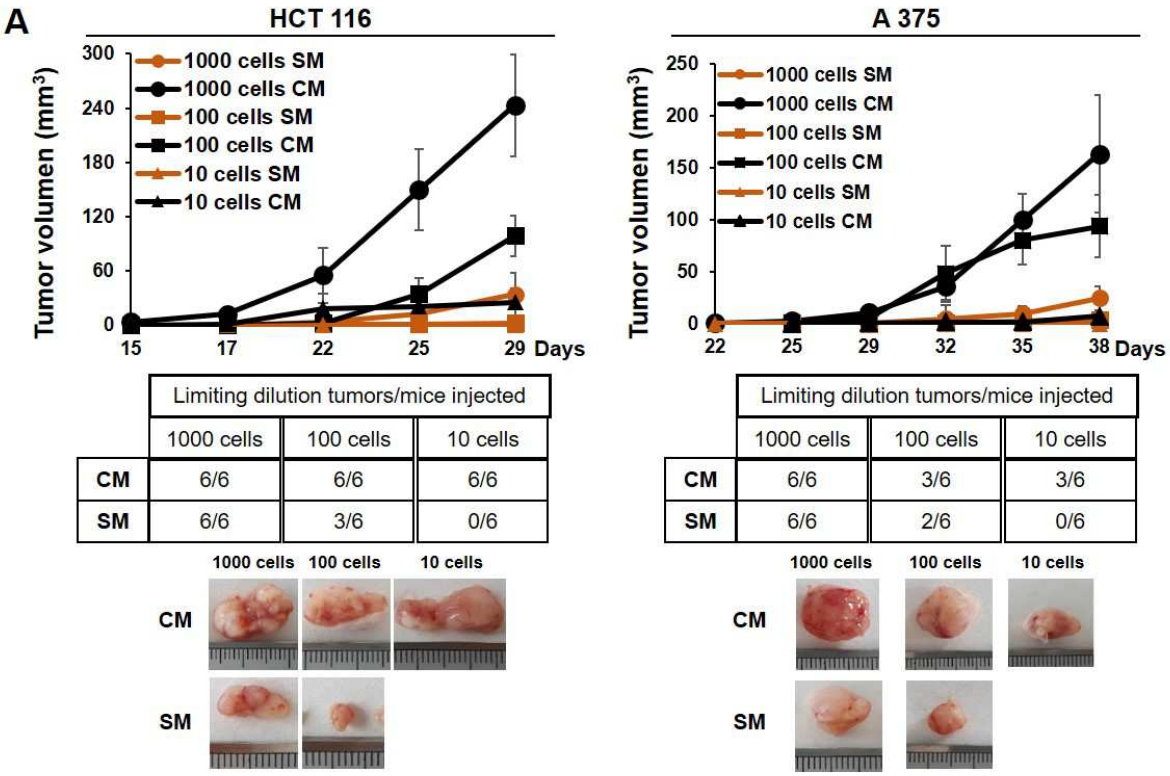
(B) Spectral karyotyping (SKY) shows the ploidy and complex genomic rearrangements among related HCT116 and A375 cell lines.

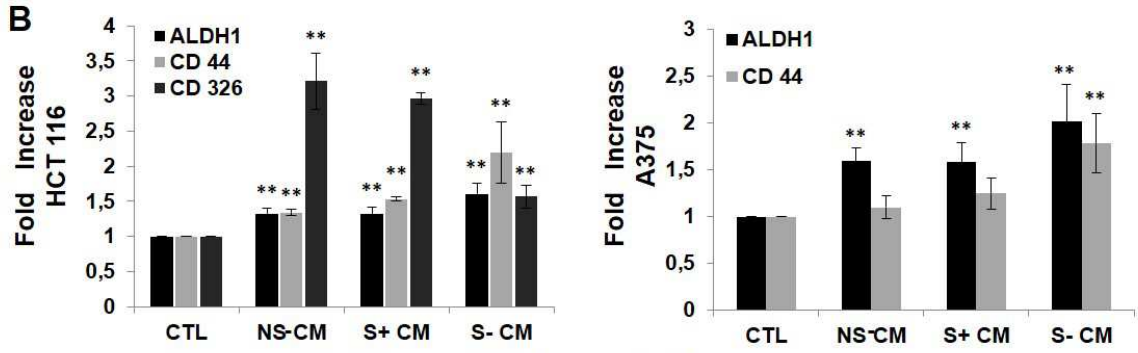
Fig. 6. Functional *in-silico* analysis of genes and microRNAs in 17q25. (A) The 10 KEGG pathways with most significant enrichment of hsa-mir-636 and hsa-mir-657/hsa-mir-338 target genes. (B) Wnt-signaling pathway and target genes: those genes regulated by one microRNA are marked in yellow, those with more than one in orange. The genes that are not regulated by one of the 4 microRNA sequences are shown in green. (C) A graphical representation of 17q25 showing the localization of the microRNAs (top) and genes (bottom). (D) The protein interaction network of 5 important genes located in 17q25 (marked with a red rectangle). The different colours mean different evidence for the physical interaction: turquoise lines (curated databases), pink lines (experimental evidence), and yellow (textmining).

