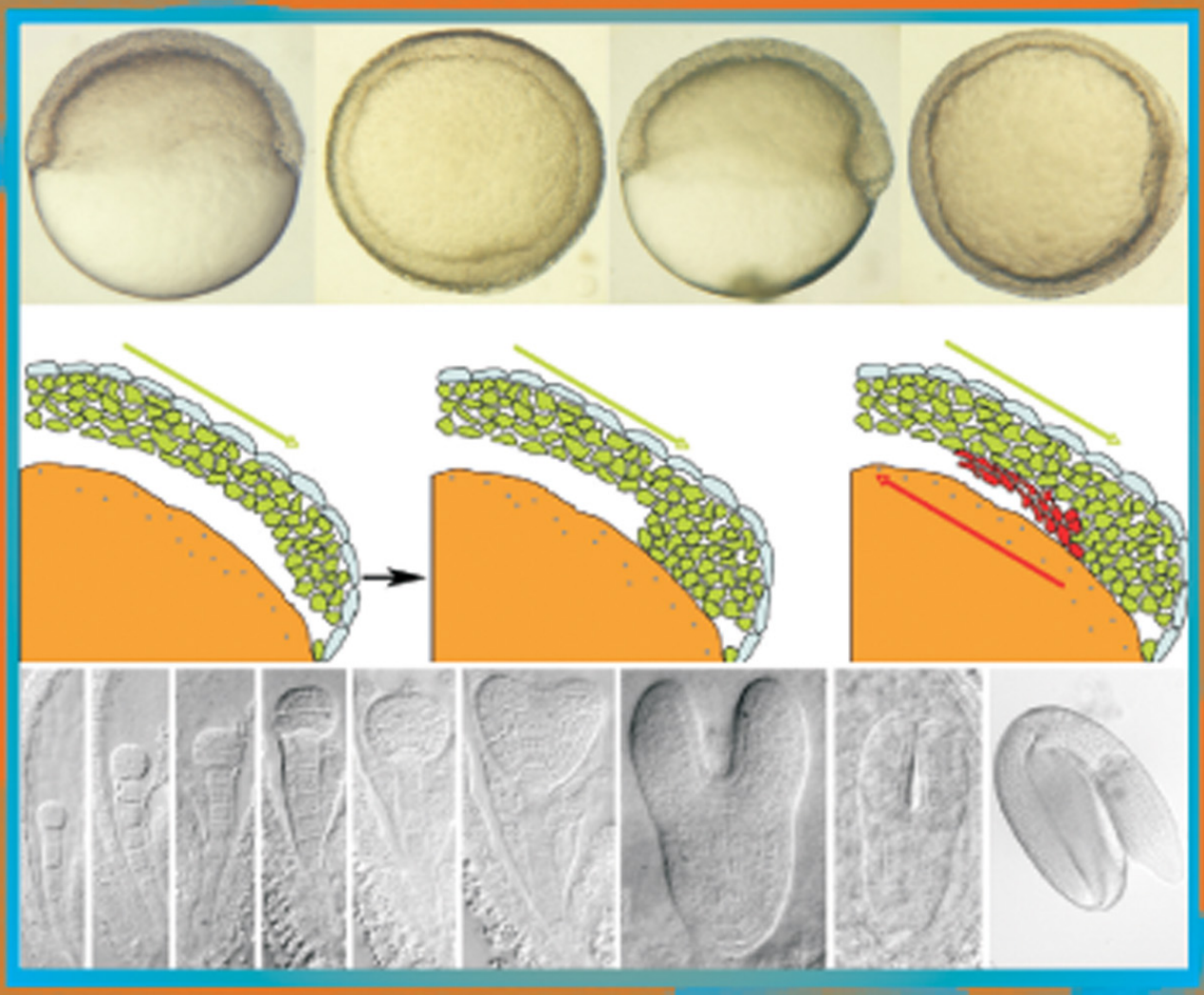
Topics in Animal and Plant Development: From Cell Differentiation to Morphogenesis

Editor
Jesús Chimal-Monroy



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Topics in Animal and Plant Development: From Cell Differentiation to Morphogenesis

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Editorial

One major challenge of developmental biology is to explain how multicellular organisms arise from the fertilized egg. During embryonic development, the egg divides to give rise to all the cells of the organism; cell differentiation involves molecular mechanisms through which a cell is able to choose its fate. Also, cells are not randomly organized in the embryo; on the contrary they are organized into complex tissues and organs. In many animal species when an egg begins to divide itself there are cytoplasmic determinants that are segregated and are able to direct cell fate. Rosa Navarro and colleagues show an example of segregation of cytoplasmic determinants, the determination of germline lineage in *C. elegans*, *Drosophila* and zebrafish. In these organisms in addition to cytoplasmic determinants there are germ granules that are involved in germline formation and embryogenesis. Juan Antonio Montero and Nuria Torre-Pérez illustrate the process of gastrulation in the zebrafish embryo. Once zygote divides, it forms a mass of cells that at specific time in development begin the first morphogenetic movements to establishment the body plan, this process is called gastrulation. Martín García-Castro describes the early development of neural crest cells (NCC) lineage, their origin and their differentiation potential. One of the most studied systems of cell differentiation is the determination of the NCC lineage. These cells contribute to the formation of the peripheral nervous system, cells of the craniofacial skeletal system such as bone and cartilage. Furthermore NCC contribute to tooth forming cells, pigmented cells, muscle, and endocrine cells. Horacio Merchant-Larios and Verónica Díaz-Hernández explain gonadal development; they show an integrated conception of classic concepts with recent results to explain gonadal determination and morphogenesis. Jesús Chimal-Monroy and colleagues depict limb formation. This complex structure represents an excellent model to understand the basic concepts of cell differentiation, cell death, morphogenesis and evolution. Jean-Phillipe Vielle-Calzada and colleagues approach to the genetic basis and molecular mechanisms that regulate female gametogenesis and early embryo formation in flowering plants. *Arabidopsis thaliana* and *Zea mays* are two excellent model systems to explain plant development. Elena Álvarez-Byulla and colleagues illustrate the role of MADS-box genes as transcriptional regulators of plants to elucidate how developmental decisions are taken and how these integrate multiple signal transduction pathways, as well as the relationship between molecular and morphological evolution in plants. During development after

several cell divisions there is restriction about the potency of the cells giving rise to specialized cells that will form adult tissues. However, when damage occurs many cells are unable to replace or regenerate the lost tissue, but some tissues have cells with the ability to do it. These cells are called stem cells and are maintained in specific locations called stem cell niches. Acaimo González-Reyes and colleagues describe the importance of stem cell niches in the control of maintenance of self-renewal of stem cells whereas Iván Velasco and Héctor Mayani illustrate some basic aspects of biology of stem cells that might make them competent for possible therapeutic applications. Finally Félix Recillas-Targa and Mayra Furlan-Margaril show the importance that epigenetic has at numerous processes during organism development as cellular plasticity, imprinting, differentiation, self-renewal, ageing and its abnormal counterparts.

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1. Germ granules: Vehicles for germ cell determination

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Abstract. Two main pathways for germ cell formation are currently accepted. In the first pathway, the preformation model, the germline is established by germ cell determinants inherited by the oocytes. In the second pathway, the epigenetic or inductive model, germ cells are established by inductive signals coming from surrounding tissues. Most animals, including mammals, use the inductive model to generate their germline. Despite the pathway that is used to specify the germline, it is clear that many proteins and mechanisms are conserved in several organisms. Most of our knowledge about germ cell preservation comes from model organisms that use the preformation model like *C. elegans*, *Drosophila* and zebrafish (among others). In these organisms, germ cell determinants are accumulated in the cytoplasm and in some

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electrodense granules commonly denominated germ granules. These granules aggregate RNAs and proteins important for the formation of the germline and embryogenesis. In this review, we will describe the components of germ plasm and its germ granules and their roles in germ cell development and embryogenesis.

Introduction

A key still unsolved question in developmental biology is how cell fate is established during early embryogenesis, and one of the best systems to study this is the germline. Two forms of germline segregation are clearly documented. Organisms recurring to the "preformation" form develop their germline through the localization and maintenance of maternally provided determinants before, or immediately after, fertilization. In the **"**epigenesis or inductive" form, germ cells arise later in development through a series of inductive signals from surrounding tissues [1].

Germ cells from many organisms possess specialized cytoplasm composed of electrodense granules that lack membrane and contain RNAs and proteins (RNPs) generally known as germ granules. Germ granules are essential for germline preservation and function and have been invoked for decades as germline determinants. In animals where the germline is preformed, germ granules are present continuously in germ cells (with the exception of mature sperm) and are inherited maternally with the germ plasm. As early as 1910, Theodor Boveri showed that in Ascaris, a localized region of polar plasm, the germ plasm, determines which cells become germline precursors [2].

Historically, germ granules have been called by a variety of names reflecting their different morphology at different developmental stages and in different organisms. For example in *Drosophila*, they are called polar granules; in *C. elegans* they are called P granules, germ granules in zebrafish, mitochondrial cloud in *Xenopus* oocytes, and chromatoid body in mammalian spermatocytes [3]. However, the morphological similarity between these structures suggests that they represent a common component present in germ cells throughout the life cycle. The ubiquity of such germline elements has led to suggestions that they serve a role in determination, identification, and differentiation of germ cells [4].

In this chapter, we will review germ cell development in the preformation model during the development of two invertebrates: *C. elegans* and *Drosophila*, where a fair amount of information has been documented. We will also review the preformation model in zebrafish, a new emerging vertebrate model that occupies a strategic position because it links invertebrates with higher organisms. For each model, we will review germ cell development; we will also describe germ cell determinants and their

possible function. Finally, we will compare the similarities and differences in germ cell development between these model organisms.

Germ cell determinants in *C. elegans*

Germline development

C. elegans animals can exist as hermaphrodites or males. In the hermaphrodite gonad, oocytes enter the spermatheca where they are fertilized and squeezed into the uterus. The position of entry of the spermatozoid into the oocyte determines the posterior part of the embryo. After fertilization, maternal provided mRNAs and proteins are redistributed differentially along the anterior-posterior axis before the first asymmetric division occurs. The bigger anterior blastomere is known as AB while the posterior is P1 (Fig. 1A). RNPs containing germline determinants, known in *C. elegans* as P granules, are segregated to the posterior side of the zygote before the first embryonic division. P1 divides asymmetrically to give rise to EMS and the germline blastomere P2; where P granules are accumulated. P granules are segregated later to germ cell precursors in two more cell divisions that will give rise to blastomeres P3 and P4. The P4 blastomere is known as the primordial germ cell (PGC) and divides symmetrically, in the 100-cell embryo, to create the PGCs Z2 and Z3 (Fig. 1A).

L1 larvae hatch with a gonad primordium formed by cells Z1 and Z4, which will form the somatic gonad and the PGCs Z3 and Z4 (Fig. 1B). During larval stages L1 through early L3, germ cells proliferate under the influence of the distal tip cells (DTC) from the somatic gonad, to form the adult hermaphrodite gonad, which has two identical tubes that are bound at the uterus (Fig. 1B). At the late L3 larval stage, the first 40 germ cells that move away from the influence of the DTC, in each gonad arm, will enter meiosis and will produce approximately 160 spermatozoids. After the L4 larval stage, hermaphrodites will produce exclusively oocytes for the rest of their reproductive life. Males, which only possess one gonad arm, initiate sperm production at the L4 larval stage continuing throughout adult life [5, 6].

In this section, we will review several aspects of P granules; the determinants that form the *C. elegans* germline, their function and their mechanism of action. Other aspects of germ cell biology can be found elsewhere [3, 7].

P granule definition and structure

In contrast to *Drosophila* where the germ plasm is polarized during oogenesis, in *C. elegans*, as we just described, the germline is established

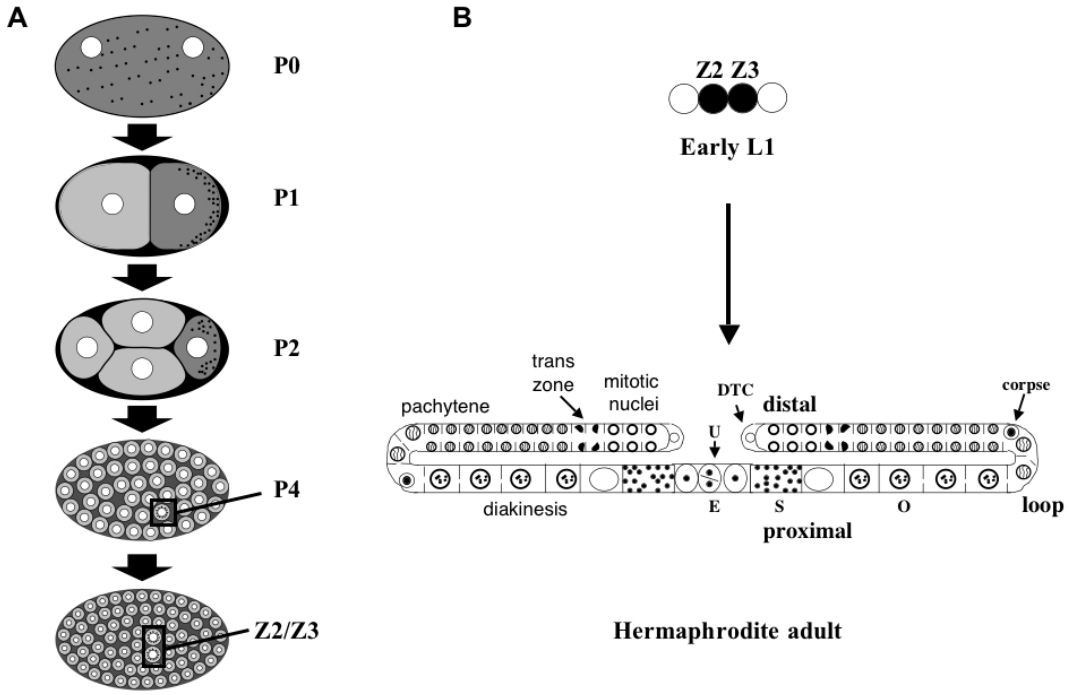

Figure 1. Cartoon representation of germ cell development in *C. elegans*. A) P granules (black dots) are contributed by the oocyte and are equally distributed in the cytoplasm. After fertilization, P granules segregate to the embryo posterior before the first embryonic division occurs. P granules are inherited to the P cells after few more divisions. The PGC P4 divides equally one more time to form the germ cell precursors Z2 and Z3. B) Newly hatched L1 larvae have four cells that will proliferate and differentiate to form the adult gonad. Z1 and Z4 (white circles) will give rise to the somatic gonad while Z2 and Z3 (dark circles) will form the germ cells. C) The hermaphrodite gonad consists of two U-shaped tubes that are bound by the uterus (U). At the distal end of each gonad arm, the somatically derived distal tip cell (DTC) maintains a self-renewing mitotic stem cell population through direct contact. The first forty germ cells that enter meiosis form 160 sperm (S); afterwards only oocytes (O) are produced. Cellularization occurs in the gonad loop, as germ cells progress from the pachytene to the diakinesis stages. Fertilization occurs in the spermatheca (S) while eggs leave through the proximal gonad end. Embryos (E) are transiently stored in the uterus (U).

during early embryogenesis after a series of asymmetrical divisions. Germ granules in *C. elegans* were first detected by two different methods; immunofluorescence [4] and by electron microscopy [8]. Because germ granules associate with P cells during *C. elegans* embryogenesis they were named P granules. These granules are present constitutively throughout *C. elegans* life cycle, and are dynamic structures. During germ cells development, P granules change in size and distribution. In oocytes and one- to four-cell embryos, they are numerous, small, and randomly distributed throughout the

cytoplasm (Fig. 2). In older embryos, fewer and larger granules are located around the nucleus, where they remain until oogenesis [4]. During embryogenesis, localization of P granules is achieved primarily through their directed movement toward the germline daughter cell, and their disassembly or degradation in the cytoplasm of somatic daughter blastomeres [9].

P granules' shape is irregular, ellipsoidal and variable in size. Some P granules have flat surfaces facing the nucleus while others are arch shaped, contacting the nuclear surface at one point and extend into cytoplasm before contacting the nucleus again. At the distal part of the gonad, P granules appear smaller while in the proximal area they seem bigger suggesting that the amount of P granule material around the nuclei increases with time [10].

P granules are associated directly with nuclear pore clusters in most of the gonad. During oogenesis, P granules detached from the nucleus and nuclear pore components associate with them in the cytoplasm. These observations suggest that P granules might bind to, or assemble on, nuclear pores [10]. The close association of P granules and nuclear pores suggests a relationship between RNA synthesis and RNA loading into these structures. Nuclear pore assembled P granules appear under electron microscopy more dense than cytoplasmic granules possibly due to material that they are receiving from the nucleus [10].

Mitochondria are also thought to be closely associated with germ line granules in several animals. In amphibian oocytes, mitochondria are frequently in direct contact with, or even embedded in, the germ granules [11]. In *Drosophila*, polar granules are attached to mitochondria in oocytes and embryos, and polar granule components like Tudor have been found in mitochondria [12]. However, it is not clear whether P granules and mitochondria associate in *C. elegans* [10].

Association between cytoplasmic P granules and centrosomes or microtubules is not clear in early embryos [8] [13, 14]. Nonetheless, microtubules are important for P granule localization during early embryogenesis [9] and electron microscopy studies show that a substantial fraction of P granules in adult gonads contained or contact microtubules [10]. In nuclear associated P granules, centrioles appeared to be embedded in, or adjacent to, P granules in a few cases [10]. Using a GFP reporter attached to the P granule component PIE-1, it was observed that during P blastomeres division this protein binds preferentially to the centrosome of the future germ cell by an unknown mechanism [15].

P granules contain RNAs

Using probes to detect poly(A) mRNAs and the SL1 leader (a 21-23 bases transcribed sequence added to 5´ mRNAs during edition), it was

observed that P granules contain a great proportion of total mRNA during embryogenesis [10, 16]. Another evidence that P granules contain RNA is that these structures stain positively for the nucleic acid dye Syto-14 [17].

Nuclei in the *C. elegans* gonad are partially surrounded by membranes and share their cytoplasm forming a syncitium (Figure 1B). The major level of transcription in the gonad occurs at pachytene nuclei. In gonads of animals treated with [3H]uridine, mRNA appeared rapidly and sequentially in germ cell nuclei, the cytoplasm and then the central core of the gonad. Low labeling was detected at the cytoplasm or nuclei of maturing oocytes. The perinuclear zone, including P granules, showed large amounts of labeling suggesting that at least some newly synthesized RNAs are associated with P granules [17].

Few specific mRNAs have been shown to associate to P granules. The first mRNA detected in P granules was *nos-2* [18], which is also present throughout the cytoplasm of the early embryonic blastomere P3. Five more mRNAs encoding developmental related proteins were later found to associate with P granules *mex-1*, which is translated later during oogenesis; *pos-1*, *skn-1* and *par-3*, which are translated in early embryogenesis. *pos-1*, *mex-1*, *nos-2* and *gld-1* genes encode for proteins required for germline development, *skn-1* is required for somatic development, and *par-3* whose function determines cell polarity [17]. Several mRNAs are rapidly degraded in the soma during early embryogenesis but they are protected from degradation in the germline [16].

mRNAs association to P granules appear to be specific and not every mRNA can associate with them, as an example we have the actin or ß-tubulin mRNA [17]. 5S, 5.8S, 18S or 26S ribosomal RNAs are barely observed in P granules [17]. These data suggest that despite many components of the translational machinery can be found in P granules (see below), actual translation is not likely to be occurring in these structures.

Several proteins associate specifically with P granules

The DEAD box RNA helicases GLH-1 and GLH-2 were the first proteins detected in P granules [19]. These proteins are highly similar to VASA, a polar granule component from *Drosophila* [19]. Two other proteins belonging to this family were found later in the genome, GLH-3 and GLH-4, which are also constitutive components of P granule [20]. These four proteins are redundantly required for fertility. GLH-1 is critical for fertility at elevated temperature (26^{o} C) while GLH-2, GLH-3, or GLH-4 are not required at any temperature [21]. The lack of GLH-1 and GLH-4 causes the most severe phenotype, however GLH-2 and GLH-3 might contribute subtly to fertility.

Hermaphrodites and males that lack GLH-1 and GLH-4 produced under-proliferated gonads [20]. All GLH proteins interact with each other in GST pull-down assays; despite this, all localize independently to P granules [22].

Another *C. elegans* DEAD box RNA helicase that is closely related to *Drosophila* proteins Vasa and Belle is VBH-1. This protein is germline-specific and associates with P granules (Fig. 2) [23]. *vbh-1* is important for embryogenesis, spermatogenesis, and oocyte function. Animals lacking VBH-1 produce few or no sperm because they enter oogenesis earlier than wild type individuals suggesting that VBH-1 participates in the sperm/oocyte switch in the *C. elegans* gonad [23].

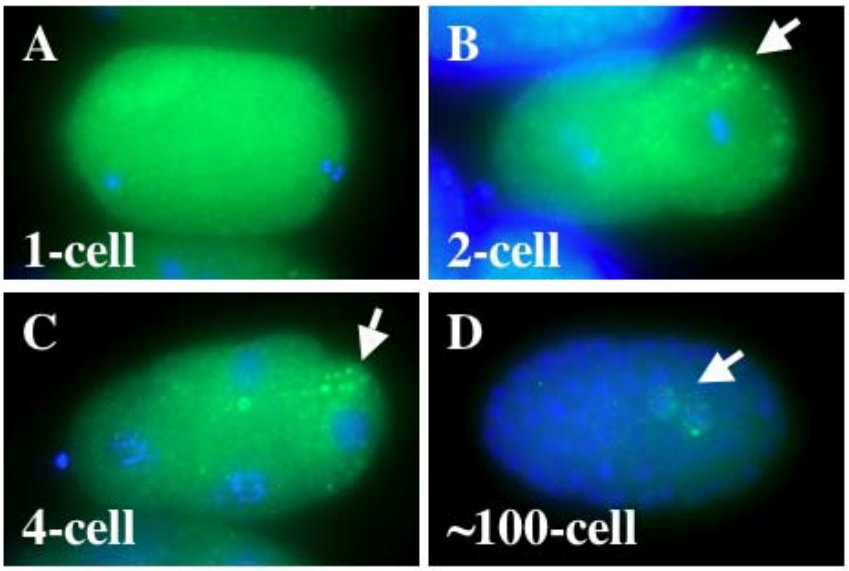

Figure 2. VBH-1 expression pattern during embryogenesis. VBH-1 is expressed diffused in the cytoplasm of all blastomeres, and in P granules in germ cells. A-D) Fixed embryos were incubated with an antibody to detect VBH-1 expression (green) and DAPI to observe nuclear morphology (blue). Embryo developmental stages are indicated in each picture. White arrows point towards P granules in B and C, and the PGCs Z2 and Z3 in D.

PGL-1 was discovered in a genetic screen were mutants with altered P granule were searched [24]. This screening was performed using an antibody that stained P granules (K76) than later was found to recognize PGL-1. However, *pgl-1* mutants have normal P granules when other markers like GLH-1, GLH-2, MEX-1 and MEX-3 are used; indicating that PGL-1 is not required for the assembly of P granules [24]. PGL-1 is a predicted RNA binding protein with RGG box motifs. The lack of *pgl-1* produces temperature sensitive sterility; some *pgl-1(bn101)* animals showed underproliferated gonads with no gametes and other animals had gonads with

defective gametes [24]. PGL-1 associates to P granules at all stages of development. Gonads from animals *glh-1(bn103)* have PGL-1 expression however not associated with P granules suggesting that GLH-1 might have a role in P granule assembly [24].

Later two PGL-1 homologs, PGL-2 and PGL-3, were found by sequence analysis, and by a yeast two-hybrid screen [25]. PGL-2 and PGL-3 associated with P granules throughout life cycle, however PGL-2 is absent in P granules during embryogenesis. Loss of PGL-2 or PGL-3 does not cause sterility. However, loss of both PGL-1 and PGL-3 results in more severe germline defects at elevated temperatures, and in sterility at lower temperatures as well, indicating that PGL-3 functions redundantly with PGL-1. Loss of PGL-2 does not produce any obvious germline defect [25]. As PGL-1, PGL-3 contains an RGG box therefore is a predicted RNA binding protein while PGL-2 lacks this motif. The three PGL proteins associated with each other as tested by yeast two-hybrid, and by *in vitro* binding assays [25]; however they associated to P granules independently of each other in a GLH-1 dependent manner. PGL-1 and GLH-1 interact genetically but fail to interact physically in GST pull-down assays [22].

In a yeast two-hybrid screen it was found that PGL-1 interacts with IFE-1, one of the five eIF4E isoforms of *C. elegans* [26]. IFE-1 is expressed in the cytoplasm of germ cells and associates to P granules in a PGL-1 dependent manner. Animals that lack *ife-1* have normal P granules, but are sterile at high temperatures due to spermatogenesis defects [26].

A new P granule-associated protein, DEPS-1, was discovered in a screen for mutations that phenocopy the PGL-1 localization defect observed in *glh (lf)* mutants [27]. DEPS-1 encodes a novel protein with a serine-rich C-terminal domain of low amino acid complexity that does not resemble any proteins in other organisms. *deps-1* mutant animals deprived of both maternal and zygotic protein are sterile at 24.5^{o} C but fertile at higher temperatures. Most of these animals fail to make embryos or oocytes and have underproliferated gonads; a phenotype similar to that observed in *pgl-1* mutant animals suggesting that they might have some roles in common [27]. At lower temperatures *deps-1* mutant animals show an embryo lethal phenotype with effect variable penetrance and no uniform stage of development arrest [27].

DEPS-1 is important for PGL-1 localization to P granules, and for the accumulation of *glh-1* mRNA and protein. GLH-1 protein localizes to P granules in *deps-1* mutants but its levels appear to be significantly reduced. Loss of DEPS-1 does not dramatically affect the overall mRNA accumulation profile in the germline suggesting either that the compromised P granules present in *deps-1* mutants are largely functional or that P granules do not play a major role in stabilizing mRNAs in the *C. elegans* germline [27].