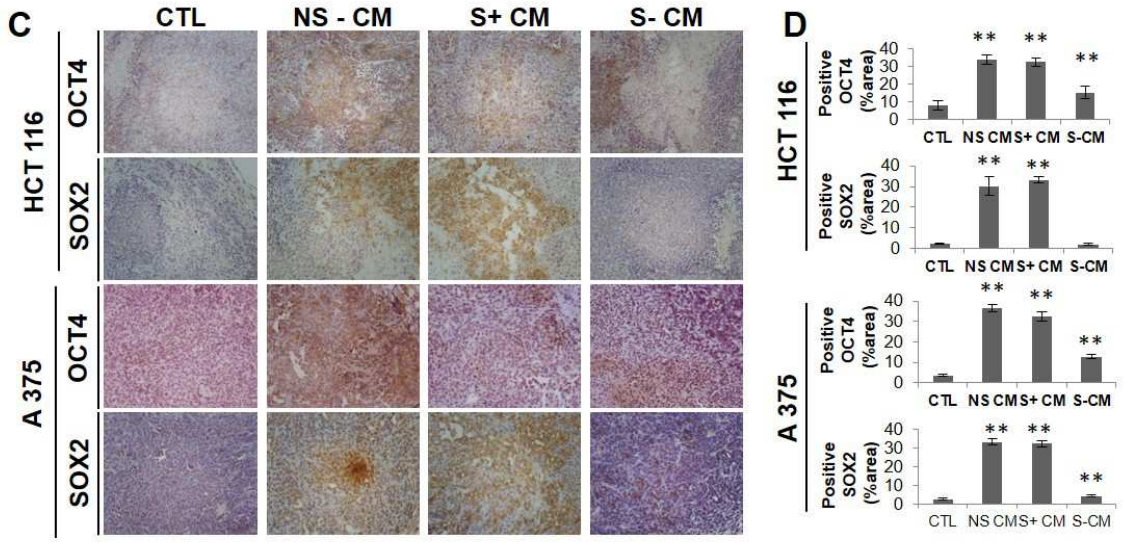
Fig. 7. Analysis of the enhancement in CSCs properties by concentrated conditioned medium (CCM) and secretome-selected cytokines and growth factors. (A) Fold increase of ALDH1 activity, CD44 and CD326 expression in HCT116 cell line. Values were normalized with SM cultured cells. Statistical significance indicated *($p < 0.05$) or **($p < 0.01$) when CM was compared with SM, and #($p < 0.05$) or ##($p < 0.01$) when 100% CCM was compared with CM. (B) Fold increase of ALDH1 activity in cells cultured with cytokines and growth factors, and (C) the potential combinations of these molecules selected by multiplex assay. Values were normalized and compared with SM cultured cells. Statistical significance indicated *, ($p < 0.05$) or **($p < 0.01$).