A genetic interaction has been observed between proteins PGL-1, GLH-1, MEG-1 and MEG-2 [28]. MEG-1 and MEG-2 associate transiently to P granules during embryogenesis, and their expression fades when Z2 and Z3 appear at the 100-cell stage. These proteins are not detected in the adult germline [28]. Lost of *meg-1 and meg-2* results in sterile animals that have underproliferated gonads due to failure of larval germ cell proliferation. Animals that lack these proteins also present ectopic P granules in somatic blastomeres [28].

Several zinc finger proteins associate transiently with P granules during embryogenesis and among them are PIE-1, MEX-1, POS-1, OMA-1, OMA-2 and OMA-3. Mutations in any of these genes cause the germline blastomeres to adopt somatic fates, suggesting that they have a role in maintaining the totipotency of the germline blastomeres. The CCCH finger motif that this group of proteins shares was first identified in the mammalian protein TIS11/Nup475/TTP. This motif has a different primary sequence from the other zinc finger domains found in transcription factors related to TFIIIA or GATA factors [29]. TIS11/Nup475/TTP (tristetraprolin or TTP) is an RNA-binding protein that interacts with 3´-untranslated AU-rich elements of mRNAs and negatively regulates their translation.

Transcription in germ cells is delayed during early embryogenesis due to PIE-1 repression [16]. PIE-1 is a maternally encoded protein that inhibits mRNA transcription and somatic development in germline blastomeres [30, 31]. In the absence of PIE-1, germline blastomeres initiate early mRNA transcription, adopt a somatic fate and in consequence, these embryos lack germ cells [30, 32]. PIE-1 inhibits transcription directly, possibly by targeting a complex that interacts with the CTD of RNA PolII [33]. PIE-1 protein is initially found throughout the cytoplasm of newly fertilized embryos and becomes enriched in the posterior cytoplasm before the first cleavage [34, 35]. PIE-1 also accumulates in the nuclei of germ line blastomeres and P granules [34]. PIE-1 is a bi-functional protein that represses transcription and regulates maternal mRNA translation in germline blastomeres. It is required for *nos-2* mRNA maintenance and NOS-2 protein expression [36].

POS-1 is expressed predominantly in the germline blastomeres. It is first detected in the P1 blastomere and remains in the germline until P4 divides [29]. POS-1 is expressed in the cytoplasm of germline blastomeres P1, P2, P3 and P4 where it also associates with P granules [29]. POS-1 and SPN-4, an RNA binding protein that is also expressed in P granules (see below), repress *glp-1* mRNA translation in the germline [37]. GLP-1 encodes for a Notch like receptor that is expressed in the ABa and ABp blastomeres (Fig. 1).

POS-1 is not only required for germ cell maintenance but also for the proper development of certain somatic tissues. POS-1 mutant embryos

undergo approximately the normal number of cell divisions and produce several tissues, however terminal stage of *pos-1* mutant embryos lack intestinal cells and germ cell progenitors. These embryos have more pharyngeal tissue and fail to hatch due to defects in body morphogenesis [29]. *pos-1* mutant embryos lack the PGC progenitors Z2 and Z3. In the absence of POS-1, P4 undergoes multiple rounds of division and instead of producing germ cells produces descendants that adopt muscle fate [29].

POS-1 interacting protein, SPN-4, is an RNA binding protein expressed in the cytoplasm of oocytes and early embryos. Its expression is enriched in the posterior blastomeres and disappears by mid-embryogenesis [37]. SPN-4 associates with P granules in a manner that resembles POS-1. *pos-1* mutant animals are embryonic lethal due to misexpression of the notch receptor protein GLP-1 [37]. SPN-4 binds to the 3´UTR region of the *glp-1* mRNA and it might regulate its translation.

MEX-1 is a two-zinc finger domain protein distributed unequally in early blastomeres and is a P granule component [38]. *mex-1* mutant embryos have somatic defects and have abnormal germ cell formation. P granules accumulate in the posterior of newly fertilized *mex-1* embryos, but failed to associate properly with the cortex resulting in a mislocalization of P granules to somatic cells and its progressive loss in germ cells [30, 39].

The mechanism by which proteins accumulate in P granules is still unknown. However, the first zinc finger domain of PIE-1, POS-1 and MEX-1 target these proteins for degradation in the soma while their second zinc finger domain is sufficient to localize them to P granules [15, 40].

The zinc finger proteins OMA-1 and OMA-2 associate with P granules in developing oocytes. OMA-2 expression is rapidly lost after fertilization in the somatic blastomeres, but continues to be present in the germline during early embryogenesis [41, 42]. The absence of OMA-1 and OMA-2 proteins results in a dramatic reduction in the offspring and size enlargement of the gonad, oocytes, and germline nuclei. These mutants also show scattering of condensed chromosomes, abnormal structure of the microtubule network and an atypical distribution of P granules in the developing oocyte [41, 42].

OMA-1 and OMA-2 are redundant positive regulators of a key step downstream of a sperm signal that controls oocyte cytoplasmic grow during prophase progression causing an arrest of meiotic progression necessary for oocyte maturation [43]. Oocytes from *oma-1*, *oma-2* mutants continue to grow sometimes becoming up to ten times larger in volume compared to wild type mature oocytes. OMA-1 and OMA-2 also repress transcription in the germ cell precursor cells P0 and P1 by binding and sequestering TAF-4 in the cytoplasm, a critical component for the assembly of TFIID and the pol II pre-initiation complex [44].

In the gain of function allele *oma-1(zu405)*, embryos have extra pharynx and intestinal cells due to abnormal high levels of the SKN-1 protein in the C blastomere (a condition known to cause C blatomere transformation to a EMS-like fate) [45]. In this mutant, OMA-1 is not degraded after fertilization, and its abnormal persistence during embryogenesis prevents other maternally supplied proteins like PIE-1, MEX-5, MEX-3, POS-1 and MEX-1 from being degraded as they normally are [45].

MEX-3, an RNA binding protein with KH domains, is another P granule component also present in the cytoplasm with dynamic changes in its distribution within the embryo during early cleavage stages. MEX-3 protein is first detected in oocytes shortly after cellularization, and its levels appear to increase in oocytes as they mature. After fertilization, MEX-3 is distributed evenly throughout the cytoplasm of the 1-cell stage embryo. By the end of the 2-cell stage, MEX-3 protein appears more abundant in the AB blastomere than in the P1 blastomere. After the 4-cell stage, MEX-3 protein disappears from the embryo in a spatial and temporal pattern similar to its mRNA degradation [46]. MEX-3 associates with P granules during early embryogenesis, but it is no longer detected in late stage embryos or adult gonads. MEX-3 protein association with P granules may play a role in their segregation, and is particularly required for proper development of the germ cell blastomere P3. This protein also plays an important role in the development of the somatic blastomere AB [46].

Another RNA binding protein, with a KH domain, expressed in P granules is GLD-1. GLD-1 expression is observed in the cytoplasm of germ cells from the transition zone trough the end of the pachytene region in the gonad; its expression is undetectable in the oocytes. GLD-1 expression in early embryos derives exclusively from maternal *gld-1* mRNA and is first detected in the P2 germline progenitor and its sister, the somatic blastomere EMS. Rapid GLD-1 degradation in the somatic blastomeres results in the accumulation of this protein exclusively in the germ cells where it associates with P granules [47]. GLD-1 has multiple functions during germ cell development including regulation of meiotic prophase progression of female germ cells, the mitotic to meiotic switch, and the promotion of the male fate in the hermaphrodite germline [48, 49, 50].

GLD-1 is a translational repressor with multiple targets that regulates the expression of several mRNAs including: the transmembrane protein that inhibits downstream male determinants *tra-2*, the oocyte yolk receptor *rme-2*, the notch receptor *glp-1* and the glucosamine phosphate N-acetyltransferase GNA-2. GLD-1 also protects GNA-2 from degradation [51, 52, 53, 54]. These mRNAs are expressed in the most distal part of the gonad, where GLD-1 expression is low, or in the oocytes where GLD-1 is not expressed at all.

gld-1 mRNA translation is promoted by GLD-2 and GLD-3. Polyadenylation of *gld-1* mRNA depends on GLD-2 and its mRNA co-immunoprecipitates with both GLD-2 and GLD-3 [55]. GLD-2 is the catalytic subunit of a cytoplasmic poly A polymerase (PAP). GLD-3 is a Bicaudal-C homolog that possesses five KH motifs and is predicted to bind RNA [55, 56, 57]. GLD-2 and GLD-3 associate with P granules during embryogenesis and together with GLD-1 are involved in several aspects of germline regulation such as inhibiting stem cell proliferation, promoting meiosis, spermatogenesis, and they even play a role during embryogenesis.

Nanos, in *Drosophila*, is required to specify abdominal cell fates. Participates in germ cells migration to the somatic gonad, and is required to maintain viability of germ line stem cells. In *C. elegans* three *nanos* homologs have been identified. NOS-1 and NOS-2 are cytoplasmic proteins that are expressed sequentially in the embryonic germ lineage. The first to appear is NOS-2, which is expressed transiently in the germline founder cell P4 and its two daughters, the PGC Z2 and Z3, around the time of gastrulation [18]. NOS-1 is expressed from embryonically transcribed RNA in mid-embryogenesis after Z2 and Z3 have joined the somatic gonad, and continues to be expressed in these cells through the first larval stage [18].

NOS-2 is required maternally for efficient incorporation of PGCs into the somatic gonad, and functions redundantly with NOS-1 to regulate survival and proliferation of PGC descendents during larval development [18]. NOS-3 expression is uniform and diffuse in the cytoplasm of germ cells in the adult gonad. NOS-3 is also detected in the germ cells cytoplasm during embryogenesis, although the distribution of NOS-3 in P cells is punctate it did not precisely coincide with P granules [58]. Like its homolog in *Drosophila*, NOS-3 interacts with the *C. elegans* Pumilio homologs FBF-1 and FBF-2, but in the nematode this interaction regulates the sperm/oocyte switch in the hermaphrodite gonad [58]. As mentioned earlier, *nos-2* mRNA is a P granule component and its translation is tightly regulated. POS-1 is required to activate *nos-2* translation in the germ cell P4 [59]. OMA-1, OMA-2, MEX-3 and SPN-4 bind directly to *nos-2* 3'UTR to regulate its translation [60]. OMA-1 and OMA-2 suppress *nos-2* translation in oocytes while MEX-3 represses it in early embryos and SPN-4 in the germline blastomeres [60]. POS-1 activates *nos-2* translation in P4 by competing with SPN-4 for its 3'UTR.

Nanos homologs have also been identified in vertebrates. Zebrafish Nanos regulates PGC migration and survival during embryonic development [61]. In mice, Nanos2 is required to form spermatogonia and Nanos3 is required for PGC survival [62, 63].

The Sm and Sm-like (Lsm) proteins are core components of the snRNPs that catalyze pre-mRNA splicing and other mRNA processing events. The

Sm and Sm-like proteins may have multiple functions in cells and promote a variety of RNA:RNA interactions. Sm proteins are expressed in the nuclei and in the cytoplasm of many cell types in *C. elegans* consistent with the localization of snRNPs in other organisms. They are also found in P granules constitutively [64]. Disruption of some of the Sm proteins has effects on the subcellular distribution and size of P granules. After Sm depletion, P granule association with nuclei was disrupted in 16- to 40-cell stage embryos. In some Sm(RNAi) embryos, P granules remained large but were primarily cytoplasmic. In other embryos, PGL-1-containing particles were small or very diffuse within the cytoplasm. This range of effects on P granules suggests that the Sm proteins may be required both for the perinuclear attachment of PGL-1-containing particles and for the integrity of the P granule structure [64].

Sm proteins are also required for transcriptional silencing in the early embryonic germ lineage, and to maintain expression of several germ cell-specific proteins. Sm proteins together with PIE-1 control expression of GLD-1 by a different mechanism from their role in transcriptional silencing [65]. Sm proteins promote PIE-1 expression suggesting that Sm proteins may control transcriptional silencing and GLD-1 expression by regulating PIE-1 levels [65].

CGH-1 is a DEAD box RNA helicase required for embryo development, germ cell function and survival [66, 67]. CGH-1 is expressed in the cytoplasm of germ cell and P granules during embryogenesis, larval stages and adult male and germline gonad. CGH-1 is also expressed in other RNA granules found in the core of the hermaphrodite gonad, oocytes, and somatic and germ cells during early embryogenesis [66]. The CGH-1 ortholog in yeast (Dhh1p) interacts with members of the decapping-dependent mRNA degradation complex, which are found in processing (P) bodies. CGH-1 interacts with CAR-1, an RNA binding protein that also associates with P granules and other RNA granules structures that resemble P-bodies. Similarly to *cgh-1*, absence of *car-1* increases germ cell apoptosis, and also leads to defective embryonic cytokinesis [68, 69, 70]. Additional P granule components that bind other RNA granules and participate in RNAi will be described below.

P granules assembly

In comparison to *Drosophila*, where it is well known how the polar granules assemble, in *C. elegans* we are only beginning to understand this process. Mutants without P granules have not been isolated to date. This suggests that P granules might be essential for survival or that the mechanism that assembles P granules is redundant and multiple proteins are required to form these structures.

Recently, epistatic studies have shown that GLH-1/-4 proteins are upstream of the PGL-1/-3 proteins in the P granule assembly pathway [21]. Lack of *glh-1* and *glh-4* causes PGL-1 to dissociate from P granules and to become localized to the cytoplasm [24]. However *pgl-1; pgl-2; pgl-3* triple mutants display an apparently normal concentration of GLH-1 in P granules [25]. This evidence suggests that GLH-1 and GLH-4 may participate directly or indirectly in the recruitment or retention of PGL proteins on P granules.

P granules are involved in mRNA transport, stability and translational regulation

P granules function is still unknown, but it is generally accepted in several systems that germ granules store RNA and proteins to regulate their expression. Several pieces of evidence suggest that P granules are involved in mRNA trafficking, translation, stability, and RNAi-related processes [3, 17, 21, 27].

During the majority of germ cell development, P granules associate in clusters in direct contact with nuclear pores. P granules dissociate from the nucleus taking some nuclear pore complex proteins with them into the cytoplasm [10]. This close association of P granules to nuclear pore complexes suggests that P granules might be involved directly in receiving mRNA from the nucleus for transportation and/or storage.

The DEAD box RNA helicase Vasa in *Drosophila*, a polar granule component, promotes the translation of specific mRNAs [3, 71]. Vasa homologs in *C. elegans* such as GLH-1/-4 and VBH-1 [19, 20, 22, 23] could also be promoting mRNA translation but no direct evidence supporting this model is available at this moment.

The association of IFE-1 to P granules further supports the notion that these particles participate in translation related processes [26]. Nevertheless cannot be rule out that IFE-1 is only being stored transiently in P granules while it is not needed in the cytoplasm. An additional possibility is that IFE-1 is competing with other members of its family to repress the translation of specific mRNAs in these structures. More evidence for translational regulation comes from the protein POS-1, which activates *nos-2* translation in the germ cell P4. We do not know if *nos-2* mRNA translation by POS-1 occurs in P granules [60].

In *C. elegans* no ribosomal RNAs have been detected in P granules suggesting that no mRNA translation occurs in these particles [17]. Indeed, several translational repressor factors associate with P granules, and although it has not been shown that they specifically inhibit mRNA translation in these structures, this suggests that P granules are places where mRNA is repressed.

Two cases of translational repression by P granules components are proteins POS-1 and SPN-4, which repress *glp-1* mRNA translation in the germline during early embryogenesis [37]. GLD-1 in the adult germline represses the translation of several proteins like: *tra-2*, *rme-2*, *glp-1* and *gna-2* [51, 52, 53, 54], and also protects GNA-2 from degradation [54].

Like we mentioned previously, *nos-2* translation is repressed by several proteins such as OMA-1 and OMA-2 in oocytes, MEX-3 in early embryos, and SPN-4 in germline blastomeres [60]. Another example is NOS-3, which interacts with FBF-1 and FBF-2 in the hermaphrodite gonad to repress *fem-3* translation inhibiting spermatogenesis [58].

P granules and their relationship to other RNA granules

Recently it has been shown that P granules share proteins with other two RNP complexes known as processing bodies (P-bodies) and stress granules. P-bodies are present in growing, unstressed cells, and are sites of mRNA degradation and/or storage of non-translated transcripts [72]. Stress granules are other type of RNA granules larger than P-bodies whose assembly is induced by various environmental stresses, such as heat shock, UV irradiation and oxidative conditions [72]. P-bodies and stress granules are highly dynamic structures that are simultaneously assembled in cells subjected to environmental stress and disassemble rapidly once stressful conditions have past. These structures associate transiently, presumably to exchange mRNAs for storage or degradation.

Both P-bodies and stress granules share some components, but they also differ in others. For instance, stress granules are defined by the translation initiation factors comprising the non-canonical 48S preinitiation complex (eIF3, eIF4A, eIF4G and poly(A)-binding protein 1) and small ribosomal subunits [72]. On the other hand, P-bodies are defined by components of the mRNA decay machinery like the decapping enzymes DCP1a DCP2 and hedls (human enhancer of decapping, large subunit)/GE-1 [72].

In *C. elegans* the P granule component CGH-1 is also present in other granules that might resemble P-bodies [66, 67]. The CGH-1 homolog in yeast, Dhh1p, interacts with components of the decapping machinery. In *C. elegans*, CGH-1 interacts with CAR-1, a classic P-bodies components [69]. Like P-bodies and stress granules, P granules aggregate in response to stress conditions like oogenesis arrest, heat shock, anoxia, and osmotic stress suggesting a common role for all three types of granules. Under stress conditions, other RNA granules appear in the cytoplasm of germ cells suggesting that three types of RNA granules co-exist in the *C. elegans* germline [73, 74]. Under stress conditions, P granules associate stress granule

components like PAB-1 and TIA-1 but also P-bodies proteins like, DCP-2 and CGH-1 [74, 75].

CGH-1 has a dual role in RNA granules that depends on its localization. In RNA granules in the soma, CGH-1 associates with decapping components and appears to participate in maternal mRNA degradation. However, in RNA granules in germ cells CGH-1 associates with stress granule components like PAB-1 and appears to protect mRNAs from degradation [76]. Particularly, CGH-1 seems to protect maternal mRNAs like *pos-1* and *nos-2* from degradation [76].

CAR-1, a P-bodies component, and its interacting protein CGH-1 are required to repress maternal mRNA from degradation [77]. CAR-1 is required for the formation of RNA granules that appear during arrested oogenesis in germ cells, however this function is independent of its role during mRNA repression [77].

P granules and stress granules have some common characteristics, for example: 1) They share some stress granules components like TIA-1. 2) They are dynamic structures that assemble and dissemble in response to stress conditions. 3) They storage mRNA. 4) P-bodies dock with stress granules, a similar phenomenon occurs between P granules and P bodies [75]. These RNA granule structures also differ in some other aspects. P granules do not associate with ribosomes, and unlike stress granules, P granules interact with nuclear pores [75]. Although apparently P granules are distinct RNA granules than P-bodies or stress granules; it is evident that P granules share some of their functions with these RNA granule structures. All three types of granules are very dynamic systems that, depending on the environmental conditions or the developmental stage, exchange components to cope with the circumstances.

P granules and RNAi

Germ cells from several organisms express a distinct class of RNAs that are 24- to 30-nucleotide-long, known as piRNAs (Piwi-associated RNAs). piRNAs are produced by a Dicer-independent mechanisms and associate with Piwi-class Argonaute proteins [78]. In *C. elegans* two Piwi proteins homologs are known, PRG-1 and PRG-2. PRG-1 associates with P granules throughout development [79, 80]. Absence of PRG-1 leads to sterility, low stem cell production, and defects of female and male gametes [79, 80, 81]; these defects are more evident at higher temperatures. *C. elegans* genome encodes approximately 15,722 piRNAs (previously know as 21U-RNAs) that are expressed in the germline and required PRG-1 for its expression. The function of these piRNAs in the *C. elegans* germline and its relationship to P granules are still unknown.

Mutations in the P granule components PGL-1 and DEPS-1 lead to a germline RNAi-defective phenotype. However, other P granule components like GLH-1 and GLH-4 are not required for RNAi [21, 27, 82]. Apparently P granules are not necessary for RNAi since in *glh-1* and *glh-4* double mutant, where PGL-1 protein is diffused, RNAi is operating.

Interestingly, RNAi is another function that might connect P granules to stress granules and P-bodies because these RNA granules associate with the RNA-induced silencing complex (RISC) and with double-stranded RNA [72, 83].

Germline determinants in *Drosophila melanogaster*

Here we discuss the preformation model during oocyte development in *Drosophila melanogaster*. How does oocyte polarization determine germ cell formation? What are the major morphological and functional aspects acquired by germ cells? What can we learn from this model to understand aspects of germ line biology in vertebrates?

Our approach to these questions begins with a summary of *Drosophila* oogenesis and early embryogenesis until primordial germ cell formation. To illustrate the strategies used in germ cell formation we will preferentially discuss recent findings on transport, anchoring and expression control of germ plasm components. In conclusion, we discuss the transcriptional quiescence and totipotency of germ cells.

Overview of germ cell specification in *Drosophila*: A case for preformation

A common feature in embryonic germ cell formation is the production of a cell type that will give rise exclusively to germ cells by clonal mitotic divisions. These cells are known as PGCs and populate the somatic developing gonad [84]. Fruit flies provided the most comprehensive data set on the molecular mechanism of PGC specification driven by preformation. Oogenesis begins at the anterior tip of the *Drosophila* ovary as each germ line stem cell divides asymmetrically to originate cysts of 16 interconnected germ cells (reviewed by [85, 86]). Of these cells, only one acquires the oocyte fate whereas the remaining 15 (nurse cells) become polyploid and transcriptionally active. Along a network of polarized microtubules shared by all cells in the cyst, the gene products synthesized in the nurse cells are then transported to the oocyte, to maintain its fate, establish its polarity and form the yolk (Fig. 3) [87].

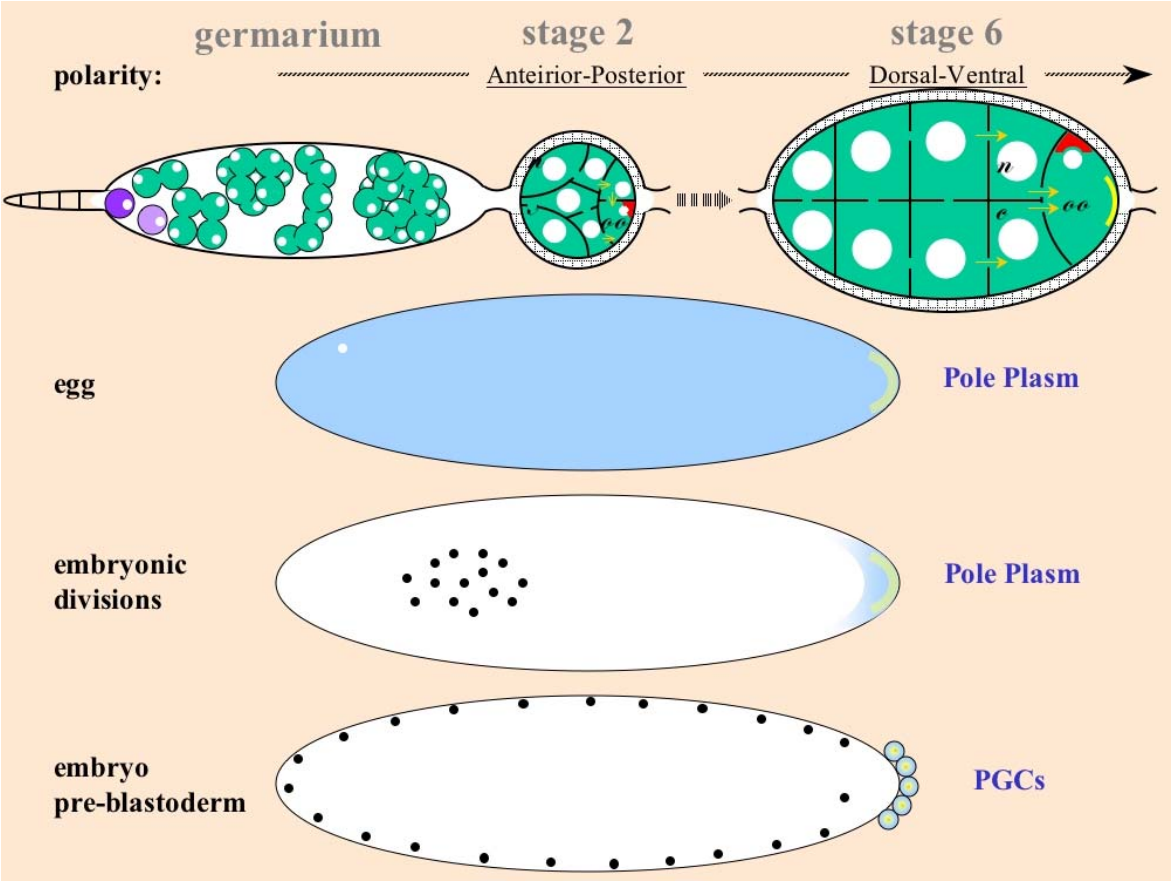

Figure 3. Preformation model of germ cell formation in *Drosophila*. In the germarium, germ line stem cells (dark purple circle) originate cystoblasts (light purple circle), which by mitotic division give rise to cists of 16 germ cells. In early stages of oogenesis the anterior-posterior polarity of the oocyte (oo) is established through the activity of the *gurken/TGFa* signal (red). Gurken is also required for the dorsal-ventral polarity later (stage 6). In these stages RNAs and proteins are synthesized by the nurse cells (nc) and transported through cytoplasmic bridges (yellow arrows) to the oocyte. Among these, *oskar* mRNA localizes at the posterior pole of the oocyte and assembles the pole plasm with other germ plasm determinants that subsequently localize to that region (yellow). Localization to the posterior pole is achieved both by anchoring and posterior-specific translational and transcriptional regulation (see text). Instead of being localized, some pole plasm mRNAs are homogeneously distributed throughout the oocyte and egg (blue). Following fertilization, the fly embryo divides by nuclear rather than cellular divisions and maternal RNAs begin to decay. However, unlocalized mRNAs become differentially protected in the germ plasm (blue crescent). The nuclei (black circles) that enter the posterior germ plasm are the first to cellularize. These cells inherit the germ plasm and become PGCs (blue/yellow circles). At this stage PGC nuclei are quiescent through the activity of germ plasm specific components such as Pgc, Nanos and Pumilio, and their transcriptional program will initiate later in development when PGCs begin their migration to the embryonic gonad (not shown).

The polarized distribution of proteins and RNAs in the oocyte is a conserved way to control protein expression locally during early embryonic development [88]. It recurs to a complex network of processes not entirely understood, which include: i) microtubule polarization through signals mediated by the Gurken/TGFa ligand; ii) active microtubule-based transport by kinesin and dynein motors; iii) molecular anchoring, and iv) specific translational and transcriptional regulation [89, 90, 91]. This sorting process leads to the formation of an electron dense cytoplasm enriched in germ line determinants known as germ plasm [92]. Because the *Drosophila* germ plasm is assembled at the posterior pole of the oocyte before fertilization, it is also known as pole plasm (reviewed by [93]. Following fertilization, embryos initiate rapid cycles of protein synthesis and nuclear division originating a syncytium of nuclei regularly aligned around the cortex (reviewed by [94]). Before these nuclei synchronously cellularize to give rise to a blastoderm, from which all somatic tissues originate, 4-5 nuclei cellularize at the posterior pole of the embryo (Fig. 3).

These pole cells harbor most of the pole plasm, and undergo two or three more rounds of division to form a cluster hanging from the posterior pole of the blastula. Although pole cells are known for nearly a century, only recently have they been shown to be PGCs [95, 96]. Gastrulation will then internalized PGCs and these actively will migrate to the developing gonad [97]. PGC loss during this process causes sterility, as the somatic gonadal cells cannot be reprogrammed to enter the germline.

The pole plasm is a bona fide germ cell determinant

Two major data sets demonstrate that the pole plasm is not simply a germ cell marker but a key germ cell determinant. Firstly, transplantation of pole plasm to the anterior tip of embryos induces functional germ cells in that region [98]. Secondly, forcing pole plasm assembly in ectopic sites in the embryo also results in PGC specification in these sites [99].

A distinctive mark of the pole plasm is its granular morphology, which at the ultrastructural level corresponds to the accumulation of organelles known as polar granules [12]. Polar granules are dynamic structures both in size and morphology, composed of a meshwork of fibrils and first seen at the posterior pole of the oocyte in late oogenesis. Following fertilization polar granules become round and hollow, frequently found associated with polysomes [100]. The identification of polar granules led to an intense search for its components by biochemical and genetic means. Direct biochemical approaches to isolate polar granules have been taken for the last 30 years although only recently yielding results with significant resolution [101, 102]. The genetic approach

Table 1. Genes required for the formation of germ plasm.

Function	Gene[a]	Molecular Nature
establishment of the posterior pole of the oocyte	*gurken*	TGFα-like ligand
	kinesin dynein	Microtubule-based motor
	spire	Novel actin nucleation factor
	cappuccino	Formin-like actin nucleation factor
	*staufen**	RNA-binding. Microtubule-based transport
	mago nashi tsunagi	Exon-exon junction complex
specifically for posterior germ plasm formation	*oskar*[1]	Novel. Drosophila-specific
	*bruno**	RNA-binding. Translational regulation
	*tudor**	Novel. 11 putative Tudor domains
	*valois***	MEP50-like. Methyl transferase.
	*capsuleen***	PRMT5-like. Methyl transferase.
	*vasa**	Helicase. RNA-binding.
	*hsp83***	Molecular chaperone
nonspecific for germ cell formation	*aubergine** *piwi**	AGO-1-like. RNA interferance.
	*miR-6***	microRNA
transcriptional silencing of germ cell	*pgc**	Pol II elengation inhibitor.
	nanos	Zn-finger. Translational regulation
	pumilio	PUF RNA-binding. Translational regulation.
PGC survival and guidance	*wunen*	Membrane-bound lipid phosphatase
	*pgc**	Pol II elengation inhibitor.

a - gene productos are marked according verified (*) or presumed (**) polar granule localization.

1 - only short form of Oskar protein localizes to polar granules (see text)

has been by far the most valuable in identifying germ plasm genes, some of which will now be discussed (Table 1).

Tudor and the *grandchildless* phenotype

The first genetic screens were aimed to identify the *grandchildless* phenotype, i.e., mutations specifically causing degeneration of the pole plasm and hence originating completely sterile progeny. However, most *grandchildless* mutations are pleiotropic [93]. One of such mutants is *tudor*, whose phenotype is not only lack of pole cells but have abnormal abdominal phenotypes [103, 104]. Necessary for the formation of polar granules, this peptide is a concatenation of 11 Tudor domains, known by their affinity to methylated proteins and nucleic acids [100, 105]. In fact, Tudor localization to the posterior pole depends on the activity of two methyltransferase components Valois/MEP50 and Capsuleen/PRMT5 [106, 107]. However, no methylated partners of Tudor are known and its role in polar granule formation remains unclear [100].

Oskar is the master key for germ plasm assembly

A major advance to the limited success of *grandchildless* selections occurred when saturation screens for female sterile mutants identified a large class of maternal-effect lethal mutations causing abnormal abdominal segmentation [104, 108]. Many of these mutants, such as *oskar*, *vasa*, *cappuccino*, and *spire* also cause pole plasm assembly defects and lack of pole cells [109, 110, 111]. Elegant studies manipulating the product of the *Drosophila*-specific gene *oskar* have shown that *oskar* mRNA is necessary and sufficient for pole plasm assembly. For example, driving the expression of *oskar* RNA to the anterior tip of the embryo was sufficient to induce the formation of a functional germ plasm [99]. Furthermore, the effect of *oskar* in pole plasm assembly is dosage-dependent, i.e., the higher the number of *oskar* copies, the larger the size of the germ plasm and, consequently the greater the PGC number [99, 112]. Directing *oskar* expression to the anterior allowed the identification of genes specifically required for pole plasm formation among all the other maternal-effect posterior segmentation mutants. The prediction was that expressing *oskar* mRNA at the anterior pole of an embryo mutant for a purely posterior segmentation gene would not interfere with germ plasm assembly in the anterior region. In contrast, anterior expression of *oskar* RNA in mutants for germ plasm components will affect germ formation in both poles [99].

On the other hand, mutations causing mislocalization of *oskar* mRNA rarely lead to ectopic pole plasm assembly, suggesting that *oskar* RNA localization and translation are tightly coupled [113, 114]. Two Oskar protein isoforms, the long and short forms, result from the translation from two in-frame alternative start codons [115]. However, no obvious homology has been found to suggest their molecular function. Although *oskar* mRNA localization during oogenesis is the first observable event in pole plasm assembly, later short Oskar translation requires Tudor, the germ cell marker *vasa*, and *oskar* RNA itself, suggesting a feedback mechanism in pole plasm control [115, 116]. Short Oskar protein interacts with Vasa and like Vasa and Tudor, localizes at the polar granules and seems to be involved in transport [117]. In contrast long Oskar associates with endosomes, to maintain the posterior localization of pole plasm components [118, 119]. The significance of the link between long Oskar and the endocytic pathway remains unclear although recent data suggest that it promotes reorganization of actin fibers at the posterior pole [120]. Such reorganization is necessary to anchor the germ plasm to the oocyte cortex [121].

Vasa is a "universal germ cell marker"

A conserved germ cell ATP-dependent RNA helicase, Vasa localizes to the posterior pole of the *Drosophila* oocyte in an *oskar*-dependent manner, concomitant with Tudor [122, 123]. However, other *grandchildless* mutations appear to have little effect in Vasa localization to the posterior pole, suggesting that Vasa function is not sufficient for PGC formation [124]. Both *vasa* mRNA and protein are constitutively present in the germ line throughout the *Drosophila* lifecycle but its function remains unclear. There are two major obstacles for our understanding of Vasa function. First, no directly bound target RNAs are known and, second, the pleiotropy of *vasa* mutant phenotypes [123]. Yet, Vasa is required for the translation of a number of mRNAs related to pole plasm assembly such as *gurken*, *oskar*, and the Zn-finger RNA-binding *nanos* [115, 116, 125, 126, 127]. Vasa has also been related to transposable element silencing in the germ line through the activity of repetitive-associated small interfering RNAs (rasiRNA) [128]. Vasa could regulate translation by binding to other factors. The best known candidate is the translation regulator RNA-binding protein Bruno, with which Vasa interacts *in vitro* and is known to repress the translation of mislocalized *osk* mRNA [129]. Vasa also binds to other ovarian proteins such as Oskar and the novel protein Gustavo, although these are more likely related to the control of Vasa transport than to its function in the germline [117, 130]. Vasa interacts directly with translation initiation factor eIF5B, and this interaction is essential for its function [71].

Mechanisms of pole plasm formation

We have exposed a number of results suggesting that the pole plasm is enriched in ribonucleoprotein complexes (RNPs) [131]. In the context of germ plasm and PGC formation, what are the possible strategies to localize RNPs to the pole plasm and restrict them to germ cells?

Localization and anchoring of germ plasm components

The most evident model to explain germ plasm transfer to PGCs predicts that germ cell RNPs localize and are held during oogenesis around the region where pole cells will later form. We gave some examples of localized mRNAs (*oskar* and *nanos*) and proteins (Tudor, Valois, Caplsuleen and Vasa). To achieve localization one needs to consider a polarized transport based on elements of the cytoskeleton (e.g. microtubules) requiring affinity

between cargos and motors [132]. In addition to these *trans* interactions, mRNA transport relies upon *cis*-acting elements conferring mechanisms of selection in a large pool of maternal mRNAs. These *cis* elements are frequently found on the 3' untranslated region (3'UTR) of mRNAs [129, 133]. Once at the posterior pole, mRNAs must be immobilized through anchoring mechanisms. Moreover, selection, transport and anchoring must be coupled to efficient transcriptional repression mechanisms to avoid premature expression (reviewed by [134]).

Localization of the primary germ plasm organizer *oskar* mRNA illustrates best these mechanisms [92, 93, 105, 123, 133]. Fine deletion mapping revealed a complex *cis* localization signal in *oskar* mRNA independently governing distinct steps of the process [135]. For example, some deletions in the *oskar* localization signal block the movement of the transcript from the nurse cells to the oocytes, while another deletion only interferes with the localization to the posterior pole, but not the earlier steps. The sensitivity of *oskar* mRNA localization to treatment with microtubule destabilizing drugs indicates a microtubule-based transport. However, several observations suggest that *oskar* transport cannot be a straightforward kinesin ride [89] (reviewed by [136]). First, *oskar* mRNA transport takes longer that the processivity of kinesin [91]. Second, *oskar* mRNA appears at the anterior and posterior poles of the oocyte before being restricted to the latter [135]. A clearer view of *oskar* mRNA localization came from real time visualization of *oskar* mRNA in cultured *Drosophila* oocytes [137]. The asymmetrical localization of *oskar* mRNA to the posterior pole is promoted by a slight bias in the direction of its transport over a period of 6-10 hours. Although kinesin mutations abolished the bias towards posterior, the role of the minus-end-directed dynein motor remains unclear.

As part of its selection *oskar* mRNA is imprinted upon splicing inside the nurse cell nuclei with the two exon-exon junction complex proteins: Y14/Tsunagi and Mago nashi. Both proteins co-migrate and co-localize with *oskar* mRNA in the pole plasm (reviewed by [138]). Clearly, the anchoring of *oskar* mRNAs at the posterior pole is also required as flies lacking the anchoring protein Staufen localize *oskar* mRNA only transiently [139, 140]. Live cell imaging has revealed another localization mechanism. The germ plasm product *nanos* mRNA, localizes to the posterior pole after *oskar* mRNA, when microtubule polarity is not as well defined and thus less likely to sustain polarized transport [141]. *nanos* RNA appears in relatively small RNPs, which move posteriorly through an apparent diffusion-entrapment based mechanism.

Differential stability and protection

In contrast with the examples given above, some maternally provided germ plasm mRNAs are homogeneously distributed throughout the oocyte. This is for example the case of the molecular chaperone *hsp83* and the lipid phosphatase *wunen* mRNA, which is required later for PGC survival and guidance [97, 142, 143]. However, a few hours after fertilization a maternal-to-zygotic transition (MZT) takes place during which developmental control is transferred to the zygotic genome. The stability of this class of germ plasm mRNAs is challenged by two observations linked to MZT: first, a subset of the maternal mRNAs is degraded; second, the zygotic genome is transcriptionally activated. Thus products such as *hsp83* and *wunen* mRNAs must be "protected" in the germ plasm. Recent genome-wide quantifications of the fraction of maternal mRNAs eliminated during MZT, have identified molecular mechanisms of mRNA instability and determined the *cis* elements involved [87, 144, 145]. In spite of this advancement, little is clear about protection in the germ line [145, 146].

One hypothesis proposes that polar granules could serve as "protective granules" for the RNPs journey from the nurse cell nuclei to the posterior pole of the oocyte [12, 100, 147]. Another possibility is that the premature PGC formation may also provide protection to germ plasm components before the MZT events take place (Fig. 3).

A new model involving mRNA stability has recently been proposed based on the observation that like Oskar, Tudor and Vasa, the *Drosophila* Piwi/Argonaute protein also localized to the pole plasm and is required for PGC formation [148]. Piwi is required for producing regulatory RNAs used by both the small interfering RNA and the micro RNA pathways (reviewed by [149]). Another Argonaute-like protein, Aubergine localizes to the pole granules whereas Vasa functions is now linked to the rasiRNA pathway [101, 128, 150]. How these proteins control pole plasm and PGC formation remains however a matter of speculation. For example, an indirect mechanism involving Piwi, Aubergine and Vasa proteins could be inhibiting transcript destabilization factors active elsewhere in the oocyte or embryo. Consistent with this idea, a miRNA (miR-6), appears to be essential for pole cell formation [151]. Furthermore, many germ granule proteins are found in P-bodies, which have been shown in yeast and mammalian cells to participate in mRNA decay, RNA interference and translation inhibition by microRNAs [69, 148]. This suggests that polar granules and P-bodies are functionally related.

Germ cell fate and transcriptional control

Germ cells must also be protected from the initiation of the zygotic transcriptional program during MZT. The transcriptional quiescence of germ

cells serves two goals: first, to maintain germ cell totipotency as PGC populate the gonad as stem cells and, second, to avoid PGCs expression of somatic differentiation genes, cause of sterility and teratomas (reviewed by [3]). One likely role is to repress the program that regulates the core transcriptional machinery as well as chromatin states.

In flies, the peptide Pgc and the translational repressors Nanos and Pumilio are germ plasm components that can play these roles [152, 153]. Germ cells lacking either Pgc or Nanos activity show elongating Polymerase II activity and express genes characteristic of somatic cells. However, the mechanism (or mechanisms) by which Pgc and the Nanos and Pumilio translational repressors prevent elongation and the relationship between these regulators is unclear (reviewed by [154]).

Another level of trancriptional control is the epigenetic modification of histones that are associated with transcriptional competence, such as methylation of lysine-4 of histone H3 (H3K4me). In flies transcriptional activity and H3K4me appear as germ cells initiate their migration and Pgc disappears [155].

Germ granule components in zebrafish

The establishment of tools to understand zebrafish germ cell development

The germline in some fishes, like zebrafish (*Danio rerio*), is established by the inheritance of maternal determinants through oocytes, which are later segregated throughout embryogenesis. Some aspects of germ cell development and function in zebrafish are indeed similar to those in invertebrates (like *Drosophila* and *C. elegans*) and at the same time other mechanisms are similar to those in vertebrates. For its strategic position in the phylogeny, zebrafish is emerging as a model organism to study germ granule components in vertebrates.

Adult zebrafish are relatively easy to maintain and breed. The zebrafish embryos are highly suitable for experimentation because multiple mutants with specific defects in organogenesis are available. The embryos are translucent allowing organ formation to be observed as it happens, in addition techniques for RNA and protein localization, cell transplants, cell labeling, cell ablation, gene expression and transgenesis are feasible and reproducible [156]. As an example that we will discuss further, the zebrafish PGCs migration behavior has been tracked *in vivo* using GFP-fusions and time-lapse fluorescence microscopy [157].

In 1891, using the fish *Micrometrus aggregatus* it was observed an early segregation of PGCs from somatic cells that were identified as four large cells on the 5th and 6th cleavage [158]. Using only mutagenesis, genetic analysis and a remarkable insight, Charline Walker and George Streisinger predicted the existence of only five PGCs during the cleavage period of zebrafish development [159]. Indeed it was an accurate calculation because later, in 1997, it was confirmed by *in situ* hybridization, using a zebrafish *vasa* (*vas*) probe, that there are four PGCs during the first ten cell cycles of zebrafish development [160, 161].

Like in other organisms, zebrafish *vasa* mRNA localizes specifically to germ cells and particularly to germ plasm, which allowed the specific label and study of zebrafish germ cells. At the 2-cell stage, *vasa* transcripts are found extended along the cleavage plane (Fig. 4B). Their localization depends on cytoskeleton, initially on actin [162] and on alpha-tubulin later on. Indeed alpha-tubulin becomes a germ granule component [163]. At the 4-cell stage, *vasa* labeling appears also on the second cleavage plane (Fig. 4C) but later at the 8-cell stage *vasa* gathers in four cytoplasmic masses (Fig. 4D), which by the 16- to the 1000- cell stage they remain localized to only four cells (Fig. 4E – F). This indicates that, during early development, germ granules are asymmetrically segregated and later are inherited to only one of the two dividing blastomeres. In 1000 to 4000 cells embryos (Fig. 4K) (3 to 4 hpf), an unknown signal induces PGCs to divide symmetrically and germ granules are inherited to daughter cells. Over the next 20 hrs a total of 25 to 50 PGCs are produced by proliferation [164].

At 4-5 hpf PGCs migrate from their site of specification to the site of the developing gonad where they will differentiate into sperm or eggs [164]. One hour later on the shield stage (Fig. 4M), PGCs are located near the blastoderm margin forming four evenly spaced clusters [165]. At the 3 somite stage PGCs form two groups of cells, each of them lateral to the midline of the endodermal layer moving along the somites flanks between the pronephric ducts and the yolk syncytial layer (Fig. 4P) (ysl). PGCs are shifted to a more posterior position moving towards the region where the gonads will develop [165].

In zebrafish, *vasa* mRNA is located on germ granules; a sequence inside its 3' untranslated region (UTR) is required for this specific localization, and is sufficient to drive GFP expression to PGCs [166, 167]. Actually, the 3'UTR of zebrafish *nanos1* (*nos1*) mRNA and the 3'UTR of *Xenopus laevis* Xcat mRNA also localize to PGCs [61, 168]. A transgenic zebrafish expressing a GFP protein in PGCs (*Kop*-EGFP-F-*nos*1-3'UTR) was created to visualize and track by *in vivo* fluorescence microscopy the migration of these cells during development [169]. Another tool to visualize on higher magnifications

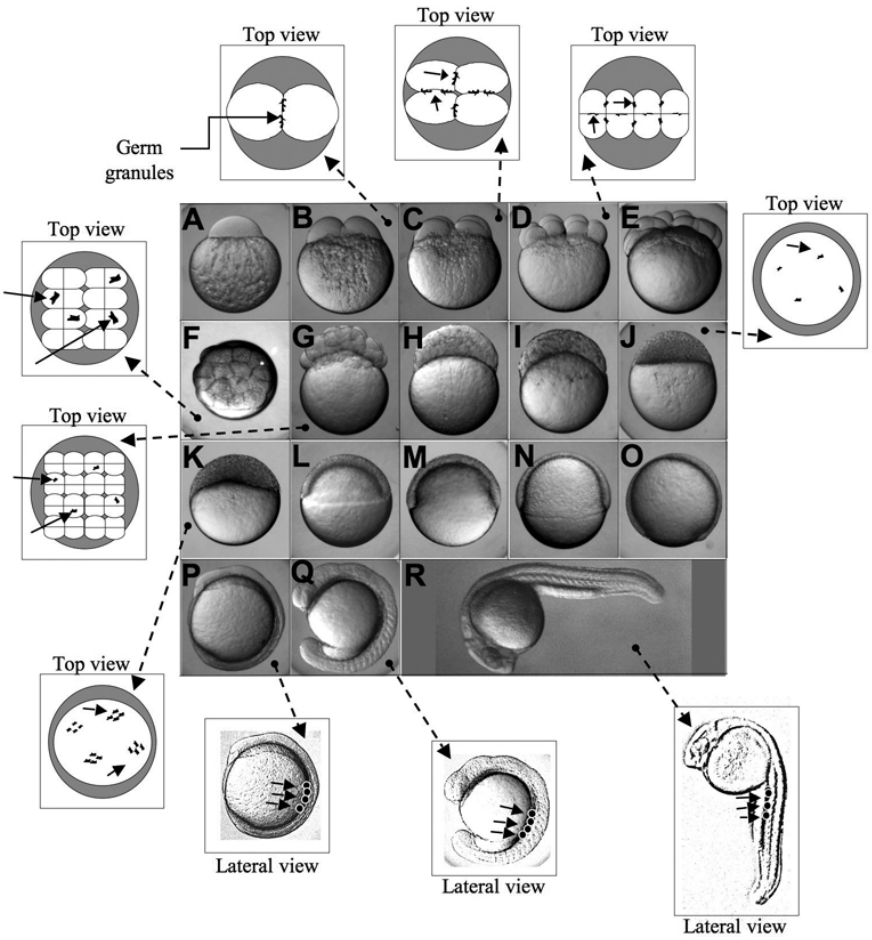

Figure 4. Germ granule distribution during zebrafish development. A to R represent the following developmental stages A) 1-cell, B) 2-cell, C) 4-cell, D) 8-cell, E and F) 16-cell, G) 32- cell, H) 64-cell, I) 128-cell, J) 512-cell, K) dome, L) 50%-epiboly, M) shield, N) 70%-epiboly, O) 90%-epiboly, P) 3-somite, Q) 14-somite and R) 24 hpf. All are lateral views, except F), which is a top view. Dashed arrows connect the photographs with drawings representing the germ granules localization in some developmental stages. Germ granules gather on the cleavage planes of cell division on the 2-cell to 8 cell stages. Solid arrows indicate germ granules. From the 16-cell stage to 512-cell stage (E – J) germ granules are distributed to only four cells. Germ cells mitosis initiate at 3 hpf (J) and soon after they start migrating in clusters along both sides of the trunk (L – R) moving towards the region where gonads will be formed.

germ granules was the construction of a transgenic zebrafish; which expresses a fusion protein between *granulito* (*gra*) and a red fluorescent protein (*granulito-dsRedEx-nos*1-3'UTR) [163]. Both transgenes are under the control of the *askopos* (*kop*) promoter and have the 3'UTR element of *nos1* mRNA. The role of genes *nos1*, *gra* and *kop* as germ granule components will be discussed later.

Germ cell migration

The *kop*-EGPF transgenic animal showed that zebrafish PGCs have a round shape and are static at 3 hpf, 15 minutes later PGCs extend multiple protrusions in all directions but remain in the same position. This behavior is inhibited when PGCs undergo mitosis (4.5 hpf) and it is regained afterwards [169]. From 4.5 to 6 hpf, PGCs migration movements initiate, which are known to be guided by the Cxcr4b receptor expressed by the PGCs and the environmental chemokine SDF-1 [157]. The migration of PGCs depends on active transcription (by RNA pol II and III) and requires normal expression of Dead end, E-cadherin, HMGCoAR (Hydroxymethylglutaryl coenzyme A reductase) and GGT1 (Geranylgeranyl transferase 1) [169, 170, 171]. Using the *kop*-dsRed transgenic fish it was observed that germ granules in PGCs are highly variable in size while migrating; however, they became very homogenous in size by the time that PGCs reach the region where the gonad will develop. It was also found that Dynein regulates germ granule size during cell cycle, which suggests that germ granules use microtubules to move and be distributed [163].

Germ cell determinants in zebrafish

Some experiments suggest that in zebrafish germ granules are also required for germ cell specification. In zebrafish like in *Xenopus*, *Drosophila* and *C. elegans* the elimination of the germ granules induce the gradual loss of PGCs. On the other hand, germ plasm components microinjected into somatic blastomeres switched these cells fate into a germline [172]. To understand how germ plasm regulates germline differentiation and survival several groups are working toward the identification of the mRNA and protein that are components of these granules.

Using whole mount in situ hybridization it has been showed that the mRNAs from *dead end* (*dnd*), *nanos1* (*nos1*), *askopos* (*kop*), *granulito* (*gra*), *deleted in azoospermia-like* (*dazl*), *bruno-like* (*brul*), *h1-type linker histone* (*h1m*) and the *tudor-repeat containing gene* (*Tdrd7*) have localization patterns similar to vasa or co-localize with vasa mRNA, indicating that these are also germ granule components [163, 169, 171, 172, 173]. germ cell less (gcl) and zebrafish oogenesis related gene (zorg) mRNAs also have expression patterns resembling vasa but exclusively for the late stages of zebrafish development [174, 175].

Germ cell less (gcl) is an essential component for initiating the germline differentiation in *Drosophila* [176]. The zebrafish homolog of gcl has a widespread mRNA expression on somatic blastomeres during embryogenesis but later is changed to a specific PGC expression during late developmental

stages. In adult zebrafish, the gcl mRNA is widely expressed on male and female gonads, and its localization suggests its participation on germline development [175]. Other genes that are also expressed on the adult zebrafish gonads are *staufen*, *dmrt1*, *gdf9*, *zpc*, *z-otu*, *zfgcnf*, *zsrg* and *zorg*, but more work is still required to determine if those mRNAs or proteins are germ granule components [174, 177, 178, 179, 180, 181, 182, 183].