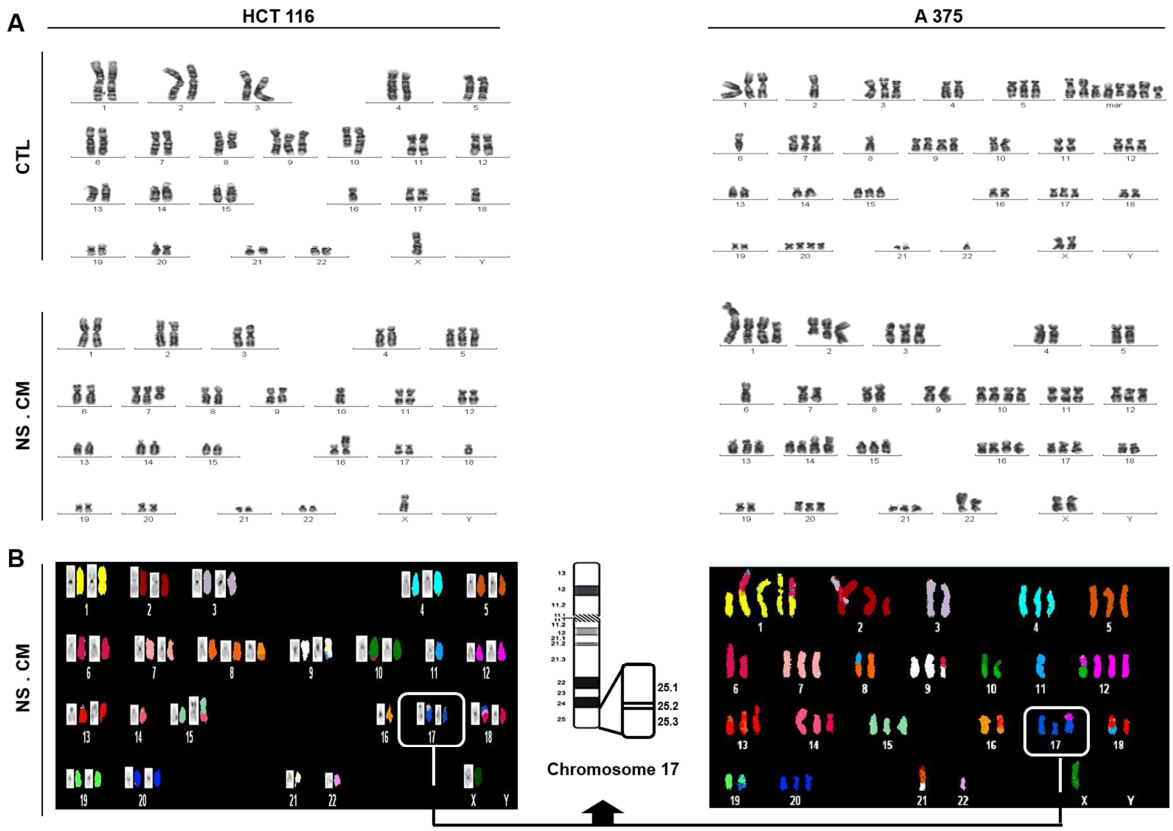

A
After 24h, conventional medium was changed by sphere medium
After 48h, sphere medium was collected and filtered
MSCs
MSCs in culture with sphere medium
Conditioned Medium (CM)
B
Colon (HCT116) and melanoma (A375) cancer lines
B1 Monolayer
HCT116/A375 Transwell
HCT116/A375 SM
HCT116/A375
HCT116/A375 CM
B3 Sorter cell suspension
B2 No-sorter cell suspension (NS)
ALDH1 positive (S+)
ALDH1 negative (S-)
NS Transwell
NS CM
NS SM
S+/- Transwell
S+ CM
S+ SM
S- CM
S- SM




A
HCT 116
A 375
Tumor volumen (mm3)
1000 cells SM
1000 cells CM
100 cells SM
100 cells CM
10 cells SM
10 cells CM
Days
Limiting dilution tumors/mice injected
1000 cells
100 cells
10 cells
CM
SM
6/6
6/6
6/6
6/6
3/6
0/6
6/6
3/6
3/6
6/6
2/6
0/6


B
Fold Increase HCT 116
Fold Increase A375
ALDH1
CD 44
CD 326
CTL
NS-CM
S+ CM
S- CM

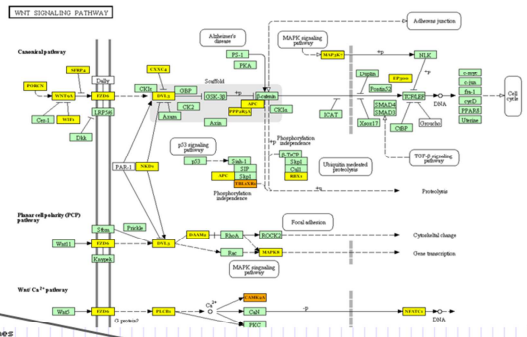

C
D
CTL
NS - CM
S+ CM
S- CM
HCT 116
A 375
OCT4
SOX2
Positive OCT4 (%area)
Positive SOX2 (%area)