Several proteins and their respective mRNAs like Vasa, Nanos1, Dead end, Tdrd7 and Bruno-like are germ granule components. For example, in zebrafish embryos from 2 to 16 cells the brul mRNA and its protein Bruno-like coexist on the germ granules, afterwards *brul* mRNA remains in germ granules whereas Bruno-like protein is widespread on somatic cells [184]. Blocking the expression of Nanos1 and Dead end proteins induces germ cells loss, indicating that these proteins are essential for germline survival [61, 171].

In contrast microinjections of *vas*, *kop* or *gra* anti-sense morpholinos, which effectively blocked their protein expression did not affect the establishment of the germline, keeping unanswered the question about its function [163, 169, 185]. In *Drosophila*, tudor function is crucial to maintain the structural integrity of germ granules. When the zebrafish protein Tdrd7 (that contains a tudor repeat domain) is knocked down by a morpholino, only big or small germ granules are observed while medium sized granules are absent. This suggests that Tdrd7 is required for the germ granules to achieve a homogenous medium size; therefore, this protein is important but not essential for germ granule formation [163].

Dead end is an RNA binding protein that associates with germ granule and is required for germ cell viability. Dead end orthologs in Xenopus, mice and chicken are also expressed in the germline [171]. Morpholino knock down of zebrafish dead end induces PGCs motility problems, which causes the lost of these cells, while no soma effect is observed [171].

Some germ granule components are involved in RNA silencing

In mice lacking *dead end* expression, a testicular tumor similar to human TGCT tumors is developed [186]. *nos1* and *tdrd7* are exclusively found in germ cells mainly because they are degraded in somatic cells by the micro RNA miR-430. In the germline, this miRNA-mediated silencing pathway is repressed by Dead end [187, 188]. Piwi an Argonaute are germ cell specific proteins in mice and its zebrafish orthologs Ziwi and Zili bind to germline specific small RNAs known as piRNAS (Piwi interacting RNAs). Ziwi is essential for germline survival and have a role in silencing repetitive elements

in vertebrates [189], whereas Zilli is required for germ cell differentiation and meiosis [190].

Conclusion

Germ granules in several species seem to storage several RNAs and proteins important for germ cell function and embryogenesis. A fair amount of germ granule components are conserved during evolution; however some of their proteins are specific for a particular group of organisms. Despite this, the role of some of these specific germ granule components is conserved. Among their conserved roles are germ cell transcriptional silencing, mRNA translational control and RNAi.

The low level of preservation of germ cell components among species suggest three scenarios: 1) A multiple origin for germ granules during evolution or 2) A rapid evolution of germ granules. 3) Germ granules are dynamic structures that depending of their components could function in multiple scenarios. To understand further the function of these important RNA granules, more genetic and biochemical approaches need to be done. An obstacle in understanding these structures has been the failure of several groups to purify these RNPs. This problem could be solved now by taken immunoprecipitation approaches that will allow us to isolate more germ granule components.

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2. Morphogenetic cellular rearrangements during early zebrafish development

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Shortly after fertilization the vertebrate embryo starts an intensive cycle of cell division that often do not imply growth in size. This process originates a mass of cells known as blastoderm, where all the cells named blastomeres are identical to their neighbors and at least morphologically, the whole embryo does not give any clue of organization within the three axes of the space. Morphogenetic movements during vertebrate gastrulation guide to the organization of a trilaminar embryo from this initial mass of blastodermal cells. We know much about vertebrate gastrulation thanks to the use of animal models with ex-uterus development including frog or fish embryos. In fact zebrafish embryos have been revealed as a powerful tool to understand vertebrate gastrulation due to the embryo accessibility and the easiness in application of optical and genetic technology for investigation. In these organisms ectoderm, mesoderm and endoderm are formed after the coordination of basically three different processes occurring during vertebrate gastrulation: epiboly, cell internalization and convergence and extension movements. In concert with cellular rearrangements cell determination mechanisms are operating and each progenitor is brought to its appropriate position within the embryo accomplishing the establishment of the body plan in the gastrula.

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Cleavage

Zebrafish eggs consist on a rounded cell of around 500µm diameter made out of yolk and cytoplasm. The newly fertilized egg present a small thickening free of yolk named ooplasm that contain the maternal pronucleus at the animal pole, while the rest of the ooplasm is intermingled with yolk granules thorough the rest of the egg [1]. Preparation of the egg for development requires the separation of organelles and maternal components (i.e. mRNAs or proteins) from the yolk. Rapidly in a mechanism known as ooplasmic segregation cytoplasm separates from the yolk being preferentially accumulated at the prospective animal pole of the embryo [2] to form the blastodisc. Separation of ooplasm from vitelloplasm usually is favored by activation of the egg upon sperm penetration. The cortex of normal eggs contains a meshwork of F-actin based microfilaments associated with the plasma membrane, whose contractility is totally required for ooplasmic segregation to occur [3, 4]. Striking work has been performed in zebrafish embryos by monitoring Ca^{2+} accumulation within the cells. Sperm penetration into the oocyte cytoplasm causes free calcium transients that move from the forming blastodisc into the peripheral cortex of the animal hemisphere correlating with ooplasmic segregation [5, 6]. It has been proposed that these elevated transient of free calcium modulate F-actin microfilaments contractility squeezing the non-yolky cytoplasm. Additionally it is known that microtubules efficiently transport foreign microinjected particles as well as cytoplasmic determinants including maternal mRNA [7-10]. Thus, the egg has at this point a central bulk of yolk surrounded by non-yolky cytoplasm (yolk cytoplasmic layer) preferentially accumulated at the animal pole of the embryo (see Figure 1).

Shortly after fertilization embryonic cells begin to divide in a developmental process known as cleavage (Figure 1). This process is characterized by a series of cell divisions without an increase in cell mass controlled by mechanisms still to be characterized. Zebrafish embryos posses a meroblastic cleavage where divisions are incomplete during the first rounds, thus the big yolk cell remains indivisible and the blastomeres located on top of the big yolky cell undergo cleavage. In zebrafish cleavage proceed symmetrically with a periodicity of around 15 minutes during the first divisions where cell cycle of the blastomeres consists on mitosis and a short interphase [11]. The division pattern can be predicted only for the first set of synchronic division. Thus, the first cleavage furrow (2 blastomeres) is followed by a second perpendicular one (4 blastomeres). Afterwards, three rounds of division take place in the daughter cells where the cleavage furrow align on the same orientation and parallel respect to the first one (8-16-32 blastomeres).

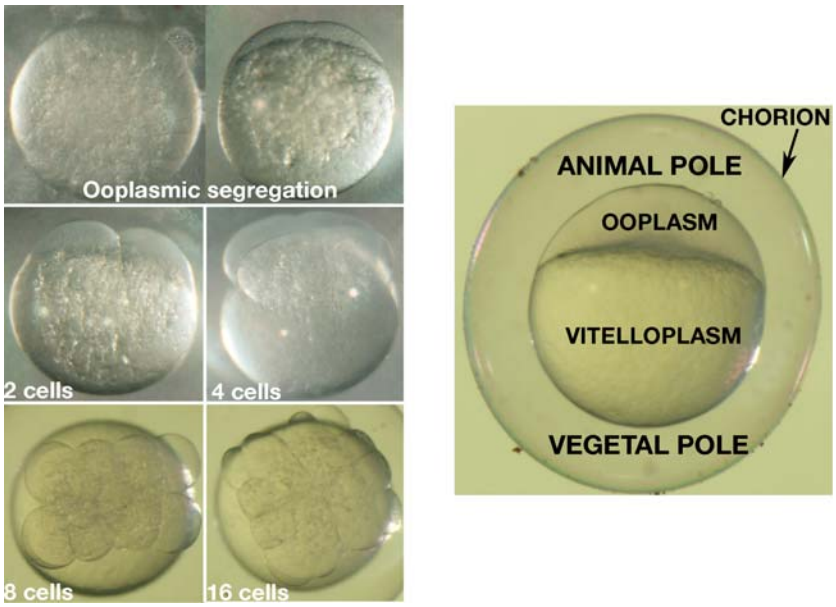

Figure 1. Ooplasmic segregation and cleavage. Shortly after laid, the zebrafish egg accumulates most of the non-yolky cytoplasm at one edge of the oocyte in a mechanism known as ooplamic segregation. After ooplasmic segregation the zebrafish egg consists on a big mass of yolk located on the vegetal pole, with most of the non-yolky cytoplasm located on top, the animal pole. After fertilization meroblastic cleavage begin displaying synchronized cell division for almost 10^{th} cycles in the cell at the animal pole. Yolk cell do not divide but keep cytoplasmic contact with most vegetal blastomeres of the blastoderm.

Next, equatorial divisions undergo (64 blastomeres), but at this point they are much difficult to predict into the blastocist. During the first 3 rounds of division all blastomeres remain communicated with the yolk cell. Staging of the embryo at this early period is based on the number of cells [11, 12].

Ca^{2+} release from internal stores within the cell seems to be involved in modulation of the early events of development. Ca^{2+} accumulation at the forming cytokinetic furrow is totally necessary for cell division to occur and predict the division plane within the blastomeres during cleavage [13-16]. As for ooplasmic segregation, intracellular $Ca2^{+}$ appears to be modulating the F-actin based cytoskeleton controlling the contractile machinery and vesicle trafficking facilitating cell division and providing a source of cell membrane for daughter cells after cytokinesis [17].

In addition it is known that at these early stages of cleavage the molecular mechanisms that are controlling embryo dorsalization are already functioning [18, 19]. It is thought that around the third round of cleavage dorsal specification has been already activated. Thus, dorsal determinants that are initially located at the vegetal pole of the yolk are lately transported animalwards by a special microtubule array. In this regard, ablation of

vegetal regions of the yolk cell causes alterations in the dorsal organization of the embryo when performed during early cleavage stages and not later [7, 20-22].

Concerning dorsal specification, in an embryo as young as at two cell stage there is an asymmetric distribution in the pattern of activation of the MAP Kinase P38 within the blastomeres. This asymmetric activation seems to be induced by the same mechanisms that dorsal specification but it is related with the maintenance of the proper and synchronic cleavage in the prospective dorsal blastomeres with respect to the others. Blocking P38 activation does not cause lost of dorsal fate acquisition but dramatically alters and impairs cytokinesis in cells of the dorsal region. In this regard it has been proposed that synchronic cleavage is an advance acquired in vertebrates, which requires being stimulated. Thus invertebrates usually display asynchronic cleavage and asymmetric P38 activation would keep cells in the dorsal region dividing in the same way than at ventral positions [23].

Along the cleavage period the blastodisc is a mass of non-motile blastomeres on top of a big yolk cell [24, 25]. However around the 512 cells stage, which corresponds to the 10^{th} cell division cycle, a mechanism known as midblastula transition (MBT) takes place and the three major cell lineages of the early embryo start to develop. In general at this point cells slow down the cell cycle and activate their transcriptional machinery [24]. The outer layer of cells behave distinctly from the rest, thus they lose synchronicity of the cell cycle and dramatically slow down cell divisions and acquire pseudoepithelial characters forming a monostratified layer. In this layer, cells are rich in tight junctions and a posses a specialized cortical actin cytoskeleton. This tissue is known as the enveloping layer (EVL) that will become the periderm, an extraembryonic protective covering that is eliminated during late development.

By 3 hours after fertilization, when the embryo is composed of around 1000 cells (1k-cell stage), cells of the blastoderm located at most vegetal positions (marginal blastomeres) that have remained with cytoplasmic communication with the yolk cell, collapse into the yolk cell forming a multinucleate layer (yolk syncytial layer; YSL) in the non-yolky cytoplasm underlaying the blastoderm [26]. Firstly the YSL form a narrow ring along the edge of the blastoderm called external YSL (E-YSL) but rapidly it spreads bellow the blastoderm organizing a complete internal yolk syncytial layer (I-YSL). Nuclei here will maintain cariokinesis only for two to three more cycles and will undergo morphogenetic movements [12]. Between the EVL and the YSL, which are firmly bounded by tight junction along the confluence border, the third lineage of cells form the deep cell layer (DEL) that will later originates during gastrulation all three embryonic germ layers:

ectoderm, endoderm, and mesoderm. In these cells the general efficiency of synchronycyty decreses.

Basically overall morphological changes within the egg provide the clues to establish some transitional stages after MBT and before epiboly. Thus 1k cell stage is followed by high blastula stage and shortly after that the big yolky cell and the overlying blastoderm have reorganized its overall appearance close to a sphere in the so called sphere stage. Around one hour after MBT, following sphere stage, doming of the yolk cell initiates epiboly, a mechanisms by which the blastoderm spreads vegetalwards over the yolk cell increasing its surface area by decreasing its thickness. Morphologic characteristics of the different stages of zebrafish embryonic development have been carefully described by Kimmel et al., 1995 [27].

Epiboly

As mentioned, initiation of epiboly characterizes the dome stage of zebrafish development, a zebrafish stage in which blastodermal cells become increasingly motile. All three early lineages, EVL, YSL, and DEL, participate in this morphogenetic process [28]. The consequence of epiboly movements is that blastoderm overgrows the yolk cell surface, but importantly, accompanied by the EVL and the YSL (see Figure 2). Doming of the yolk helps in cell mixing during epiboly and a mechanism known as radial intercalation is one of the most important pushing force. This process consists on cells from deeper regions moving outwardly to intercalate between more superficial cells. Attempts to establish cell fates on blastoderm cells have failed until shortly before gastrulation as epibolizing morphogenetic movements cause unpredictable cell mixing [29, 30]. However, probabilistic fields of fate have been established [31, 32].

Blastoderm epiboly and, in general, gastrulation movements seems to be partially independent of YSL movements. Epibolic expansion of the YSL is critically dependent of a microtubules array in the yolk cell. Two different arrays of microtubules exist in the cortical cytoplasm, thus the anuclear yolk cytoplasmic layer (YCL) possesses microtubules aligned in the direction of epiboly, extending toward the vegetal pole, and the YSL display the organization of intercrossing interphase or mitotic microtubules. The oriented microtubule array is visible in the yolk cell within the embryo as early as at two cell stage [7]. As epiboly progresses the YCL array of microtubules reorganize to the YSL configuration. Importantly, external disruption of microtubules using UV light or nocodazole considerably alters YSL epiboly but only partially inhibits epiboly in the EVL and DEL [33, 34]. During epiboly it seems that YSL is promoting the stabilization and organization of

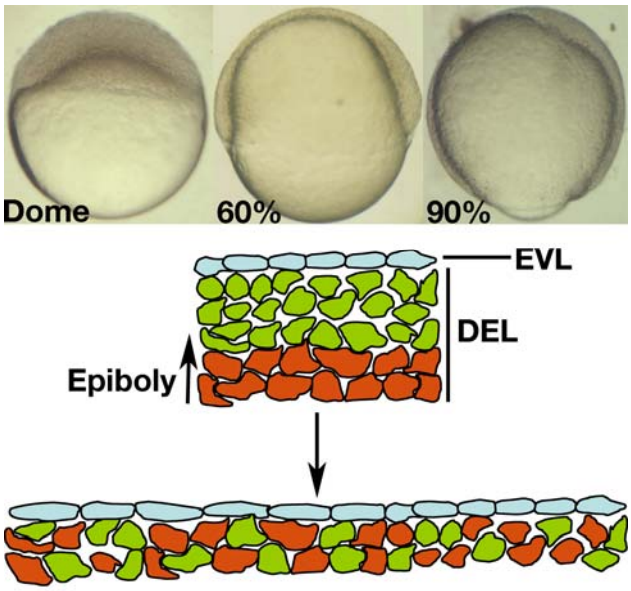

Figure 2. Epiboly. Shortly after midblastula transition the doming of the yolk into the blastoderm causes the initiation of epiboly. Epiboly consists on the covering of the yolk cell by the blastoderm, which spread on its surface. Radial intercalation is a morphodynamic process based on cell movements that cause blasmomeres of the most vegetal layers of the blastoderm to intercalate between the most animal layers of cells. This movement cause the lost of thickness of the blastoderm as it moves vegetalward "engulfing" the yolk cell. Schematic drawings in this figure illustrate the mechanism of radial intercalation representing a portion of the deep cell layer (DEL) under the enveloping layer (EVL).

the microtubules in the yolk cell. Thus the YSL produces pregnelonone from cholesterol through the expression and activation of the esteroidogenic enzyme cholesterol side-chain cleavage enzyme, P450scc (Cyp11a1), what stimulates microtubule stabilization [35].

Massive endocytosis occurs in the external YSL in the proximity of the region where EVL is firmly bounded to the YSL via tight junctions. This area is the invaded by I-YSL what seems to favor EVL movement. However there is also an important mechanism that seems to modulate EVL cell morphology and epiboly. Around 30% epiboly F-actin starts to accumulate at the YSL at the proximity but vegetally to the EVL anchorage area. Actin accumulation is modulated by phosphorilated myosin light chain 2 that organizes a contractile actin ring in this region of the YSL [36]. The MAP kinase 4 Misshapen, which is required for epiboly and gastrulation movements, is modulating the formation of this actin/myosin2 ring. Alterations in the organization of this actin/myosin2 ring formation cause epiboly abrogation affecting exclusively to the EVL, although this alteration secondarily alters deep cell epiboly. All these findings have lead to a model in which local constriction mediated by

the actin ring guide the “engulfment” of the yolk cell by the EVL. Intriguingly it seems to be an evolutionary conserved mechanism as this process based on an actin ring extraordinarily resembles what happens in dorsal closure during Droshopila development [36].

There are however more evidences that indicate that although coordinated and somehow interrelated, DEL epiboly is independent of EVL and YSL. Thus there are several mutants with arrested epiboly in the DEL that present normal EVL and YSL movements [37, 38]. One of the most characteristic epiboly mutants is defective on the gene that encodes E-cadherin [39]. E-cadherin is a cell-membrane protein involved in homotypic cell adhesion. E-cadherin mRNA is provided maternally and it has been shown to be necessary for blastomeres adhesion during cleavage, and at later stages for aspects of morphogenesis during gastrulation and epiboly [40, 41]. Indeed in the developing epiblast E-cadherin is expressed displaying a radial gradient with blastodermal cells on the external layers having higher expression that in the deeper layers. This is translated in differences in cell morphologies being able to distinguish two different layers of cells within the blastoderm with a progressively softer border in between as radial intercalation progresses. Thus, cells of the external layer with high expression of E-cadherin are organized as epithelial-like tissue with polyhedric shapes as they spread below the EVL. However cells of the internal layer with lower expression of E-cadherin are organized more loosely associated and with much rounder shapes. In the E-cadherin mutant *half baked* (*hab*) these two differently organized cell layers are not found [39]. During normal epiboly radial intercalations favor that cells from the internal layer intercalate and integrate within the external layer. However in *hab* mutant these cell intercalations are reduced in number and much slower than in wt, in fact the cells often are not maintained in the external layer of the epiblast and delaminate [39, 42].

During epiboly dorsal specification can be easily detected morphologically and molecularly in the embryo prior to gastrulation. Morphological criteria have been proposed to distinguish the dorsal side of the early embryo. Thus there are reports supporting that when looking at the circumferential boundary separating the blastoderm and the yolk cell, exactly at the point where the dorsal territory is determined, the angle of the separation boundary tends to be equal to 180°. However in the opposite region these surfaces have a different curvature, always lower than 180° [43].

There is also a cluster of deep cells below the EVL and at the margin of the blastoderm named as forerunner cells that are detectable by fluorescent labeling at late blastula and can predict the site of shield formation at the dorsal side of the embryo. At gastrulating stages when the shield forms, these cells are displaced to the leading edge of the blastoderm epiboly movements.

They are the precursors of the Kuppfer's Vesicle and extraordinarily relevant in the organization of the left/right axis of the embryo. It is known that forerunner cells organize a Ca^{2+} based flux responsible of bilateral asymmetry in gene expression [44].

Finally, early during development, microtubular mechanisms maternally orquestrated within the egg guides the accumulation at the prospective dorsal side of the embryo of high ß catenin activity and enrichment in the nodal related protein Squint [45, 46]. This occurs previously to shield formation, which is the equivalent structure of the dorsal lip of the blastopore in amphibians.

Gastrulation

Zebrafish gastrulation consists on a stereotypical set of cellular movements of the blastodermal cells leading to the formation of the three germ layers: ectoderm, mesoderm and endoderm. Gastrulation movements organize the embryo in two transitory layers of cells that will lately generate the trilaminar embryo. Thus, as epiboly reach 50% of the yolk cell, the morphogenetic process of gastrulation starts and the blastoderm begins to lose cells underneath organizing a second cell layer below. At this point upper layer of epibolizing cells is named epiblast, precursor of the ectoderm, while the cellular layer that is being organized bellow the epiblast is known as hypoblast and contains the mesendodermal precursors (that will lately segregate into the mesodermal and endodermal layer). Gastrulation imply three different type of cellular movements which are epiboly as it continues and contributes to germ layer formation, cell internalization where cells delaminate from the epiblast to organize the hypoblast beneath; and convergence and extension movement consisting on mediolateral intercalations that cause accumulation of cells at the dorsal side of the embryo and at the same time contribute to its elongation in the anterior-posterior axis. Lately its being accepted than extension is more complex than only mediolateral intercalation and it seems that once internalized cells can also migrate toward the animal pole in a directed manner as they converge and intercalate. Thus, we could talk also about a fourth movement that undergo concomitantly to convergence and extension. In zebrafish embryos, at contrary to what happen in Xenopus, all movements seem to be quite independent from each other [47, 48].

Germ ring formation and internalization

As mentioned, when blastoderm has covered around 50% of the yolk cell epibolic migration toward the vegetal pole slow down for a short period of

time. This phenomenon responds to the initiation of internalization, what means the initiation of germ layer formation. Cells at the leading edge of blastoderm stop to move vegetalward and change their direction to move firstly inward toward the yolk cell and rapidly start anterior migration toward the animal pole. Cell internalization begins at the dorsal side of the embryo and expands laterally all along the border of the blastoderm [49]. This causes cell accumulation at the marginal region of the blastoderm originating a thickened region just at this level known as the germ ring (see Figure. 3). First cells to internalize at the dorsal side of the embryo will form the embryonic "shield" (see Figure 3). The shield is easily recognizable within the dorsal side of the blastoderm margin as a group of mesendodermal cells that associate after internalization. First cells to internalize constitute the prechordal plate that will become the most anterior mesoderm and endoderm, namely the precursors of the endodermal pharynx and mesodermal derivatives such as the hatching gland [50].

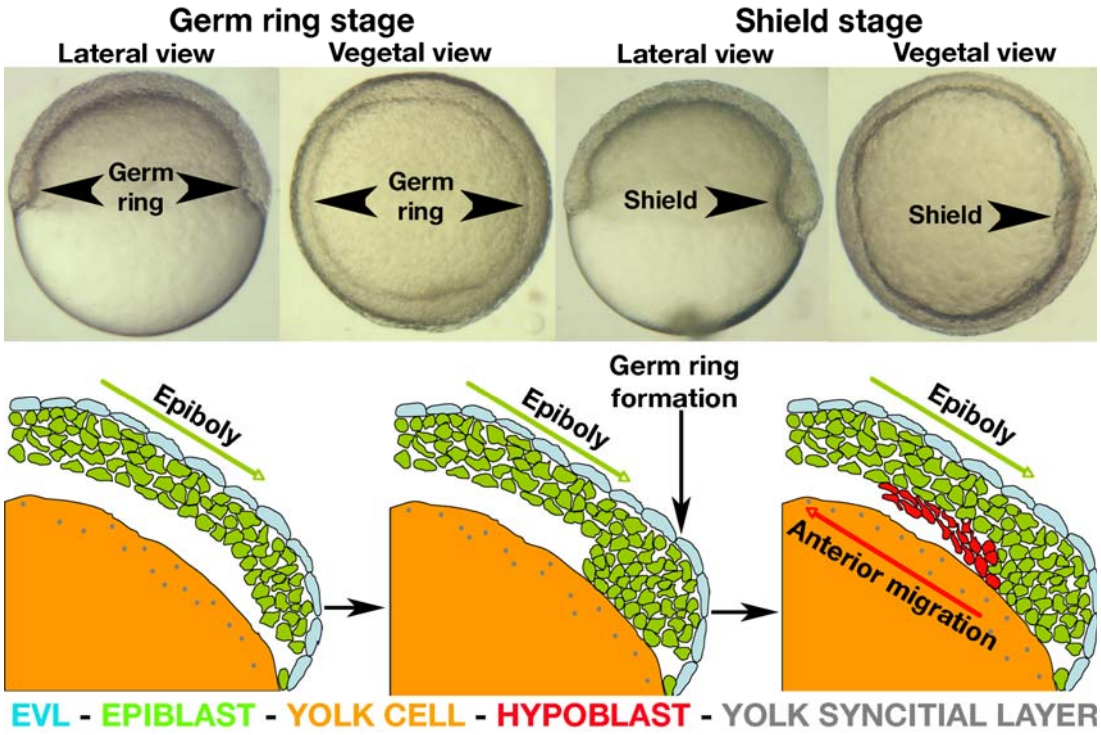

Figure 3. Germ ring formation and anterior migration. Around 50% epiboly, epibolic movements transitorily slow down and cells of the leading edge of the blastoderm margin accumulate generating a thickening all along such margin named the germ ring. Shortly after germ ring formation cells become motile and migrate animalward organizing the hypoblast layer below the epiblast layer. The hypoblast contains the mesendodermal precursor while the epiblast contains the ectodermal precursors. Schematic drawings in this figure illustrate these processes representing a lateral view on a section focusing on the shield forming region.

In amniota embryos cell internalization from the epiblast during gastrulation is mediated by epithelial mesenchyme transition (EMT). EMT involves a mechanism sharing multiple cellular and molecular aspects of tumor metastasis and basically consists on loss of epithelial characteristics of a tissue, including tight cellular junctions, to form a disperse tissue containing motile cells able to spread out and invade new regions [51]. Cell internalization in zebrafish occurs by single cell delamination at the germ ring [42, 52]. However, EMT has not been observed during gastrulation in anamniote embryos [42, 53]. In zebrafish neither the epiblast nor the hypoblast cells exhibit clear epithelial features and both cell types are highly motile showing dramatic changes in their cellular morphology, as they are rich in cellular protrusions [54]. It seems that internalizing mesendodermal progenitor cells may change their general state of adhesiveness, which allows them then to segregate from the epiblast and take on a more mesenchymal appearance [42, 53]. Recently it has been shown that not only differential cell adhesiveness but also distinct tensile forces at the cell cortex, which are modulated by the actomyosin contractile cytoskeleton, are crucial in the segregation in the different tissue progenitors during gastrulation [55]. In conjunction with cell adhesion it seems that segregation of two different tissues depend of the different tensile forces established at the cell cortex at the level of cell to cell or cell to medium interface [55].

Once internalized mesendodermal precursors migrate anteriorly underneath the epibolizing epiblast toward the animal pole (see figure 3). Shortly after internalization mesendodermal cells move upward seeking the epiblast inner surface that they will use as substrate for migration [42]. The differential adhesive properties and cell cortex tensile forces generate a border between both tissues that, in spite of being in contact, avoid cell population intermixing [55]. At this point opposite migration are taking place, thus while the epiblast continues its migration vegetalwards, hypoblast migrates underneath toward the animal pole of the embryo. Axial mesendodermal cells move as a tightly packed group of cells giving rise to axial structures such as the prechordal plate and notochord, while paraxial mesendodermal progenitor cells migrate as more loosely associated mesenchymal cells [25, 56]

Nodal related signals including Cyclops (Cyc) and Squint (Sqt), their cofactor the EGF-CFC protein One eyed pinhead (Oep) and the nodal antagonist Lefty are responsible of mesendodermal cell specification during gastrulation [57, 58]. Maternal-zygotic oep (MZoep) embryos lack response to nodal signals and are not able to induce mesendoderm from the epiblast lacking hypoblast formation. However it seems that even in absence of mesendodermal induction these cells are able to internalize. Thus, on one

hand, when a MZoep mutant cell is transplanted into the blastoderm margin of a wild type host, such cell is able to internalize with the forming hypoblast. Interestingly, this cell is not able to move anteriorly with its wild type partners and move vegetalwards with the vegetal movement of the margin, not contributing to the hypoblast formation. On the other hand if a WT cell is implanted into the blastoderm of a MZoep embryo, it internalizes independently of the absence of partner cells, being able to differentiate into mesendoderm and move toward the animal pole of the embryo [52]. In sum all these suggest that mesendodermal induction is independent of cell internalization but is required for anterior migration.

Anterior migration of the mesendodermal cells

A group of transcription factors regulating anterior migration in the hypoblast is the SNAIL family of transcription factors. Snail1a and Snail1b are expressed in the migrating hypoblast cells in an overlapping manner except at the level of the precordal plate. Loss of function of any of them causes dramatic shortening of the anterior-posterior axis what seem not to be due to cell specifications alterations. However cell migration appears to be dramatically disturbed even in the precordal plate, which is affected non-cell autonomously. It seems that in absence of these transcription factors cells may express cadherin1 (E-cadherin), a feature that does not allow cells to migrate in a dissociated manner. Indeed in zebrafish embryos has been reported that E-cadhein is required for proper anterior cell migration of axial mesendoderm [41]. In wild type embryos absence of Snail1a or Snail1b expression would allow the high level of expression of E-caherin, which is needed for proper anterior migration of this cells, however alteration in the surrounding hypoblast would cause the morphogenetic disturbances due to abnormal cell migration in a non-cell autonomous manner of the precordal plate mesendoderm [53].

STAT3 is a member of the group of signal transducers and activator of transcription that seems to promote cell motility in the mesendodermal precursors of the precordal plate in a cell autonomous manner and in the neighbouring cells in a non-cell autonomous manner. STAT3 has been shown to upregulate the Zn2C transporter LIV1 and it has been proposed that LIV1 promotes hypoblast cell migration. Phenotypes caused by knocking down either STAT3 or LIV1 reflect deficient anterior migration with shortened anterior-posterior axis [59].

Polarized anterior migration toward the animal pole is mediated by the polarized formation of cellular protrusions at the leading edge of mesendodermal cells [54, 60]. Phosphoinositide 3 kinase (PI3K) pathway has

been involved in this process [60]. PI3K is an enzyme involved in phospholipids metabolism regulating the phosphorylation of Phosphatidylinositol (Ptdlns) on the third carbon of the inositol ring. These types of lipids phosphorilated at the position 3 can be recognized at the membrane surface by enzymes containing the pleckstrin homology domain (PH), being collected and activated at the cell surface where they activate their effectors. In zebrafish it has been shown that class IA PI3K is activated at the leading edge of the migrating mesendedormal cells stimulating the production of Ptdlns-3,4,5-P and causing in consequence the recruitment of the kinase PKB(Akt) that posses the PH domain. PKB is activated at the cell surface of such leading edge where it regulates the polarized polymerization of the actin complex required to organize cellular protrusions. Absence of PI3K activity causes loss of cellular protrusions generating much less efficient motility in these rounded mesendodermal cells, which now move much slower with the consequent morphogenetic alterations in the embryo. Platelet derived growth factor (PDGF) has been proposed as the upstream extracellular signal that would activate PI3K upon binding and activation of its tyrosine kinase receptor [60].

Convergence and extension movements

After internalization and as mesendodermal cells start to migrate animalwards, both ectodermal and mesendodermal progenitors undergo Convergence and Extension (CE; see figure 4). In CE cells move towards the dorsal side by mediolateral cell intercalations that cause mediolateral narrowing and anterior-posterior extension of the developing body axis at the dorsal side of the embryo. Molecular mechanisms controlling CE movements in zebrafish are being elucidated on the basis of the analysis of multiple mutants that display typical phenotypes of deficient CE movements, including shorter anterior-posterior axis and wider embryonic structures at the end of gastrulation. Importantly many of these mutants affect the non-canonical WNT signaling pathway, which is independent of ß catenin and shares homologue proteins with the Planar Cell Polarity (PCP) pathway that guide epithelial polarization during drosophila wing development [48]. Mutants deficient in non canonical WNT pathway as pipetail (ppt)/wnt5a [61], knypek (kny)/glipican4/6 [62]; trilobite (tri)/strabismus (stbm)/Van goh-like 2 (Vangl2) [63]; or silberblick (slb)/wnt11 [64] or studies on factors involved in this pathway as Prickle (PK1) [65] or frizzled 7 (Fz7) [66] have demonstrated that this pathway is responsible of the polarized migration and mediolateral intercalation than take place during CE. Interestingly it has been shown that radial intercalation, in a similar fashion that occur in the epiblast

during epiboly, is also involved in convergence and extension movements especially at the level of the paraxial mesoderm [67]. Importantly, the non canonical WNT pathway it is also involved in regulating the polarized behaviour of these cells during such movements [67].

Much advance has been done in the analysis of the slb mutant, which shows one of the most dramatic phenotype in CE [64]. By analyzing this mutant at cellular level it has been demonstrated that Wnt11 modulates proper cell morphology and protrusive activity during CE to achieve proper mediolateral intercalations and extension [54]. Presumably this effect on the control of proper cell shape in order to perform appropriate cell movements is modulated through the activation of small GTPases of the rho family including Rac, Cdc42 or RhoA [68-70]. These proteins are key effectors in modulating cytoskeletal rearrangements and organizing fillopodia, lamellipodia and pseudopodia, which are basic for proper cell migration. However via another small GTPase known as Rab5, largely involved in modulation of the endocytic pathway, Wnt11 regulates the adhesiveness of

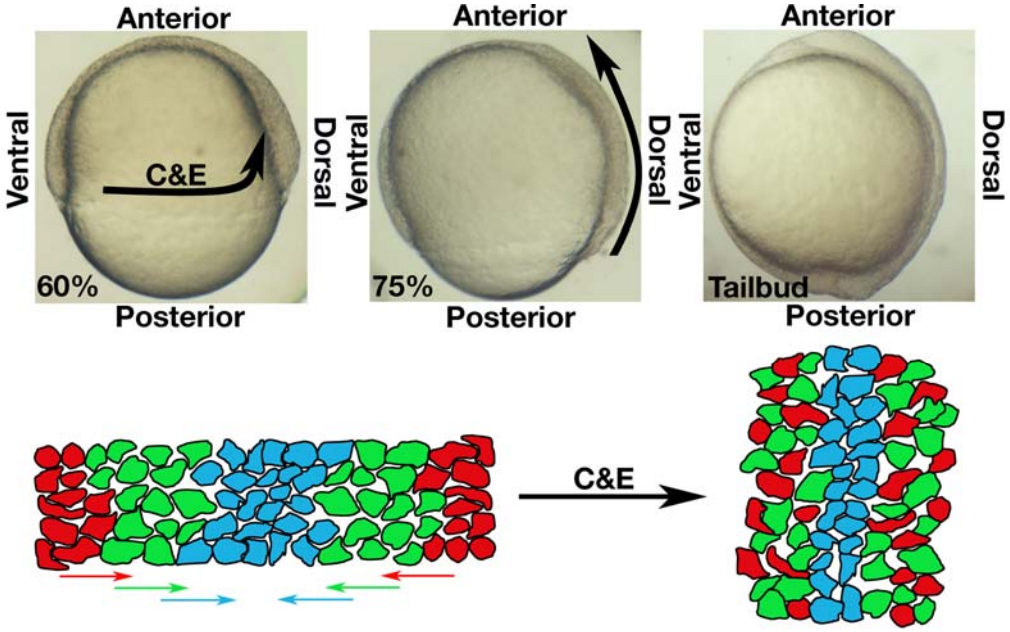

Figure 4. Convergence and extension. As the germ ring forms and anterior migration starts, the cells of the epiblast and hypoblast germ layers begin to converge towards the dorsal side of the gastrula and extend along the forming anterior–posterior body axis. Convergence movements first become apparent in the germ ring by a local thickening at the prospective dorsal side becoming the zebrafish embryonic organizer named ‘shield’. While converging, mesendodermal and ectodermal progenitors undergo medio–lateral cell intercalations, leading to a thinning of the forming body axis along its medio–lateral extent and consequently elongating along the anterior–posterior axis. Schematic drawings in this figure illustrate the mechanism of medio-lateral cell intercalation and subsequent antero-posterior elongation.

mesendodermal cells to control CE movements. Slb mutant cells present weakened adhesive properties in comparison with WT cells, thus both adhesion to extracellular matrix molecules and adhesiveness to the neighboring cells is altered [71, 72]. Wnt11 modulates cellular cohesion by controlling E-cadherin activity via endocytosis. This is modulated through Rab5c that strikingly can rescue the slb phenotype when overexpressed in mutant embryos [72]. It has been them proposed that Wnt11 through Rab5c promotes the ability of mesendodermal cells to dynamically assemble and disassemble E-cadherin-based cell-cell junctions required for effective cell cohesion and migration during gastrulation. Additionally it has also been shown that Wnt11 modulates cell contact persistence by interacting at the cell membrane with its receptor Frizzled 7 (Fz7) and the atypical cadherin Flamingo [66]. In consistence with all these results morphant embryos for E-cadherin, Flamingo (fmi) or Rab5c have been shown to display CE defects during gastrulation [41, 66, 73].

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3. Early origin and differentiation capacity of the neural crest

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This chapter is devoted to the early developmental stages of neural crest cells (NCCs), a fascinating population of migratory cells generated early in development and endowed with a remarkable differentiation potential. These cells contribute to essentially all organs and systems in the body because they play a major role in the formation of the peripheral nervous system. Additionally, they contribute to many other derivatives including: bone, cartilage, tooth forming cells, pigmented cells, muscle, and endocrine cells. The origin, migration, and differentiation potential of NCCs have prompted considerable scientific interest ever since their original description in 1868 by Wilhelm His [1]. Their vast differentiation potential and experimental amenability has made NCCs a great model to study general mechanisms of cell induction, specification, potential, migration, and differentiation. Despite constant interest and the large number of studies focused on neural crest cell development, many enigmas remain unresolved. This chapter provides a brief historical perspective on their discovery, followed by an overview of their early development and relevance to various disciplines. Next, an account of NCC derivatives and of their differentiation potential is provided. Herein will be discussed the perceived segregation and differentiation potential of neural crest cells relative to their axial position in the embryo; this will also include studies that have challenged NCC potential *in vivo* and *in vitro*. The next section provides a depiction of the

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embryonic origin of the neural crest, and the relation of early neurulation to neural crest development. An account of the difficulties to understand the origin and formation of neural crest cells is provided by acknowledging the complexities of the neural plate border, by presenting models for their cellular and molecular induction, and by exposing the tools available to identify and investigate the early development of the neural crest.

Introduction

The amazing neural crest is a multipotent population of cells that originates at the border of the neural epithelium during early development in vertebrates. Later on, NCCs undergo an epithelial to mesenchymal transition (EMT) and delaminate from either 1) the open neural folds (amphibians and mammals); 2) an ectodermal thickening at the neural plate-epidermis boundary (fish); or 3) the closed neural tube (birds). From this departure point, NCCs follow stereotypic migratory pathways permeating throughout (essentially) the entire vertebrate body. The formation of neural crest cells, and their EMT and migration, are carefully orchestrated and proceed sequentially in a rostro-caudal wave closely linked to the rostro-caudal development of the neural epithelia. Due to largely unknown signals, NCCs stop migrating at their final destinations, and in several cases they condense and differentiate; however, some evidence indicates that these cells might initiate differentiation prior to the cessation of migration.

During development, critical events endow daughter cells with different potentials, which are modulated by specific environmental signals. In order to understand how NCCs are able to execute their remarkable migration and differentiation potentials, it seems critical to unveil their cellular origin, and the possible environmental effectors, which clearly depend on the time and regions where NCC precursors appear.

Discovery and early history of the neural crest

The story of the neural crest begins in the hands of the talented Swiss scientist, Wilhelm His (1831-1904). His instrumental work in developing the neuron doctrine that established neurons as individual units is well recognized [2]; however, his reputation was gained prior to this, as His challenged the dominant evolutionary perspective of the time by promoting the study of embryology in its own right. With this, His ignited the movement of experimental embryology by calling for the need to understand the mechanisms underlying the transformation of the fertilized egg into an organism. He provided some of the earliest embryological tools by developing methods to hold, move and cut thin sections of samples with

precision (innovations that have been cited as the origin of the microtome). In a landmark study, His collected chick embryos during the first 2 days of development and generated accurate serial sections that allowed for an analysis of whole embryos for the first time in a systematic way [1].

In this initial study by His of chick embryo sections, neural crest cells are identified for the first time. However, His does not refer to them as neural crest cells. Rather, he identifies a specific set of cells as a middle furrow or groove ("**zwischenrinne"**) surrounding the neural plate in early stages, and as a middle cord or thread ("**zwischengstrang"**) of tissue in between the neural tube and the epidermis in more advanced stages of development [1]. His suggested that these zwischenrinne and zwischenstrang cells are the same, and that they migrate from their original position to generate the cranial and spinal cord ganglia. This proposal endured many years of dedicated research and controversy (see [3] for an animated discussion) to yield our current understanding of the origin, formation and extent of the contributions of the neural crest. His' proposal that the neural crest generated the cranial and spinal cord ganglia was eventually rectified (cranial ganglia actually have a dual origin, made of placodal and neural crest derivatives), and extended to incorporate NCCs as the source of mesenchymal tissues of the head [4-7] all cited in [8]), and the melanocytes of the skin [9-11].

Although neural crest cells initially fell under the umbrella of zwischenrinne and zwischengstrang, the actual term "neural crest" was first coined by Marshall in 1879 [12], and adopted soon after by the rest of the community [13,14]. Marshall referred to the borders of the neural plate as "neural ridges" (on either side of the neural plate at its rostro-caudal axis). As the neural plate closes to form a neural tube, the neural ridges on either side fuse with each other, generating a mass of cells separate from the neural plate and the overlying ectoderm. Initially, Marshall called this mass a neural ridge, but in a 1879 paper he opted for a term to differentiate between the two neural ridges lateral to the neural plate and the neural ridge above the neural tube, baptizing the latter as "**neural crest".** Both the neural ridge and the neural crest correspond closely to the zwischenrinne and zwischenstrang identified by His. Thus, the developmental biology community has embraced the term **Neural Crest** (NC) to refer to the transient cell population found at the edge of the neural plate and dorsal neural tube, which then migrates to contribute to varied derivatives throughout the vertebrate body.

Relevance of neural crest development

Neural Crest cells (NCCs) constitute a fascinating population of multipotent migratory cells that contributes to a wide range of derivatives of

the vertebrate embryo. NCC derivatives include the neurons and supportive cells of the peripheral nervous system, melanocytes, and endocrine cells. In addition, NCCs also generate a set of derivatives collectively known as mesectoderm, which includes a large portion of the head skeleton (both bone and cartilage). The astonishing capacity of neural crest cells to generate such a broad spectrum of derivatives, specifically those traditionally seen as mesodermally derived, collides with our preconceptions of the potential of the three germ lines. Perhaps the early ectoderm has an extended capacity which is lost in epidermal and neural tissues, but not in the neural crest. An alternative proposal is that a fourth germ layer -constituted by NCCs- generates both ectodermal and mesodermal derivatives [15,16]. The semantic debate about the germ layers and their capabilities aside, the extensive differentiation potential of neural crest cells is fascinating, and offers a great model for the study of stem cell biology, pluripotency and differentiation. Additionally, the contribution of NCCs to the head of vertebrates (craniates) has prompted researchers to suggest an intimate link between the appearance of this cell population and vertebrate evolution [17-19].

The diverse differentiation capacity of neural crest cells is suggestive of stem cell like properties. Furthermore, true stem cells derived from this cell population have been isolated from regions colonized by differentiated NCCs (in embryos, newborns and even adults). The isolation of neural crest stem cells was initially achieved from embryonic dorsal root ganglia [20], a tissue derived from the neural crest. More recently, neural crest stem cells have been identified from varied sources in both infants and adults, including the carotid body, the dental papillae, the skin, and the hair follicles [21]. These cells can be propagated *in vitro*, retaining cell renewal capacities, and are able to differentiate into various neural crest derivatives upon proper stimulation.

As a consequence of the robust contribution of NCCs to many specific cells, organs and systems during development, this cell population is implicated in a large number of human pathologies [22-24]. Birth defects resulting from aberrations in NCC development include craniofacial malformations (cleft lip/cleft palate, fetal alcohol syndrome), congenital malformations of the cardiac outflow tract, and Hirschsprung's disease, amongst other disorders. Additionally, neural crest related tumors include melanoma, neuroblastoma, neurofibromatosis, and pheochromocytoma. Thus, studying the basic developmental biology of NCCs is a critical step to improve our understanding of these conditions, and to generate diagnostic and therapeutic strategies. The interest of the scientific community in NCCs can be easily appreciated through the extensive literature available on the subject: more than 150 **reviews** on different aspects of neural crest development and differentiation potential were published from 1998 to 2008

(PubMed “neural crest”[TITLE] Limits: **Publication Date from 1997/01/01 to 2008/08/01, Review**), and three books devoted solely to NCC development have been published and revisited in subsequent editions or updated commentaries [16,25-28]. In the past 140 years, a large number of studies have addressed the origin, migration, and differentiation potential of the neural crest in various model organisms. Recently, within the last 20 years, great progress has also been made regarding the tissue and molecular events leading to crest induction.

Neural crest differentiation potential

Variety. The derivatives generated by NCCs are astonishing due to their variety and number. NCCs generate most of the neurons (sensory, cholinergic and adrenergic) and glia of the peripheral nervous system, all the pigmented cells in the skin (melanocytes), and endocrine cells of the thyroid and adrenal gland. Perhaps the most striking derivative of the NCCs is the mesectoderm, a special mesenchyme from ectodermal origin capable of generating derivatives once thought to be made exclusively by mesoderm. NCC-derived mesectoderm is capable of forming cartilage, endochondral bones, dermal or intramembranous bones, teeth, dermis, smooth muscle, and other connective tissues.

Number. The NCC derivatives referred to above, are only subtypes which represent many different cell types, and which contribute to several tissues and organs throughout the vertebrate body. For example, more than 20 different cranial bones are made by neural crest cells. Furthermore, many different sensory neurons expressing a different array of molecules and employing characteristic neurotransmiters are made by neural crest cells in specific locations of the embryo. The same is true for the other neuronal subtypes (parasympathetic, sympathetic, and enteric).

Regional contributions of NCCs *in vivo*. Fate map studies generated via abalation or grafting strategies have provided a very detailed picture of the specific contribution of neural crest cells from different regions of the avian embryo. Neural crest cells from the cranial region, which are subdivided into prosencephalon, mesencephalon and rhombencephalon groups, generate mesectoderm and pigmented cells. The prosencephalon makes no other crest contributions, while NCCs from a slightly more caudal location (the mesencephalon) generate, in addition to neurons and glia of the parasympathetic and sensory ganglia, mesectoderm and pigmented cells. Yet, in the caudal portion of the rhombencephalon, sensory ganglia, enteric

ganglia and endocrine cells accompany the mesectoderm and pigmented cells as NCC derivatives. In the rest of the embryo (caudal to the head), a similar display of specific derivatives has been described. The cervical spinal cord, thoracic spinal cord, and lumbosacral spinal cord regions generate a variety of derivatives according to specific locations within each territory. However, no mesectoderm or parasympathetic ganglia are generated in these more posterior locations; instead, posterior NCCs contribute to sympathetic and sensory ganglia, and pigmented cells. In addition, the anterior cervical spinal cord and the lumbosacral region (but not the interceding territories) also contribute to the enteric ganglia. Similarly, only the caudal portion of the cervical, and the anterior half of the thoracic spinal cord generate endocrine cells [28,29].

NCC-derived mesectoderm contributes to the formation of the dorsal fin in the trunk region of lower vertebrates [30,31], but in higher vertebrates the capacity to generate mesectodermal derivatives is restricted to the head region where they generate most of the cranium, dermis, odontoblasts, adipocytes, and muscle cells [28]. However, recent studies in the turtle suggest that perhaps the bones of the shell also receive trunk NCC-derived mesectoderm [32], and in mouse embryos two separate studies have recently demonstrated that trunk NCCs generate mesenchymal stem cells which are thought to be able to generate derivatives similar to those made by mesectoderm [33,34].

Experimental *in vivo* differentiation potential of NCCs. Beyond the normal *in vivo* fate of NCCs, their differentiation potential has been tested by heterochronic and heterotopic grafting experiments that placed neural crest cells at earlier or later stages of development, and in different locations of the embryo. It was found that NCCs hold an extensive “regulative” capacity that allows them to modify their differentiation, adapting to external conditions. These cells respond to “new” environments by modulating their expected fate according to the new conditions. Collectively, this research demonstrates that NCCs can generate a wide range of derivatives - much larger than the expected *in vivo* fate for a given region [35-42]. For example, NCCs from the medial trunk region that normally do not contribute to the enteric ganglia readily do so when grafted into the vagal region [42,43].

Because of the large number and variety of derivatives generated by neural crest cells, it seems valid to question whether these derivatives are generated from a heterogeneous population of cells, each with a more modest or restricted differentiation capacity, or whether single cells actually have the differentiation potential to generate the full repertoire of neural crest derivatives.

Clonal analysis of the differentiation potential of NCCs *in vitro:* Experiments launched in the late 1970's in the laboratory of Alan M. Cohen addressed the individual potential of clonally cultured quail NCCs [44,45]. These studies clearly demonstrated that single NCCs generate multiple derivatives *in vitro,* and that progeny of these clonally obtained NCCs, once injected back into normal NC migratory routes, were able to contribute to various derivatives in the embryo [46]. Several other clonal studies have contributed to our current perspective supporting the existence of multipotent NCC precursors in avians [47-52] and mammals [20,53]. The laboratory of Nicole Le Dourain has been instrumental in the identification and characterization of the potential to differentiate and to self renew of the various multipotent NCC precursors. It was only in 2007 that a long awaited NCC precursor, able to generate neurons, glia, melanocytes, myofibroblasts and cartilage (NGMFC) was identified [54]. This study detected a frequency of 6.5% for this NGMFC precursor in clonal cultures of cranial NCCs, which was increased to 18.5% in the presence of Sonic Hedgehog. Today's prevailing model ratifies the sequential restriction of potential differentiation of NCCs, such that precursors with a wide differentiation potential give rise to other precursors with more restricted potential. In general, it is believed that as NCCs emigrate from their original location, they are conformed by a heterogeneous population of cells, some of which hold a wide differentiation potential, others with more restricted potential, and yet some others with a unique potential.

Single cell labeling and the *in vivo* progeny of individual NCCs. In agreement with the *in vitro* clonal studies described above, *in vivo* studies have demonstrated the existence of premigratory and migratory NCC progenitors endowed with multiple differentiation potentials. Single cells were labeled with vital fluorescent dye (lysinated rodamine dextrans), and their fate determined after further embryonic development through morphological, antigenic and positional analysis. For example, melanocytes, dorsal root and sympathetic ganglia cells were found to be derived from a single labeling event [55,56]. Complementing these experiments, single NCCs were labeled by viral infection and their progeny determined. The results obtained confirmed the existence of premigratory NCCs with multipotential capacities to differentiate into various derivatives, and also supported the existence of some other precursors that seem to be restricted and give rise exclusively to one type of derivative [57]. Single cell analysis of NCC derivatives performed in mammal and amphibian embryos [58,59] corroborate these avian results. In zebrafish, NCC potential seems to be restricted in the cranial region at the time of migration, while in the trunk the

existence of both multipotent and restricted precursors have been recorded [60,61].