A

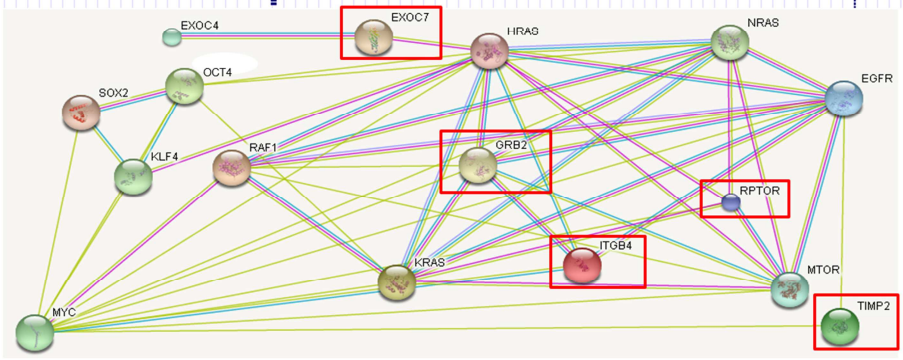
#	KEGG pathway	p-value	#genes	#miRNAs
1.	MAPK signaling pathway (hsa04010)	2.822153e-08	37 see genes	4
2.	RNA degradation (hsa03018)	1.138142e-06	15 see genes	3
3.	Protein processing in endoplasmic reticulum (hsa04141)	3.195106e-06	23 see genes	4
4.	Wnt signaling pathway (hsa04310)	1.003255e-05	23 see genes	4
5.	Adipocytokine signaling pathway (hsa04920)	3.031834e-05	13 see genes	4
6.	Long-term potentiation (hsa04720)	0.0002117702	12 see genes	4
7.	Transcriptional misregulation in cancer (hsa05202)	0.0002827774	25 see genes	4
8.	Dopaminergic synapse (hsa04728)	0.0003029792	19 see genes	4
9.	Pathways in cancer (hsa05200)	0.000545969	39 see genes	4
10.	PI3K-Akt signaling pathway (hsa04151)	0.0006910381	36 see genes	4