Embryonic origin of the neural crest cells

NCCs were identified by His as being adjacent to the neural plate of chick embryos. Since then, NCCs have been identified in a similar territory flanking the neural plate in other species, from urodels to mammals. Walter Vogt generated the first fate maps of vertebrates by applying vital dyes to salamander embryos in 1929 [62]. Vogt's maps identified a clear border between the neural plate and the prospective epidermis, and further showed some contribution of this border to NCC-derived peripheral ganglia. Vogt's fate map was later modified by Harrison to properly display the position of neural crest precursors at this neural plate/epidermis border [63]. In the chick embryo however, it was not until 1981 that Rosenquist provided definitive evidence that the origin of the neural crest was at this border [64].

To date, cell labeling techniques and grafting experiments have been used to map the precursors of the neural crest to the border of the presumptive neural plate in all vertebrate embryos examined. However, the precise identity, and location of this border region has been the subject of debate over the years [65]. A recent study has generated a map suggesting a slightly new shape for the early neural plate. More importantly, this map incorporates several molecular markers previously suggested to label neural or epidermal prospective cells [66]. The results of this study suggest that none of these molecular markers (*Ganf, Plato*, *Sox2*, *Otx2* and *Dlx5*) match precisely the shape of the neural plate at early stages; instead, a range of possible combinations might define it. Thus, the molecular nature of the early border region remains unclear.

Neurulation and early NCC development. The neural plate appears early in development after the ectoderm receives signals from the embryonic node and underlying mesoderm. These signals trigger a thickening of the ectoderm that will generate the neural plate. In tetrapods, the neural plate deepens centrally while the lateral edges (neural folds) appear to elevate until they touch each other and fuse in the middle/dorsal portion of the embryo. This is the standard primary neurulation common to most vertebrates.

The appearance of the neural plate proceeds in a rostral to caudal wave, such that while neural folds are fusing in the anterior regions of the embryo, the neural plate is just being formed in more posterior territories. It is therefore believed that as new neural plate is formed, new border territories emerge that include new neural crest precursors.

The complex neural plate border

One could imagine the border of the neural plate as being a sharp line between the thin prospective epidermis and the thick neural plate. However, the border territory is a transition zone between both tissues, and is characterized by a gradual shift from thin to thick epithelium. Traditionally, it has been proposed that the neural crest appears at the neural plate/epidermis border, and this border is a consequence of, or secondary event to, the formation of the neural plate. Initial experiments monitoring the second axis induction by node grafts suggested that neural plate border markers appeared after neural plate markers [67]. More recent experiments, however, have shown that border markers can appear in the absence of neural markers [68].

In an ideal world, the precursors of the neural crest cells would be easily identifiable in the neural plate border, at the neural folds. However, life at the border is a lot more interesting than expected. This border is apparently composed of a heterogeneous mix of cells, intermingled and moving. Within this border reside epidermal cells laterally and prospective neural cells centrally; neural crest cells are adjacent to the neural plate from the midbrain level and downwards. In the head, cranial placodal cells also reside in the border region [69]. At the caudal end of the embryo, along the open neural plate, past the node, resides a stem zone of cells capable of generating mesoderm and neural cells [70]. This stem zone is flanked by the neural plate border containing prospective neural crest cells, which in turn are surrounded by prospective epidermis. At the most posterior end of the stem zone a gastrulating primitive streak can be recognized.

Given that the neural plate border is so complex, it is easy to appreciate the difficulties earlier scientists faced defining the precise location or origin of NCC precursors. This issue has never been fully solved, in part due to our incapacity to isolate neural crest versus neural plate, epidermal or placodal precursors. In fact, during early stages of development, it is unclear whether separate precursors for each lineage exist. Single cell labeling studies suggest that cells in the neural folds are capable of generating epidermal, neural and neural crest cells at early stages; at the time of neural tube closure, both neural and neural crest derivatives are also formed [71]. This latter result suggests that the neural and neural crest lineages do not separate. Adding to this complexity, grafting experiments suggest that neural cells placed in NCC migratory paths can behave like neural crest cells, and that migratory neural crest cells placed in the neural plate acquire central nervous system properties [72,73]. These results indicate that both cell types have an equivalent differentiation potential, and are capable of responding to alternative environments by making the derivatives dictated by the environment.

Collectively, this work supports the view of a shared precursor between neural and neural crest cells.

Specific gene expression has provided molecular markers that label prospective epidermal, neural, placodal, and neural crest cells. However, these markers are often shared amongst two or more of the cell precursors found at the border, and their possible participation on the formation or development of the other cell type(s) has not been fully characterized. Added difficulties emanate from changing patterns of expression, as well as the possible movements of cells in these territories. We can clearly identify neural crest cells after they have initiated their emigration from the lateral regions of the neuroepithelium, and we are also able to identify populations of cells harboring neural crest precursors; however, identifying exclusively early neural crest precursors at this border region is still an unattained goal.

Cellular and molecular events responsible for neural crest formation

The neural folds (where prospective neural crest cells reside) are surrounded by the neural plate medially and by non-neural ectoderm (prospective epidermis) laterally. Additionally, the ectoderm of the embryo is underlined by mesoderm. Specifically, axial mesoderm lays under the central most region of the embryo, and paraxial mesoderm under the more lateral sides of the embryo (beneath the neural folds). The location of these tissues has prompted investigators to suggest their involvement in the formation of the neural crest, and a considerable body of evidence based on juxtaposition experiments performed *in vivo* and *in vitro* supports a role of these tissues in NCC development. Juxtaposition of “naïve” intermediate neural tissue (ventral to the neural folds and dorsal to the ventral midline or prospective floor plate) against prospective epidermis (lateral non-neural ectoderm) triggers crest induction. Interestingly, these experiments have shown that epidermal and neural tissues signal to each other, and both tissues generate neural crest cells. Most attention has focused on the possible signal(s) from the epidermis that trigger neural crest induction in the neural tissue [71,74-80]. However, a similarly large body of experiments demonstrates that mesoderm is the source of induction activity, and it is this germ layer that directs the overlaying ectoderm to form the neural crest [71,80-86].

The participation of both tissues in neural crest induction is possible; however, distinguishing between the real mode of induction *in vivo* and potential experimental artifacts is extremely difficult. The timing of events, which are normally perceived by the expression of specific markers, complicates things further. Inductive events occur at slightly different time

points across model organisms, and the appearance of markers that allows us to distinguish such events also varies. Furthermore, differences in the manner of neural crest induction between models challenges our capacity to resolve this problem. For example, experiments in mice and zebrafish, suggests that mesoderm is not required for NCC induction [87-89]. Similarly, in chick embryos, mesoderm-independent neural crest specification has been recognized at early gastrula stages [90-92]. In contrast to this, it has been shown that in Xenopus a dual role for mesodermally-derived signals involved in neural crest induction exists. Here, an early neural crest induction signal is generated by prospective mesoderm, and a later signal required for the maintenance of the induced state is supplied by the intermediate mesoderm that lies underneath the neural crest territory [93].

At the molecular level, several signals have been identified as neural crest inducers. The most prominent of these are BMP, FGF and Wnt. Studies from the Jessell laboratory were the first to identify, at the molecular level, a neural crest inducer. Members of the TGF-β family (Dorsalin-1, Activin, BMP2, 4, & 7) effectively induce neural crest formation from the naïve neural plate [77,94,95]. Together with studies in *Xenopus* [79,84,96,97] and zebrafish [98,99], this research suggested that BMP signals operate at intermediate concentrations during neural crest formation.

The participation of FGFs in neural crest induction has been better characterized in *Xenopus* embryos, where neuralized ectoderm can be specified to form neural crest by FGF [79,86,100,101]. It was once thought that FGFs could have an indirect role in NCC development, functioning through the induction of mesoderm and/or Wnt signals. However, it has now been shown that, in *Xenopus* embryos, FGF8 can directly induce neural crest in the absence of mesoderm [85].

In whole amphibian embryos, as well as in neuralized animal caps, Wnts are required for neural crest formation [86,102-107]. Furthermore, XWnt-8-mediated signals can establish a lateral neural plate domain, marked by Pax-3 and Msx-1, from which NCCs arise [82]. In zebrafish embryos, Wnt8 is also required for neural crest formation [108]. In chick embryos the requirement and sufficiency of Wnt signals to induce NCC formation has been demonstrated [109], and the role suggested for Wnt6 as a candidate inducer [109] was recently demonstrated [110].

In an attempt to assess the combined role of BMPs, Wnts and FGFs in neural crest cell formation, a recent study investigated the molecular interactions of these signals in *Xenopus*. This work suggested a multi-step process where BMP establishes the neural crest border, along with the expression of an early neural crest marker, Msx1. According to this model, FGF and Wnt signals operate after a BMP signal, and in various subsequent

steps during NCC development [85]. While the synergistic participation of several of these molecules has been corroborated in different organisms, this is not the case for all. Critical differences complicate generalizations about the exact mechanism of NCC induction across species. For example, while intermediate levels of BMP signaling are required for NCC induction in both fish and frog embryos, its role in amniote embryos remains unclear [111-114].

Identification of neural crest cells

Histology, grafting, labeling and antibodies. Identification of NCCs can only be performed once they have emigrated from the neural tube or its borders. This task was achieved initially on cell morphology grounds. Subsequently, scientists introduced grafting experiments [30] using amphibians, with different cell sizes or pigmentation to recognize donor from host tissues (reviewed [25]). In higher vertebrates, a major improvement was the use of grafts from a donor chick embryo "labeled" with tritiated thymidine into un-labeled chick hosts [115]. This technique was surpassed by the arrival of the "quail-chick chimera" [116], based on the ability to identify the quail nucleus after feulgen staining. It took almost fifteen years for the arrival of antibodies that allowed the identification of migrating NCCs (HNK-1 and NC-1 [117,118]). However, these antibodies recognize a sugar motif also expressed by other non-NCC cells in the embryo, preventing their wide use without additional tools. A valuable addition to this set of tools was the QCPN monoclonal antibody, which identifies perinuclear proteins exclusively in quail cells. This allowed for the identification of migratory NCCs of quail origin in chick embryo hosts. And while different labeling approaches and chimeras are available today, the quail-chick strategy still serves as a prime tool to investigate migration, specification, commitment, and differentiation of neural crest cells.

Gene expression and Molecular markers. In addition to the antibodies mentioned above, the arrival of molecular biology brought a whole new approach to the study of developmental biology. The expression of various genes became associated specifically to certain cells, allowing their use as markers. This also sparked an interest in the study of genes themselves to further understand the capacities they convey to the cells that express them. Because transcription factors and regulators play a central role in the modulation of expression of molecules that finally define the properties and capacities of cells, the identification of neural crest related transcription factors and modulators became of utmost relevance. Amongst the first neural crest markers to be identified is the gene Twist, a basic HLH protein involved

in neural crest migration [119]. During the last 15 years, considerable progress has been made towards the identification of additional neural crest-related transcription factors. This list includes, amongst many others: AP2, Zic, Msx1, Msx2, Pax3, Pax7, Snail1, Snail2, Sox8, Sox9, Sox10, FoxD3, cMyc, Ets1, and the proteins of the ID group.

In chick embryos, the transcription factors Ap2, Msx1 and Pax3 and Pax7 all participate in early NCC development. AP2 is expressed in most of the prospective epidermis in a wide, oval territory surrounding the center of the embryo where the prospective neural plate and the primitive streak are located [120]. This expression includes the neural plate border where NCC precursors reside, and excludes the most lateral prospective epidermis. Msx1 and Pax3 are expressed in a “Y” pattern on caudal portions of the neural plate border, as well as in the lateral caudal epiblast, caudal half of the primitive streak, and caudo-lateral mesoderm. Pax7 is co-expressed initially at the neural plate border with Msx1 and Pax3, but it is also expressed in the more rostral neural plate border (slightly more lateral than Pax3). In addition to these markers, the TGFß signaling molecule BMP4 is coexpressed with Msx1, Pax3 and Pax7 in the posterior “Y” pattern, but the anterior expression is extended in an inverted “U” shape that surrounds the prospective neural plate completely. Later in development Snail2 and Sox9, two additional transcription factors, appear in the anterior neural folds. These markers are followed by the expression of FoxD3, cMyc, Msx2, RhoB, and Sox10 in later premigratory and early migrating stages. As NCCs emigrate, they express specific adhesion molecules (Cad7), and surface markers such as the sugar motif recognized by the HNK-1 antibody.

The chick expression data presented above serves as an example of the progression of NCC development, which is accepted to match closely with the order of events in other model organisms. However, some differences exist. For example, Snail1 appears early in frog and mouse NCC development, while in the chick, it is Snail2 that is expressed in early NCC development. The expression of Pax3 and Pax7 is also a good example of slight divergence between species, as in fish and frogs Pax3 is expressed earlier and more anteriorly than Pax7, while the reverse is true in chicks.

Genes required and/or sufficient for NCC development

Our understanding of the regulation, function, and interactions between the NCC molecular markers is limited. Most of our knowledge is based on the study of a few transcription factors expressed by early NCC precursors, or by both premigratory and migratory NCCs. The function of these transcription factors has been investigated through overexpression and

inhibition approaches in whole embryos, or in explanted tissues. These studies have identified transcription factors that are required for neural crest development (Ap2, FoxD3, Msx1, Pax3, Pax7, Snail1, Snail2, Sox9, Sox10, Zic2, etc.), some of which are able to promote an expansion of NCCs in relatively endogenous territories (FoxD3, Pax3, Snail1, Snail2, Sox9, Sox10, Zic1, Zic3, Zic5).

A more stringent test has been to challenge the inductive capacity of certain factors to trigger the formation of neural crest cells in naïve ectoderm. To date, only FoxD3 and Snail1 have been identified as having this capability. However, Snail and/or Snail2, Sox9 and FoxD3 are all vital to neural crest formation, and are considered by the neural crest community to be *bona fide* neural crest markers. Therefore, the current understanding of the participation of these three factors in NCC development is addressed bellow:

Snail. Amongst the most prominent markers of neural crest development are the zinc finger transcription factors of the Snail family, Snail1 and Snail2 (formerly known as Slug [121]). These genes are expressed in neural crest cells, as well as in early mesodermal tissues and are associated with morphological changes and movements including those necessary for the epithelial to mesenchymal transition that neural crest cells undergo prior to their migration [122]. In the chick, Snail2 is expressed in the anterior neural folds before and after NCCs emigrate from the neural tube [123]. In *Xenopus*, both Snail1 and Snail2 are expressed in premigratory and migratory NCCs, and Snail1 operates upstream of Snail2 [124]. In the mouse, Snail1 is expressed in premigratory and migratory NCCs, while Snail2 is only present in the latter [125]. In general the overexpression of Snail genes expands neural crest formation [126], while inhibition of Snail genes prevents neural crest formation and migration [123,127]. This suggests that these factors are required and sufficient for NCC development. However, in the mouse neither of these members seem to be required for NC development [128].

FoxD3. The fork-head or winged-helix transcription factor FoxD3, is expressed in prospective and migrating NCCs in mice, frog, fish and chick embryos. Its earliest expression is concomitant with the expression of Snail genes. Overexpression experiments offer controversial results, in some contexts leading to an expansion of the neural crest territory. Furthermore, combinatorial experiments suggest that Snail genes and FoxD3 genes may act in parallel pathways leading to NCC development [129,130]. In the mouse, FoxD3 is required for implantation, and conditional removal of FoxD3 in NCCs leads to a dramatic reduction and loss of NC derivatives in cranial and trunk regions [131].

SoxE. SoxE genes (Sox8, Sox9 and Sox10) are a subgroup of transcription factors containing a high mobility group (HMG) DNA binding box. SoxE genes are also expressed by precursors and migratory NCCs, and their functions are required for different aspects of NCC development. Sox9 appears to be critical for early NCC development, and downregulation experiments demonstrate this requirement. In contrast, overexpression experiments suggest that Sox9 can trigger the formation of neural crest cells. Mice deficient for Sox9 display regional differences regarding the role of Sox9 in crest development. In the cranial region, NCCs apparently form and migrate normally and it is only later aspects of NCC differentiation into cartilage that display marked aberrations [132]. Instead, Sox9 deficient mice display increased cell death in trunk levels likely responsible for a reduction of NCCs and dorsal neural tube markers [130].

Neural crest genetic network

The expression, function and interaction of many neural crest markers has been incorporated into models as a cascade of gene regulation [133], or a neural crest gene regulatory network [111,134,135] that propose a logical progression of gene expression and function regulating neural crest development from their induction to eventual migration and differentiation.

Early induction events involve at least 5 signaling pathways triggered by BMP, FGF, Notch, RA and Wnt molecules. This induction triggers the expression of early neural crest markers, also called border specifiers (Zic genes, Msx1, Pax3/7, and Dlx), as they appear early in development flanking the neural plate. Later in development, it is proposed that the expression of these early neural crest marker genes, in association with more signaling pathways, leads to the expression of neural crest markers or neural crest specifiers (transcription factors Snail and Snail2, SoxE, FoxD3, Ap2, Id family members, etc). The expression of these neural crest marker or specifier genes, in turn, is thought to lead to the expression of late neural crest markers or effector genes, that regulate various aspects of the neural crest phenotype - including the epithelial to mesenchymal transition, the emigration from the neural plate or tube, the stereotypic migration, cessation of migration and differentiation into the various different cell derivatives (RhoB, Cad7, Col2a, cRet, Mitf, etc). It is thus apparent that signaling pathways are reiteratively used and participate in later stages of development in conjunction with corresponding sets of transcription factors to regulate later events in neural crest development.

The proposed neural crest cascade and gene regulatory network is a starting place that provides a useful framework to better understand and study

neural crest development. It consolidates available data, and facilitates the segregation of different components (signals, transcription factors and other molecules) or processes. It also facilitates comparative studies between different species enabling phylogenetic analysis. However, the available information regarding the direct or indirect regulation of these components, the minimal understanding of the existing and participating splice variants, and the lack of knowledge regarding their protein-protein interactions offers a wide and fertile working environment to improve our understanding of NCC development.

Concluding remarks

Despite intense research surrounding neural crest cells, many questions remain to be answered. It will be critical to resolve the pending issues on the maintenance, segregation, and distribution of different multipotent precursors. It will also be important to increase our knowledge regarding the function and molecular interactions between the genes and proteins that have been identified as important players in NCC development. Additionally, we are likely to identify new players as partners of these molecules, or as new independent components relevant for neural crest development. It would not be surprising either to identify new NC derivatives and a broader presence of NCC stem cells in adult tissues. After nearly 150 years of neural crest research, we have learned a great deal about their capabilities, migratory paths, and origin, and we are beginning to unravel the molecular underpinnings of their formidable capacities. The near future promises a true understanding of neural crest biology, which will lead to diagnostic and therapeutic strategies addressing the large number of human conditions derived from NCC anomalies.

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4. Gonadal development

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Sexual reproduction requires the presence of dimorphic adult individuals who are able to produce either oocytes or spermatozoa. In mammals, sexual characteristics are established at three stages along development: i. Fertilization, ii. Gonadal sex determination and iii. somatic sex differentiation. In the current chapter we focus on the gonadal determination stage integrating classical concepts with recent results obtained using new technological methods. Alfred Jost's paradigm on mammalian sex differentiation clarified that formation of functional testes cause phenotypic male differentiation by preventing the expression of a female program. Thus, the mechanisms underlying the formation of either testes or ovaries are crucial knowledge for our understanding of dimorphic sex. Most data come from the mouse as model system. Three genes acting as transcription factors play the leading role in gonadal sex determination. The gene Sry together with Sf1 act as coactivators in Sox9 upregulation, and decide the fate of the somatic supporting cells as preSertoli cells. Following the short period of Sry expression, Sox9 is maintained at the center of a complicated network of gene pathways encoding signaling proteins, receptors, transcription factors, etc. necessary for the establishment of functional testes. Simultaneously, XX undifferentiated gonads initiate the female program required for ovarian

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differentiation. Genes Wnt4, Foxl2, Dax1 and Rspo1 are part of the ovarian network of gene expression underlying the formation of the ovaries. In contrast to female somatic gonadal cells, germ cells start precocious differentiation by entering meiosis long before male germ cells. Retinoic acid (RA) produced in the adjacent mesonephros appears as the long-time sought "meiosis inducing factor". Since RA is produced in the mesonephros of both sexes, male germ cells are prevented from entering meiosis by the action of the RA-degrading enzyme CYP26B1 produced in Sertoli cells of the seminiferous cords. Although advances in our understanding of mammalian sex establishment are considerable using the mouse model system, several important cues remain unknown. Most importantly, the extent to which the molecular mechanisms found in the mouse can be applied to other mammalian species including humans, still remains to be investigated.

Introduction

Two years after the Second World War, Alfred Jost published his seminal study "Rôle des Gonades Fetales Dans la Differentiation Sexuelle Somatique" (1947) [1]. Since then, the still valid paradigm of sexual differentiation in placental mammals was established. Although the question asked by Jost was basically simple, the surgical skills he employed were so difficult that nobody has ever been able to repeat the experiment. The question was: What happens to fetal development if they are castrated before phenotypic sexual differentiation? Using the rabbit as a model, Jost castrated fetuses in utero at around 20 days post coitum (dpc) and found that Mullerian and Wolffian ducts and the urogenital sinus spontaneously developed the female phenotype. He went on and discovered that female fetuses with grafted testis developed as phenotypic males. With these results, Jost was able to postulate that fetal testis produce factors necessary for induction of male development on an otherwise intrinsic female developmental program. Since at Jost's time the sex chromosomes were still unknown, a genetic approach to elucidate the molecular mechanisms underlying testis determination was distant. In this chapter we review some of the most recent investigations that are gradually increasing our understanding of the complex process of sex determination in mammals.

I. Genetic sex, sex determination and sex differentiation

Mammalian sex involves three chronological stages of development. The first stage is when "genetic sex" is determined, and it occurs soon after fertilization when the fate of the zygote's sex is defined by the presence of

either an X or a Y chromosome in the male pronucleus. The second stage known as “sexual determination” involves molecular processes that open alternative pathways of gene expression previous to morphologic sexual differentiation of the embryonic gonad. Thus, “gonadal sex determination” is the establishment and stabilization of dimorphic gene expression pathways that lead to differentiation of either ovaries or testes. The third stage of sexual determination was named “somatic sexual differentiation” by Alfred Jost [2] and refers to the sexual differentiation of the Wolffian and Müllerian ducts and of the urogenital sinus, the embryonic precursors of the genital tracts and of the external sexual organs, respectively. However, the developmental process that leads to sexual dimorphism of the individual continues after birth, when the neuroendocrine mechanisms responsible for the secondary sexual characteristics start to function. Lastly, the sexually dimorphic pattern of behavior of each gender, which ultimately leads to the fusion of sperm and oocyte, completes the sexual reproduction cycle.

The classic experiments [1] showed that in rabbit fetuses castrated at stages previous to somatic sexual differentiation, the urogenital ducts and sinuses follow a feminizing pathway independent of the genetic sex. Jost described that fetal testicles produce two factors necessary for male somatic differentiation, testosterone (T) and antimullerian hormone (AMH). In their absence, the Müllerian ducts form the oviducts, the uterus and the upper third of the vagina, and the urogenital sinus forms the clitoris and vulva of the castrated male fetus. On the other hand, if testicles are implanted to a castrated female, the Müllerian ducts regress, and the Wollfian ducts form the epidydimis and deferent ducts and the urogenital sinus forms the glans penis, penis and scrotal bag. Thus, establishment of the somatic sex depends on the determination and differentiation of the fetal testicles. In this chapter we describe the molecular bases of “gonadal sex determination” in mammals taking the mouse as a model, always considering the intrinsic limitations of extrapolating these results to other species, including humans.

II. The mouse as model system

In the mouse, the gonadal crest is established 10-12 days post-coitus (dpc) and, the period that corresponds to 10.5-11-5 dpc is considered critical for sex determination at molecular level determination of genetic males (XY) takes place, characterized by the beginning of seminiferous cord formation. In the females, ovaries do not show morphological differences at 12.5 dpc compared to the undifferentiated gonad; nevertheless, the gonads grow due to germ and somatic cell proliferation. Hence, in approximately 48 h, a couple of genetic expression pathways are set in motion in the morphologically

undifferentiated gonad which lead to the differentiation of a testicle in type XY or an ovary in type XX embryos.

The gene cascade involved in sex determination is not a lineal process where expression of a gene leads to positive or negative regulation of the following gene. The process of sex determination constitutes a network of molecular events, in which many regulatory pathways are intertwined. Thus, the discovery of new genes has added to this complex net, which was previously regarded as simple and unidirectional when the *Sry* gene was discovered.

III. Gonadal crest establishment

The mouse gonad originates in the genital crest located in the ventral region of the mesonephros. Here, between 9.5 and 10.5 dpc, cell proliferation of the coelomic epithelium coincides with the arrival of the first primordial germ cells (PGC), whose presence marks the extension where the genital crest will be situated (Fig. 1A-D).

At 11.5 dpc, the genital crest protrudes into the coelomic cavity and may be easily distinguished as an elongated form of different color and texture to the adjacent mesonephros. Until this stage of development, the histological composition of the gonad is similar in XX and XY embryos. Two incipient tissue compartments, the epithelial and the stromal, form the histological substrate of the undifferentiated gonad. In essence, the epithelial compartment develops from the proliferation of coelomic epithelium cells, as mentioned before. Tightly associated with each other and with the PGC, epithelial cells gradually initiate the deposit of a basal lamina, which will lead to the demarcation of the gonad's epithelial compartment. In males, epithelial cells are precursors of Sertoli cells, and in females, they are precursors of follicular cells. PGCs included in this compartment will give origin to spermatogonia and oocytes in XY and XX embryos respectively. On the other hand, the stromal compartment is formed by blood vessels, mesenchymal cells, fibroblasts and an extracellular matrix which includes collagen fibers and fibronectin. Initially (9.5-10.5 dpc), the stromal compartment is distributed along the genital crest between the mesonephros and the coelomic epithelium thickening. On day 11.5 dpc, blood vessels penetrate from the mesonephros as part of the stromal tissue and are distributed among the epithelial clusters, in such way that the deposit of the basal lamina becomes apparent simultaneously with the presence of incipient epithelial tissue. From this stage on, the undifferentiated gonad will begin a transformation, which will lead to the sexual dimorphism evident at 12.5 dpc.

IV. Morfological differentiation

Two morphogenetic processes occur in XY embryos, which reveal the formation of a testis: separation of the epithelial compartment from the coelomic epithelium, and penetration of abundant stromal tissue from the mesonephric region. Both events call attention to the presence of a transitory epithelial reticulum, which will generate the seminiferous cords also known as testicular cords. Leydig precursor cells make their appearance in the stromal tissue and, later, myoid cells will consolidate the assembly of the seminiferous cords. On the other hand, in 12.5 dpc XX embryos, few changes occur regarding the histology of the undifferentiated gonad. However, in contrast with males, PGCs initiate meiotic prophase I at 13.5 dpc. Since, in mice, migratory PGCs that are not incorporated into the genital crest start meiosis regardless of the genetic sex of the embryo, it follows that the somatic cells of the male crest inhibit this process [3]. Thus, it may be postulated that, besides determining Sertoli cell formation and the morphogenetic changes that lead to the development of the testis, the sex determination cascade includes inhibition of fetal meiosis in males.

It should be emphasized that the process of sexual determination takes place before the morphologic differentiation of the gonad. Indeed, microarray results of genital ridges showed dimorphic expression of numerous genes at 11.5 dpc. While one study reported 61 and 25 upregulated genes in male and female genital ridges respectively [4], other laboratory reported 266 upregulated and 55 downregulated genes in males and 243 upregulated in females [5].

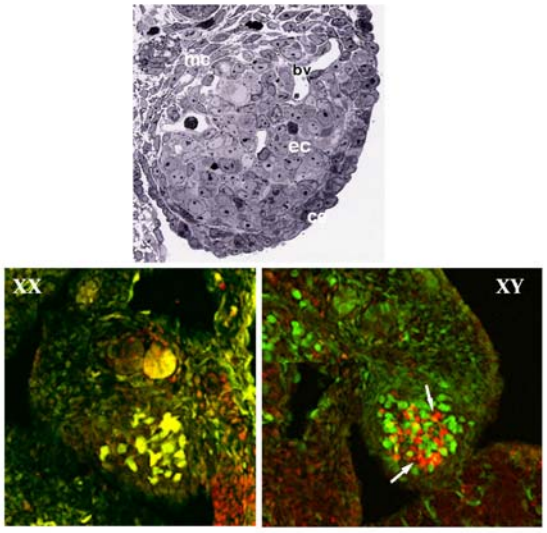

Figure 1. Cross sections of mouse genital ridges at 11.5 dpc. Immunoflourescence with an antibody against Sox9 reveals red stained preSertoli cells among the core cells of the genital ridge (see description in text).

Figure 1 shows cross sections of mouse genital ridges at 11.5 dpc. Top panel in Fig. 1 is a plastic embedded sample showing several kinds of cells: coelomic epithelial cell (ce), mesenchymal cells (mc), epithelioid cells (ec) and blood vessels (bv). The two bottom panels correspond to frozen sections of Rosa26 mouse strain which express green fluorescent protein in most cells. Immunoflourescence with an antibody against Sox9 reveals red stained preSertoli cells among the core cells of the genital ridge (arrows).

V. Genes implicated in the establishment of the bipotential gonad

Several genes have been implicated in the bipotential gonad formation. Among them are *Wt1* and *Sf1*, which were initially proposed as key genes to start the formation of the genital crest. However, studies with null mice allowed determining their role as important genes in crest maintenance, and not in its formation [6, 7, 8]. As will be discussed later, both *Wt1* and *Sf1* are also central to the processes of sexual determination and differentiation of the gonad. The genes *Lhx9* [9], *Emx2* [10] and *M33* [11] are also expressed in the undifferentiated genital crest. As in the case of $Wt1^{-/-}$ and $Sf1^{-/-}$, when embryos develop with these three knock-out genes, they do not maintain the genital crests. Nonetheless, their exact function remains to be determined.

A. The testis-determining genes: *Sry* and *Sox9*

In mammals, the Sry (**S**ex-determining **R**egion of the **Y** chromosome) gene is located on the short arm of the Y chromosome. The product of the *SRY/Sry* gene has been described as a transcription factor. The coding region of the human *Sry* gene corresponds to a single exon which codes for a protein of 204 amino acids. The SRY protein is characterized by three regions. The central region, which is the only domain extensively conserved among species, corresponds to the HMG box. Several mutations have been found in the HMG box in XY women who develop gonadal dysgenesis. The N-terminal domain does not possess a conserved region whose function can be deduced, however, it is susceptible to phosphorylation, which could increase the affinity of SRY to the target DNA. Regarding its structure, in the mouse it possesses a glutamine-rich region, which is not found in the human SRY nor in the proteins of other mammalian species [12]. Gene *Sry* is expressed in the testicle during the critical period of sex determination. In the murine model, *Sry* transcripts are detected since 10.5 dpc; gene expression peaks at 11.5 dpc and subsides at 12.5 dpc [13]. In transgenic mice, 14 Kbs fragments of genomic DNA containing the *Sry* sequence were enough to direct testicular differentiation and generated sexual

reversion in XX mice [14]. This experiment conclusively showed that the gene *Sry* corresponded to the testicle-determining factor (TDF), which had been previously postulated by several authors.

The *Sox-9* (**S**ry-like HMG b**ox 9**) gene is an autosomic gene of the *Sry* family. The SOX9 protein possesses two transcriptional activation domains located downstream of the HMG box. One of these, the PQS domain, is rich in proline-glutamine-serine residues and is indispensable for transcriptional activity. The adjacent domain (PQA) is rich in proline-glutamine-alanine, and is required to maintain maximal transcription activity, since mutations in this domain have been found to diminish the transactivation capacity of *Sox9* [12] (Figure 2B). In mice embryos of 10.5 dpc, low levels of *Sox9* expression are detected along the urogenital crest in both sexes. However, this low expression level is diffuse along the urogenital crest, i.e., it is not specifically located in testicular pre-Sertoli cells or in ovarian granulose cells. At 11.5 dpc, *Sox9* expression becomes clearly dimorphic, since strong expression is detected in the male urogenital crest, while none is observed in the ovary. At 12.5 and 13.5 dpc, Sox9 expression in the testicle is limited to the sexual cords [15, 16, 17]. It has been suggested that *Sry* regulates *Sox9* expression due to the space-time pattern of these two genes. Pre-Sertoli cells positively regulate *Sox9* expression four hours after *Sry* expression is initiated [15, 17, 18, 19].

Sekido and Lovell-Badge [20] recently found an element that acts as enhancer involved in the regulation of *Sox9* expression in the mouse gonad. It is a sequence of 1.4 Kb conserved in placental mammals, humans included, named TESCO (testis-specific enhancer of *Sox9* core). They found that SRY synergically interacts with SF1, activating TESCO and positively regulating *Sox9* expression. The authors propose that SF1 plays a key role in the determination and differentiation of the testicle during the early stages of development. During the first stage, its presence in the bipotential gonad “sensitizes” the *Sox9* enhancer facilitating the interaction with SRY. They also speculate on the mechanism of “sensitisation” as a change in chromatin structure by removing repressors that allow low levels of transcription. Then, SRY/SF1 bring the enhancer closer to the promoter and/or generate a platform which permits the interaction of other coactivators with SRY and with their DNA-binding sites. In the second stage, SRY and SF1 bind to their target sequences in TESCO, increasing the enhancer activity and levels of *Sox9* expression. Finally, the third stage takes place when SOX9 has accumulated beyond a critical threshold in which several loops independent of positive feedback are triggered, which in turn increase Sox9 expression and maintain it after *Sry* has turned off. These feedback loops include the SOX9/SF1 protein-protein interaction and its direct binding to TESCO, thus increasing its activity.

Figure 2 illustrates the key role played by SF1 in the sexual determination and differentiation of the testicle. *Sf1* expression at 9.5 and 10.5 dpc in the genital crests of both sexes suggests its participation in the establishment of the bipotential gonad. Transgenic null *Sf1* mice initiate the formation of the genital crest which then disappears approximately at 11.5 dpc [6], as has been similarly described for *Wt1* null mice (Kreidberg et al., 1993) [7]. In the genital crest of wild-type XY mice, SF1 probably interacts with WT1 to activate the expression of *Sry* during the establishment of the genital crest [21, 22, 23]. Between 10.5 and 11.5 dpc, the interaction of SF1 with SRY increases SOX9 expression in Sertoli precursor cells, thus preparing them for physiologic differentiation. SF1 interacts with SOX9, activating *Amh* expression some time between 11.5 and 15.5 dpc to inhibit the development of the Müllerian ducts, an indispensable step in male somatic sexual differentiation.

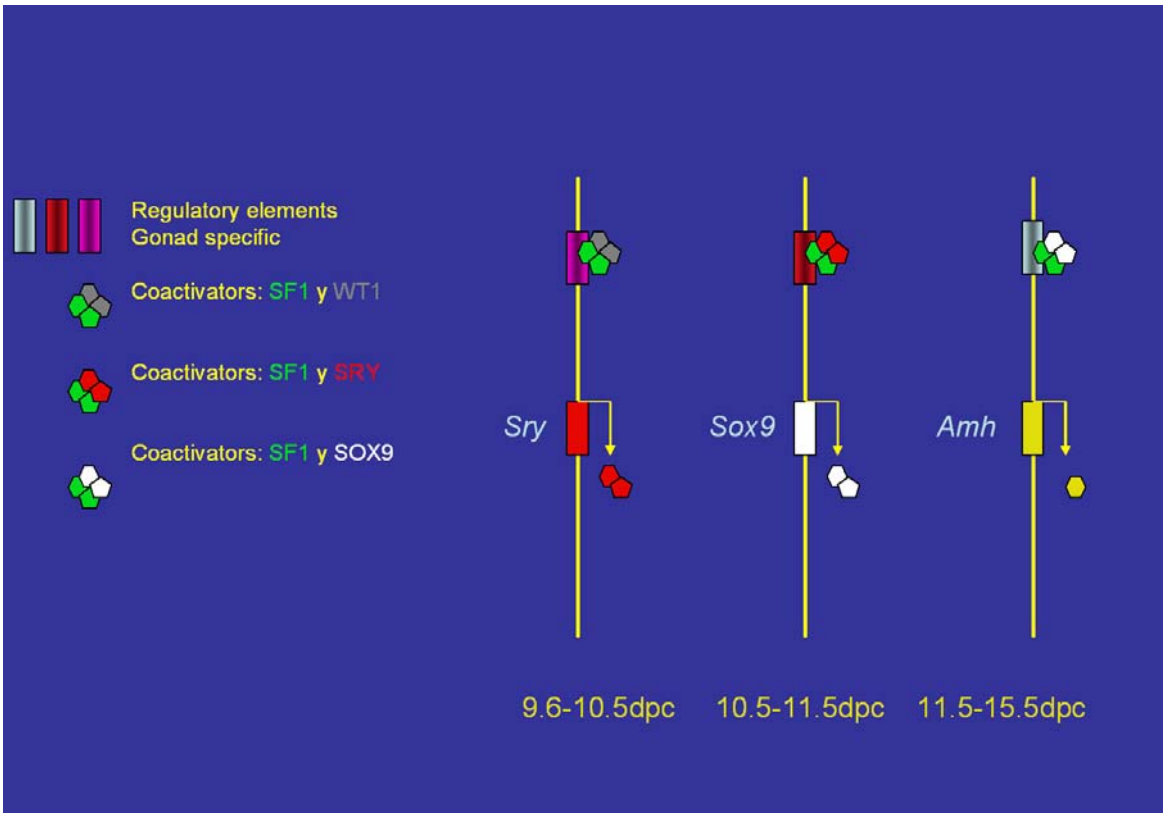

Figure 2. Schematic representation of the role played WT1, SF1, SRY and SOX9 in the sexual determination and differentiation of testicle. The SF1 and WT1 regulating positively Sry expression. Thus, SRY interacts with SF1, to positively regulating Sox9 expression. SF1 interacts with SOX9, activating Amh expression to inhibit the development of the Müllerian ducts as an indispensable step in male somatic sexual differentiation.

As in any developmental process, gene expression occurs simultaneous to cell proliferation and tissue morphogenesis. Figure 3 shows a schematic representation of the position of cells in a transversal section of the genital crest central region. At 9.5 dpc, the thickening of the coelomic epithelium becomes evident. Its cells express, among others, transcription factors common to the two sexes. As they proliferate, coelomic epithelial cells disintegrate the basal membrane that separated them from mesenchymal cells (not shown). Daughter cells accumulate and remain closely associated; they are now known as "supporting cells" that surround primordial germ cells (not shown). In XY embryos, these supporting cells maintain *Sf1* expression and initiate *Sry* expression, and subsequently, the onset of *Sox9* expression can be detected. It should be stressed that the expression sequence is asynchronous, so that pre-Sertoli cells expressing *Sf1* coexist with other such cells expressing *Sry* and/or *Sox9* in the undifferentiated gonad. In addition, other cells which precociously express *Amh* initiate the physiologic differentiation of pre-Sertoli to Sertoli cells. Finally, at 12.5 dpc, the morphologic differentiation of the testis becomes evident as the seminiferous tubules form.

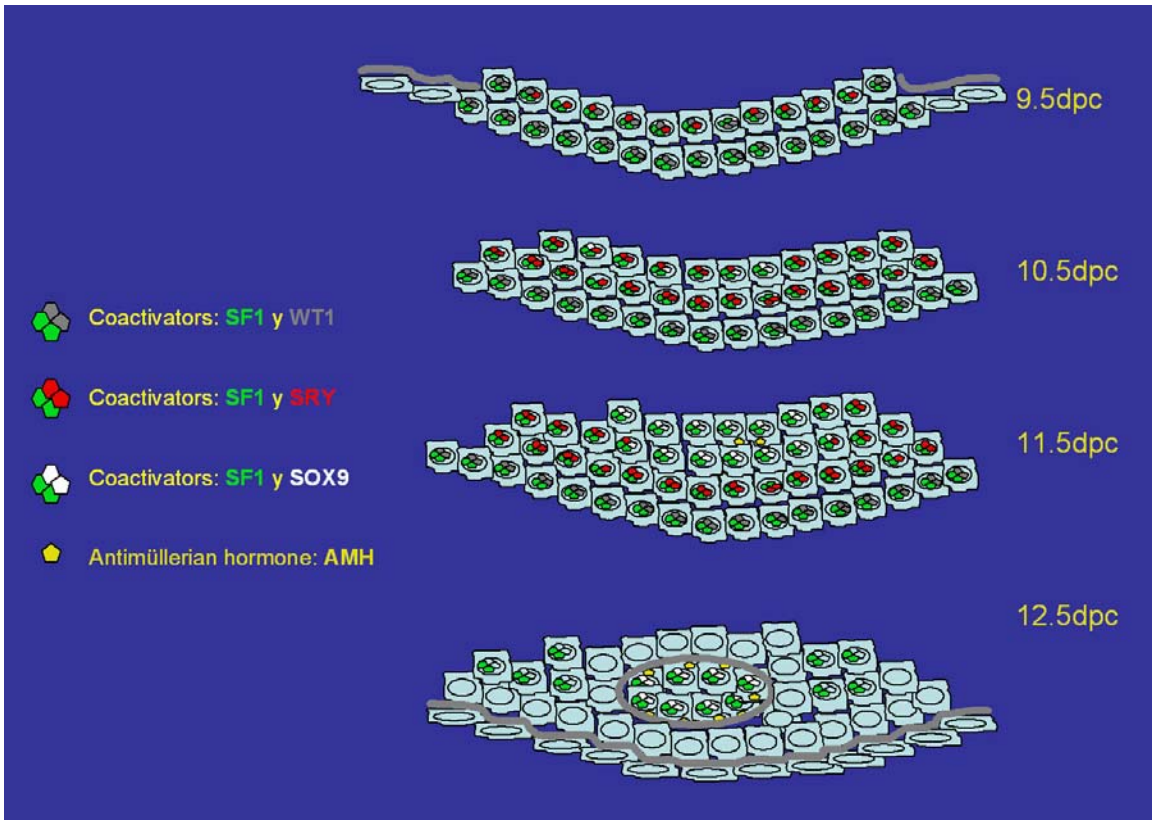

Figure 3. Schematic representation of position of cells in a cross section of the genital crest central region. In XY embryos, these supporting cells maintain Sf1 expression and initiate Sry expression. Subsequently the onset of Sox9 expression can be detected.

B. Hypothetical genetic network of testis determination

Figure 4 proposes a molecular cascade of testicle determination. The presence of the *Sry* gene is the key to the regulation of this process. The hypomethylated regulating region of *Sry* is an important point of access for transcription and co-activating factors, which together with the transcription machinery, positively regulate *Sry* expression [24]. In addition to SF1, WT1 is able to bind directly to regulating elements of the *Sry* promoter region and positively regulate its expression [21]; [22, 23]. Another component of the sexual determination cascade is GATA-4 and its cofactor FOG2. Interaction of these two factors is necessary to reach peak expression of *Sry* at 11.5 dpc; however, its mechanism of activation is not known [25]. The different members of the tyrosine-kinase insulin receptors (IR, IRR, IGF1r) cooperate to positively regulate the expression of *Sry* since the triple knockout for these receptors ($Ir^{-/-}$, $Irr^{-/-}$, $Igf1r^{-/-}$) shows male to female sexual reversion [26].

Before the discovery of TESCO, it was known that *Sox9* expression is positively regulated in the testicle four hours after *Sry* expression is initiated [15, 17, 18, 19]. Once SRY levels are abated, WT1 could be relevant keeping up *Sox9* expression, and therefore Sertoli cell maintenance [8]. *Fgf9* expression is critical to maintain *Sox9* expression, as a feedback loop is established between these two genes. This ensures the maintenance of the differentiated testicle, as shown in $Fgf9^{-/-}$ knockout mice [27, 28].

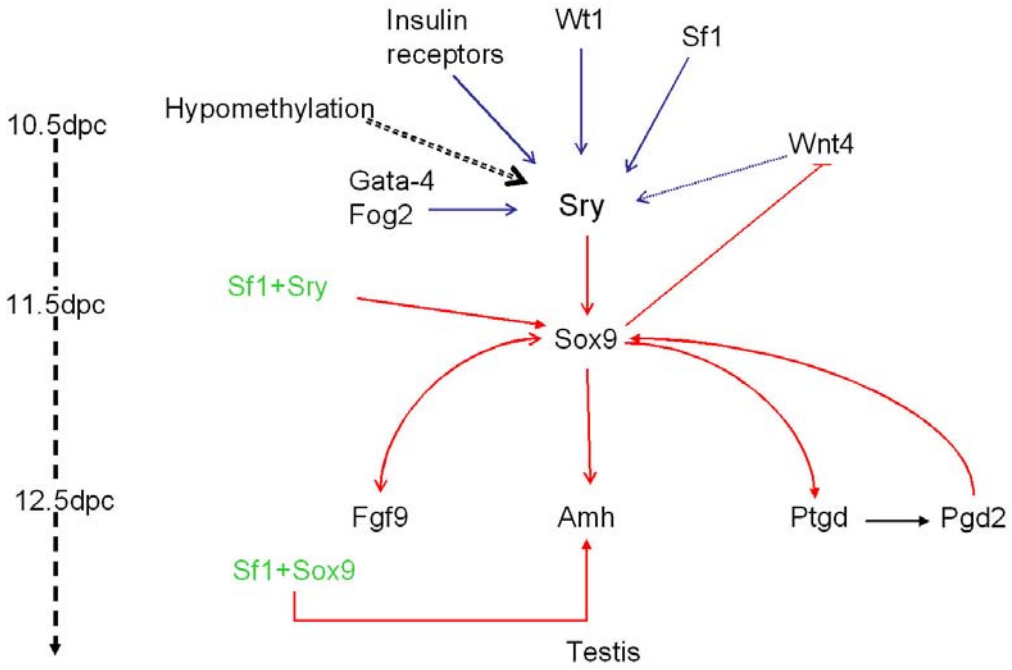

Figure 4. Hypothetical genetic network of testis determination.

In addition, Fgf9 also represses *Wnt4* in Leydig cell precursors [27]. The signaling molecule Pgd2 is involved in Sertoli cell differentiation, since it induces *Sox9* expression in cells that did not express Sry [29, 30]. At cellular level, the mechanism consists of inducing nuclear tanslocation of SOX9 [31]. *In vitro* experiments have shown that SOX9 is able to bind to and transactivate the promoter prostaglandin D-synthase (*Pgds*) producing Pgd2 [29]. So far, the only target gene of SOX9 shown in Sertoli cells both *in vivo* and *in vitro* is *Amh* [32, 33] *In vitro* experiments show interaction of SOX9 with SF1 on the *Amh* promoter [32].

C. Hypothetical genetic network of ovary determination

On the other hand, Figure 5 shows the network of molecular events that could be implicated in the determination and differentiation of the ovary. The role of these genes in ovarian development needs to be clarified: to fulfill the role of an ovarian determinant (Od), the hypothetical gene should be able to direct the molecular pathway which leads to ovarian determination, as the *Sry* gene does with the testicle, before morphologic differentiation of the ovary. Genes *Dax1* [34, 35], *Rspo1* [36], and *Foxl2* [37] have been proposed as ovarian determinants since mutations in each of them produce sexual reversion of the female to male in humans and *Foxl2* in goats. However, this sexual reversion in humans is incomplete and not reproducible in null mice. Besides, it is not clear if the determining role of these genes is positioned in the testicle-inhibiting pathway (Z genes) or in the ovary-organizing pathway (*Od* genes).

Since XX *Wnt4*$^{-/-}$ gonads show an invasion of mesonephros endothelial cells similar to that observed in XY gonads of control mice, it has been suggested that the role of Wnt4 is to repress the male signalization pathway, blocking endothelial cell migration to XX gonads [38]. However, XX *Wnt4*$^{-/-}$ mice are not positive to Sertoli cell markers and, at birth, no testicular tissue has been formed.

Initially, *Dax1* was postulated as an ovary-determining factor, based on the sexual reversion phenotype of XY individuals with double copy of *Dax1* [39]. However, knockout mice revealed that it is also necessary for testicular development [40]. Dax1 has been reported to inhibit synergic interaction of SF1 and WF1 (For Review see [41]).

Gene *Foxl2* specifically expresses in female gonads of mice, chicken, turtles and fish during sexual determination (For Review see [42]). The dimorphic expression led to the postulation of *Foxl2* as an important factor in the ovarian determination pathway. Experiments with XX *Foxl2*$^{-/-}$ mice showed partial sexual reversion at birth, and over-expression in XY gonads

led to disorganization of testicular cords. These results agree with an anti-testicle function of *Foxl2*. On the other hand, *Fog2* expression in the ovary inhibits *Gata-4* necessary for positive *Amh* regulation. Absence of *Amh* in females allows the differentiation of Falopian tubes, neck of the uterus and upper third of the vagina [43] as would be expected according with the paradigm forwarded by Alfred Jost. Recently, the *Wnt4* and *Foxl2* double knockout was reported, which shows sexual reversion of female to male. In the newborn, the gonads show an ovarian cortex with oocytes in late prophase. However, the medulla develops testicular cords with cells that express *Sox9* and *Amh*. Germ cells found in the medulla differentiate as spermatogonia; thus, the *Wnt4*$^{-/-}$ *Foxl2*$^{-/-}$ double knockout develops ovotestes at birth and does not show complete sexual reversion [44].

Up until now, the genes that could function either as ovarian (Od) or anti-testicular (Z) organizers have not been clearly defined. Results with *Wnt4* and *Foxl2* double knockout mice suggest that the anti-testicle function of *Wnt4* and *Foxl2* is clear during postnatal life [44].*

Parma et al., [36] described female to male sexual reversion in patients who present mutation in the gene *R-spondin 1 (RSPO1)*. As in the mutations described above, *RSPO1* has been proposed as the "ovarian organizer". RSPO1 is part of a family of orphan ligand proteins that act by activation of the Wnt and β-catenin signaling pathways. Regarding chondrocytes, the signaling pathway through β-catenin leads to Sox9 degradation) [45]. Assuming that this can happen during gonadal development, *RSPO1* has been

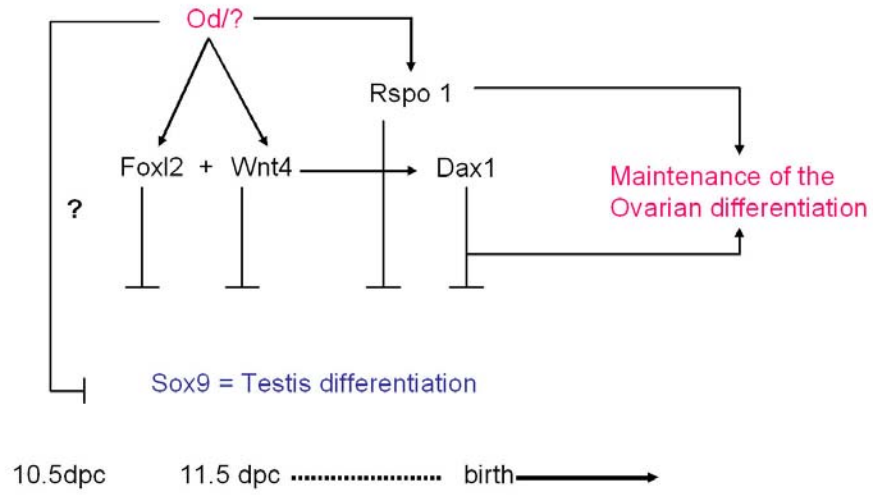

Figure 5. Hypothetical genetic network of ovary determination

*Recently Uhlenthaut et al. (2009) showed that FOXL2 is required to prevent Sox9 expression in adult ovaries and thus, to prevent transdifferentiation of ovaries into testes in adult mouse. Uhlenthaut et al.2009, Cell 139, 1130.

postulated as *gene Z*, since it represses the testicular determination pathway as it degrades Sox9 by the β-catenin pathway [46]. However, Tomizuka et al., [47] found that *Rspo*$^{-/-}$ mice females show pseudo-hermaphroditism as it retains derivatives of both Wolffian and Müllerian ducts and *Sox9* is not expressed. Additionally, at 14.5 dpc the XX gonads of *Rspo*$^{-/-}$ females have the normal form of a wild-type ovary of the same stage. It is thus clear that the absence of Rspo1 does not cause primary sexual reversion; it is a secondary ovarian dedifferentiation due to germ cell death.