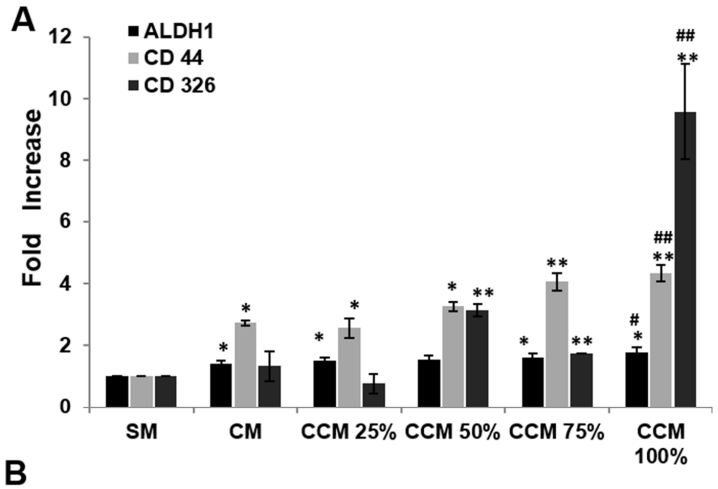
B

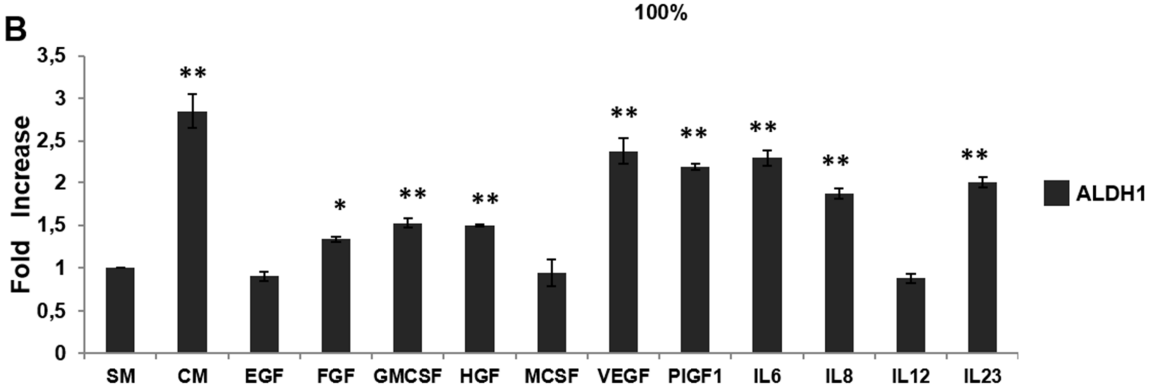


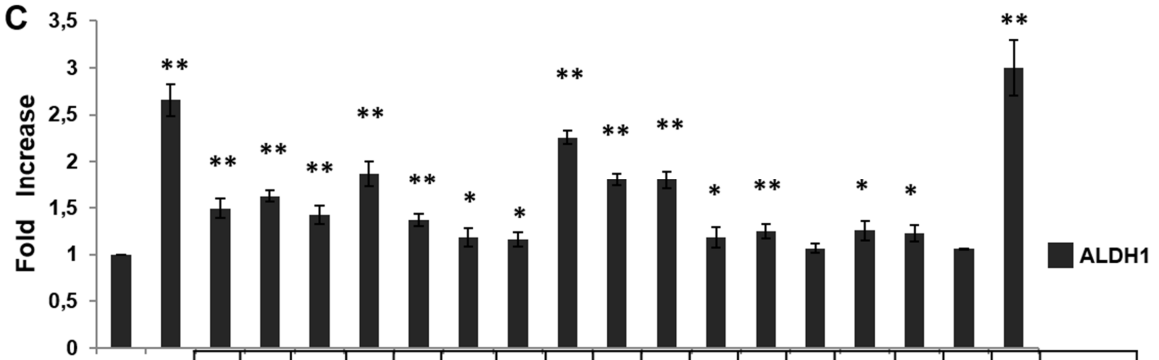
C

D

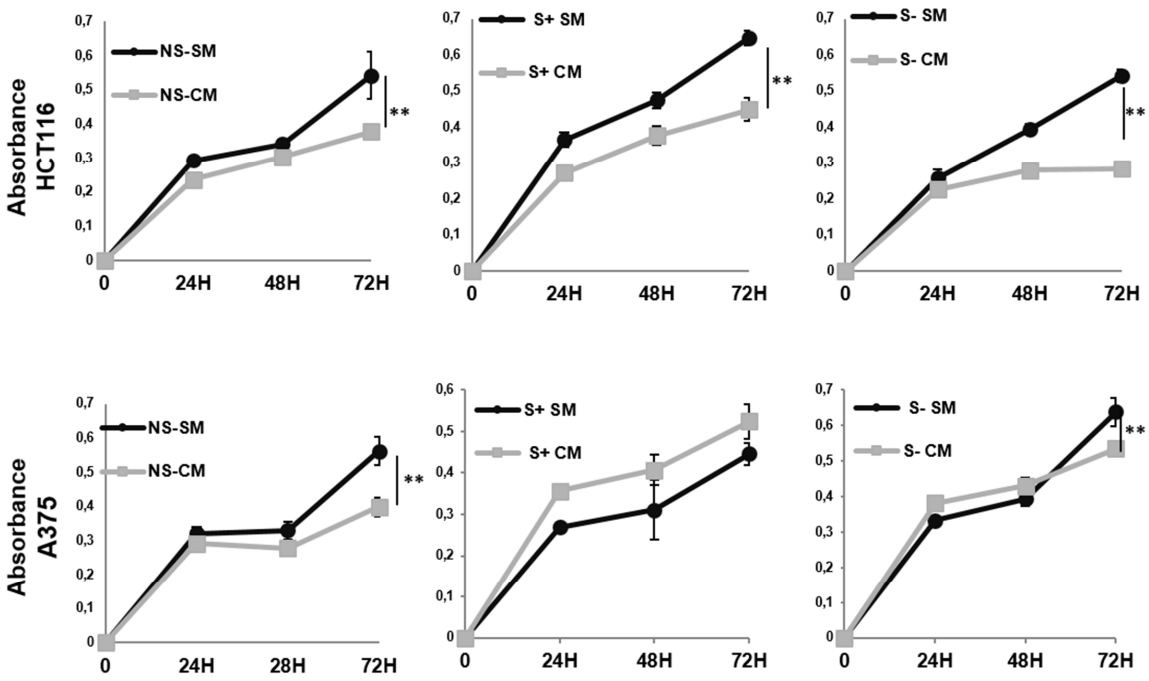








	+		+												+	EGF
	+		+												+	FGF
+	+			+			+			+			+			+	IL6
+	+				+			+			+			+		+	IL8
+	+															+	IL12
+	+					+			+			+			+	+	IL23
		+	+	+	+	+										+	GMCSF
		+	+													+	MCSF
		+	+				+	+	+							+	HGF
		+	+							+	+	+				+	PIGF1
		+	+										+	+	+	+	VEGF



Highlights

- MSCs secretome select and maintain cells with CSCs-like phenotype and behavior
- Cells cultured with MSCs secretome retain *in vivo* CSC-like characteristics
- MSCs secretome selects cells with cytogenetic alteration on chromosome 17 (17q25)
- The combination of IL6 and HGF improves the CSCs enrichment