D. Sexual diferentiation of germ cells

In contrast with testicular determination and its morphological differentiation, which depend on somatic cells, in females, germ cells play a determinant role in ovary differentiation) [48, 49]. In XX gonads, germ cells start meiosis during fetal or perinatal life, stop at diplotene and reinitiate just before ovulation. The process then stops again at metaphase II and is only completed if the oocyte is fertilized. In the testis, germ cells do not start meiosis in fetal life, but enter mitotic arrest (G1/G0) to start meiosis during puberty [50]. This difference in meiosis initiation time has led to the notion of the presence of a factor produced in the *rete ovari*, which promotes meiosis in the ovary [51]. The observation in mouse embryos that ectopic XY germ cells initiate meiosis at the same time as XX germ cells in the ovary led to postulate that germ cells have a cell autonomous program of meiosis initiation independent of chromosomal sex, and that a meiosis-inhibiting factor is produced in Sertoli cells that inhibits initiation of meiosis in germ cells inside the seminiferous cords [3].

Recent studies in mice show that retinoic acid stimulates the expression of the gene *Stra8* to induce meiosis in germ cells of the fetal ovary [52]. These authors propose that the absence of *Stra8* in the fetal testicle is due to the degradation of retinoic acid by the enzyme CYP26B1. In support of these observations, Bowles et al. [53] describe an ample expression of *Aldh1a2* in the adjacent mesonephros of the two sexes, which codes for the enzyme that participates in retinoic acid synthesis. They also identify CYP26B1 as the fetal testicle Meiosis Inhibiting Factor, since XY *Cyp26B1*$^{-/-}$ mice express *Stra8* and initiate meiosis, as do wild-type females. In males, *Stra8* expression is found in postnatal testicles, where meiosis will begin, and in adult testicles, where meiosis is a continuous process along the reproductive life of the male [54]. Thus, retinoic acid coming from the fetal mesonephros functions as a factor inducing meiosis in ovarian germ cells, as it stimulates the *Stra8* gene, which is involved in chromosome condensation to initiate meiosis. As in the testicle, retinoic acid stemming from the fetal mesonephros

is degraded by enzyme CYP26B1, *Stra8* levels are very low and therefore, meiosis cannot be initiated [53].

Final comments

Even though the mouse is the model system of choice mainly due to its well known genetics and practical experimental handling, important differences in timing and developmental patterns exist with other mammals. In contrast to mouse, *Sry* expression is maintained after sex determination in human [55, 56], dog [57], pig [58, 59], sheep [60] and rabbit [61]. While the origin of preSertoli cells in mice is the celomic epithelium, in rabbit [61] and probably in other species, contribution of epithelial cells from the mesonephric corpuscles has been found. Formation of seminiferous cords takes several days in larger mammals which may explain the evolutionary adoption of different cell-cell interactions, proliferative regulation and morphogenesis. Par example, paracrine regulation of meiosis by retinoic acid produced by the adjacent mesonephros found in mouse, cannot occur in species where germ cells enter meiosis long after gonad and mesonephros have been separated.

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5. Female gametogenesis and early seed formation in plants

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The formation of reproductive organs is a defining characteristic of sexual reproduction in flowering plants. Unlike animals, plants do not establish a germline early in development. In flowering species, specific somatic cells present in sexual reproductive organs divide by meiosis and differentiate haploid precursors (named spores) that undergo several mitotic divisions before giving rise to functional reproductive cells (gametes). It is therefore considered that the life cycle of flowering plants is composed of two distinct phases or "generations": the diploid phase that is phenotypically predominant and represented by all vegetative organs (roots, stems, flowers, etc…), and the ephemeral haploid phase represented by a limited number of cells that differentiate deep within male or female reproductive organs. Another distinctive feature of flowering plants refers to the unique sexual event of double fertilization in which two functional female gametes fuse with two sperm cells and generate two different products: the embryo and the endosperm. The correct development of these two tissues coordinated with the development of the maternal seed coat allows the formation of a viable seed.

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In this chapter we review current knowledge in the understanding of the genetic basis and molecular mechanisms that regulate female gametogenesis and early embryo formation in flowering plants, emphasizing recent results obtained in 2 model systems: *Arabidopsis thaliana* and Zea mays. We also describe some of the particularities associated with parent-of-origin effects in plants and their relation to genomic imprinting, and advance some of the crucial problems that remain to be solved for the coming years.

1. The alternation of generations

The life cycle of flowering plants is characterized by the alternation of two generations (Figure 1). The sporophyte is the predominant generation and is represented by all the vegetative organs of the plant. The gametophyte is a more ephemeral generation that is confined to just a few cells of highly specialized reproductive structures of the flower: the anthers and the ovules.

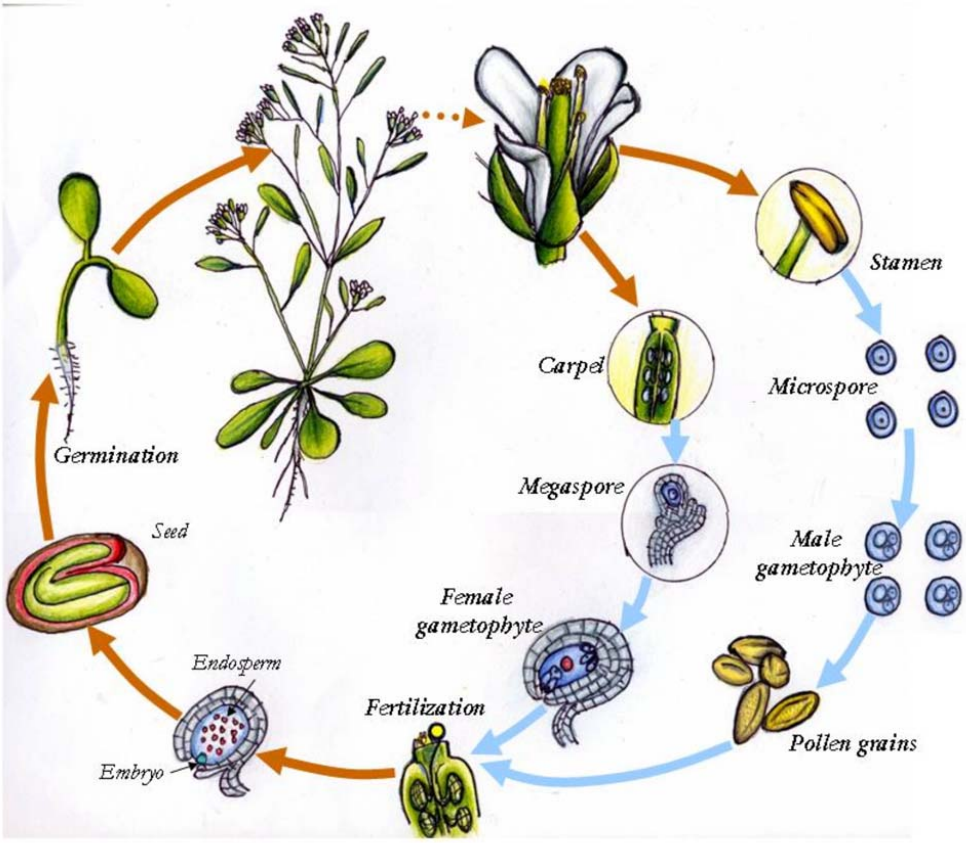

Figure 1. The alternation of generations in *Arabidopsis thaliana*. The haploid gametophytic generation (blue arrows) initiates with the precursors of the reproductive cells (microspores and megaspores) which undergo several rounds of mitosis and form the true gametes (female gametophyte and pollen grains). The diploid sporophytic phase (brown arrows) begins after fertilization of the gametes and is represented by all the vegetative organs of the plant.

The anthers produce the male gametophyte (microgametophyte), while the ovules produce the female gametophytes (megagametophyte or embryo sac). A meiotic division of specific precursors within the reproductive organs determines the transition from the sporophytic to the gametophytic phase. After gamete differentiation and pollination, a double fertilization event is responsible for re-establishing the sporophyte by giving rise to both the embryo and the transient endosperm.

2. The formation of female gametes

The female reproductive organ of angiosperms is called the *gynoecium* or more commonly the pistil. It is usually located in the center of the flower. Morphologically, the pistil is composed of three distinct tissues: the stigma, located at the aerial extremity of the pistil, is responsible for pollen grain reception, adherence and pollen germination; the style, a longitudinal tissue that separates the stigma from the ovarian cavity, is necessary to conduct growing pollen tubes towards the ovules; and the ovary that is composed of one or several cavities (locules) that harbor the ovules. An ovule consists of one or two integuments enclosing a group of inner cells that form the nucellus. The formation of the female gametophyte occurs within the nucellar tissue (Figure 2).

2a. Megasporogenesis

Female gametogenesis is arbitrarily divided in 2 developmental stages: megasporogenesis and megagametogenesis. In *Arabidopsis* as in maize, primordial ovules arise from periclinal divisions of the inner ovary wall of the young gynoecia and extend into short fingerlike projections, which are the origin of the integuments and delineate the nucellus [1,2]. During megasporogenesis, in the young ovule primordium a single sub-epidermal somatic cell differentiates into a megaspore mother cell (MMC), the precursor of the meiotic products [3,4,5]. Whereas in most of the cereals (including maize) cell wall formation occurs immediately following meiosis I, in *Arabidopsis thaliana* meiosis occurs without cytokinesis and it is only at the end of meiosis II that a cell wall isolates individual meiotic products. In contrast to male gametogenesis where many haploid precursors differentiate simultaneously within the anther, the MMC is the only cell that undergoes meiosis within the ovule primordium, giving rise to a tetrad of haploid megaspores. In more than 70% of species cytologically examined, 3 meiotically-derived cells invariably die, and only the most proximal megaspore with respect to the ovule survives, giving rise to the functional megaspore.

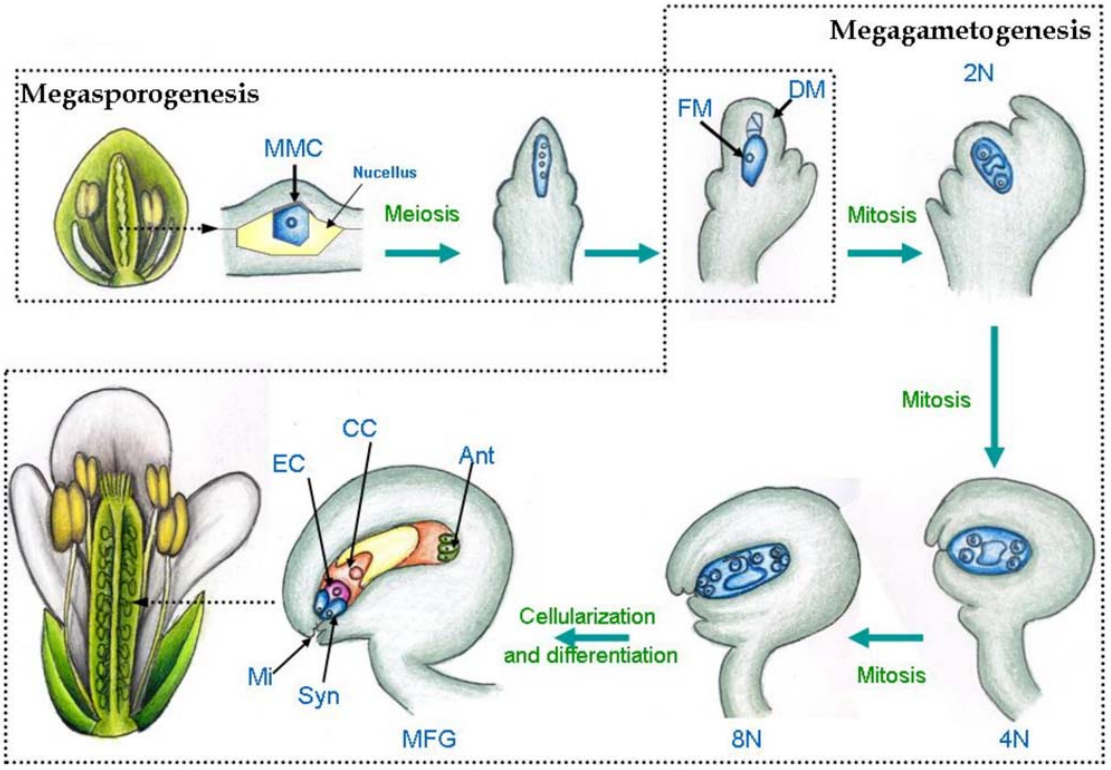

Figure 2. Female gametophyte development in *Arabidopsis thaliana*. Megasporogenesis: A single archesporial cell within the nucellus of the ovule primordium differentiates into a megaspore mother cell (MMC) that proceeds through meiosis and generates four haploid nuclei. Cytokinesis produces four haploid megaspores, three of which degenerate (DM) and only a functional megaspore (FM) survives. Megagametogenesis: the functional megaspore divides mitotically three times generating first two nuclei (2N), four nuclei (4N), and finally an eight nuclei syncitium (8N). After cellularization, seven cells differentiate into four lineages: antipodals (Ant), central cell (CC), egg cell (EC), and synergids (Syn). The ovule integuments enclose most of the mature female gametophyte (MFG) except the mycropile (Mi) through which the pollen tube penetrates the embryo sac.

2b. Megagametogenesis

The nucleus of this single meiotic product undergoes 3 consecutive rounds of mitosis without cytokinesis to form a voluminous syncitium with 8 haploid nuclei. It is only after mitotic arrest that cellularization and differentiation occurs within the syncitium. The majority of the space is occupied by a binucleated central cell. At the distal pole, 2 synergids and the egg cell give rise to the egg apparatus; at the proximal pole, 3 antipodals complement the 7 cells of the most conventional female gametophyte found in flowering plants. These cells have defined positions within the embryo sac; cell positioning, intracellular polarity as well as intercellular signaling are

thought to be determinants of female gametophyte cell specification [6,7,8]. Whereas most plants follow this “monosporic” type of megagametogenesis, in many other species either two (bisporic) or all four (tetrasporic) meiotic products survive and participate in female gametogenesis, contributing several cells to the final organization. Interestingly, the tetrasporic type of development gives rise to a heterozygous female gametophyte in which haploid cells can carry distinct allelic contributions, an attribute that to this date has not been genetically exploited to investigate haploid interactions among female gametophytic cells [7].

In *Arabidopsis* but not in maize, the antipodal cells, located at the most chalazal end of the female gametophyte, degenerate prior to fertilization. Although they are thought to be involved in the import of maternal nutrients to the fertilized embryo sac, their specific reproductive function has yet to be determined [9,10]. The absence of antipodals in female gametophytes of many species suggests that their function is not essential for sexual reproduction. The 2 synergids, located at the most mycropilar end of the female gametophyte, are characterized by the presence of the filiform apparatus, a thick finger-like projection of cellulosic and hemicellulosic material that occupies their micropylar pole, substantially increasing the surface of the plasma membrane. Contrary to antipodals, synergids are essential for fertilization since they are necessary for the attraction of the pollen tube and the release of the sperm cells [11,12,13]. The antipodal cells are, together with the synergids, two lineages of accessory cells. The two lineages of true reproductive cells are the egg cell and central cell. The egg cell is located adjacent to the synergids and is highly polarized, with a large vacuole at the chalazal end of the cell, and the nucleus and most of the cytoplasm at the micropylar end [14,15,16,17,18]. The conspicuous central cell is located in the center of the female gametophyte and is characterized by the presence of a large vacuole and many cytoplasmic organelles. It contains two nuclei that in the case of *Arabidopsis* fuse prior fertilization with the sperm cell. At maturity, the integuments completely enclose the nucellus and the differentiated female gametophyte, leaving only a small aperture (the micropyle) through which the pollen tube grows before penetrating a degenerated synergid and releases 2 sperm cells.

Recently, several studies have identified a multitude of genes claimed to be specifically expressed in female gametophytic cells either by microarray analysis [19,20,21,22, Wang, 2010; Bemer, 2010; Wuest, 2010]. Their discovery was allowed by global transcriptional comparisons in either gynoecia or ovules from wild type and mutant individuals lacking a normal female gametophyte. The functional characterization of most of these genes will remain a major task for the coming years.

3. Double fertilization

Flowering plants and a few of their closely related non-flowering seed species are characterized by a unique process of gamete fusion in which one sperm cell fuses with the egg cell while a second sperm cell fertilizes the central cell [23,24,25]. Double fertilization requires the attraction of the pollen tube into a synergid and its subsequent arrest, the deposition of the 2 sperm cells within the degenerated cytoplasm of the synergid, the transport of the sperm cells to the surface of the egg and central cell, and the fusion of male and female gametes [26]. Although some recent discoveries have improved our knowledge of the molecular genetic control of pollen tube attraction and sperm release, almost nothing is known about subsequent steps of the process.

In recent years synergids have been shown to control the mechanisms of pollen tube attraction within the ovule [11]. In *Torenia fournieri*, the establishment of an elegant experimental system allowing laser ablation of individual cells of the female gametophyte allowed to discover that synergids are essential for guiding the pollen tube through the micropyle and into the female gametophyte. On the initial basis of the experiments performed in *Torenia*, it has been suggested that diffusible pollen tube attractants produced by the synergids might provide short-range localized signals that control pollen tube guidance [27]. An interesting attribute of this attractant would be its species preferentiality, as shown by *in vitro* experiments in which pollen tubes of a specific species grow towards the female gametophyte of their own species if placed in a medium containing ovules from divergent genera [28]. This preferential attraction appears to discard the possibility that calcium ions might be the universal attractant derived from synergid cells [29]. Recently, in the synergid cell from T. Fournieri, two cystiene-rich polypeptides (CRPs) named LUREs were identified as the specific signals that attract the pollen tube. LUREs are defensin-like polypeptides secreted by synergid cells to the surface of the egg apparatus (Okuda, S. 2009). In maize, ZEA MAYS EGG APPARATUS1 (ZmEA1) is a 94 amino acid small protein that is more abundantly expressed in synergid cells and is predicted to be anchored to the plasma membrane [30]. ZMEA1 is a member of the EA1-like (EAL) family of genes that contain the so-called EA box [31]. In *Arabidopsis*, *MYB98* [32], a gene encoding a R2R3-MYB transcription factor, is also required for pollen tube guidance, a defect that could result from morphological abnormalities of the filiform apparatus, or from a reduced secretion of a putative pollen tube attractant [33].

Additional genetic evidence shows that *FERONIA* (*FER*), a gene encoding a receptor kinase specifically expressed in the synergids, is essential

for the arrest of pollen tube growth. In adidtion, *ANXUR1* (*ANX1*) y *ANXUR2* (*ANX2*), the most closely related homologs of *FER*, are also expressed in pollen. *ANX1* and *ANX2* function redundantly to inhibit pollen tube rupture and sperm discharge until reaching the female gametophyte. These results indicate that a cross-talk between female and male reproductive cells occurs before gamete fusion [34, Boisson-Dernier 2009]. In *abstinence by mutual consent* (*amc*), pollen-tube arrest is also prevented, but only when a mutant *amc* pollen tube reaches a mutant *amc* female gametophyte. *AMC* encodes a peroxin essential for protein import into peroxisomes, a finding suggesting that an unknown diffusible molecule is required for pollen tube discharge [35]. Additional genetic evidence confirms that sperm-specific genes are also important for pollen tube guidance and cross-talk; *Generative Cell Specific 1* (*GCS1*) is localized in the plasma membrane of male generative cells and sperm cells [36,37,38], and is likely anchored by a C-terminal transmembrane domain present in all members of the protein family identified to date. A second class of transmembrane proteins represented by LGC1 has been found in generative cells of *Lilium* [39]; however, a possible function in gamete recognition has yet to be determined.

4. Early seed development

Double fertilization marks the initiation of seed development. Along this process, the three intimately related tissues, embryo, endosperm, and the maternal coat undergo dramatic molecular and morphological changes that result in the formation of a mature seed, the progenitor of a new plant. Coordinated molecular interactions among these three tissues are essential for the correct development of a seed [40,41, Spencer 2007]. Given the absence of cell movement in plant development, cell fate, cell division, and cell differentiation during embryogenesis strongly depends on information provided by the position of the cells, the orientation of cell-division planes, and the direction of cell expansion. These events are tightly regulated by both genetic and epigenetic mechanisms. The development of the *Arabidopsis* embryo follows a highly reproducible pattern of sequential cell divisions that allows the definition of a series of embryonic stages: quadrant, octant, globular, heart, torpedo, and bent-cotyledon [42] (Figure 3). It takes about 9 days at 25°C from the time of fertilization to the mature embryo stage. The first one third of this time, i.e. from zygote to globular stage, establishes the basic body plant that includes the apical meristem, hypocotyl, cotyledons, root, and root meristem. The later stages of seed development are associated with further growth of the embryo, partial cell differentiation, and preparation for dormancy.

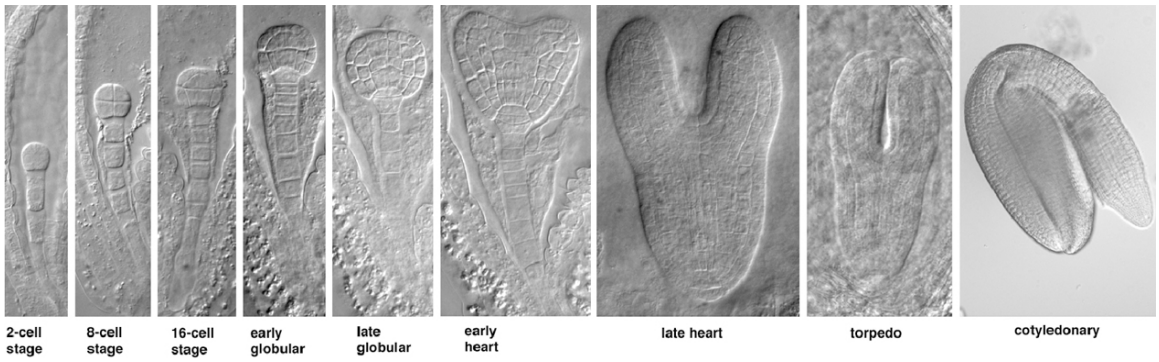

Figure 3. Embryo development in *Arabidopsis thaliana*. Whole-mount cleared seeds showing the different stages of development in the embryo of *Arabidopsis thaliana*.

4a. Embryogenesis

In *Arabidopsis*, within a few hours of fertilization, the zygote elongates about three-fold along the chalaza-micropyle axis and undergoes an asymmetric cell division resulting in a small apical cell and a large basal cell [43,44]. A series of divisions of the basal cell perpendicular to the apical-basal axis produce a final structure of 7-9 cells called the suspensor that connects the embryo to the maternal tissue. The uppermost cell of the suspensor divides transversely and the daughter cell closest to the embryo becomes the hypophysis (basal end of the embryo). The apical cell gives rise to the embryo proper through a defined pattern of divisions. Initially, two longitudinal divisions form a quadrant, and one transversal division result in an octant. The octant comprises an upper and a lower tier that are the origin of the apical and basal regions of the seedling respectively. The apical cells of the embryo are destined to generate the shoot meristem and most of the cotyledons; the basal cells will give rise to the hypocotyl and root while also contributing to cotyledons and root meristem; and the hypophysis will form the distal parts of the root meristem, the quiescent center, and the stem cells of the central root cap [44]. While maintaining a globular morphology, the octant undergoes a round of tangential divisions that originate two separate layers: the protoderm or epidermal precursor cells and the inner cells. The protoderm cells divide along the surface of the embryo while the inner cells divide parallel to the apical-basal axis. The centrally located inner cells become the primordium of the vascular tissue or procambium. At late-globular embryo stage, the upper tier of the embryo widens as a result of vertical cell divisions while the lower tier expands in the apical-basal axis. A change from radial to bilateral symmetry marks the beginning of the heart stage in which the cotyledonary primordia and most of the basic structures of the seedling (hypocotyl, primary root, vascular tissues) emerge and the

embryo turns green. The heart stage closes the first phase of embryogenesis. The later stages of embryogenesis, torpedo and bent-cotyledon are mainly associated with the growth and elongation of the different structures, and the bending of the cotyledonary primordia towards the root part of the embryo that now occupies most of the seed in *Arabidopsis*.

4b. Endosperm development

Endosperm is a unique triploid tissue product of the fertilization by a sperm cell of the female binucleated central cell. Although a clear and conclusive global function has yet to be assigned to this tissue, the classical view is that the endosperm sustains embryo development and germination [41,45]. Current knowledge extends the functional significance of the endosperm to that of a key coordinator of the signaling processes that occur among the components of the seed to ensure proper development [46]. The uniqueness of this tissue raises interesting questions related to its origin and the significance of its evolution. Two hypotheses have been proposed to explain the evolutionary origin of the endosperm [47]. One suggests that the endosperm was originated from a second embryo that evolved to become a nourishing tissue for the surviving embryo. The alternative hypothesis proposes a maternal origin for the endosperm as an extension of the female gametophyte development. Although this issue is still on debate, recent scientific work supports the maternal origin of the endosperm [48,49].

Research on endosperm development has mainly been performed in *Arabidopsis*, maize, and barley. In the case of *Arabidopsis*, endosperm is a transitory tissue as the embryo utilizes it during its latest stages of development. On the contrary, endosperm in cereals persists throughout seed development and occupies most of the mature seed. Despite this difference, studies on endosperm development have shown that both dicotyledonous and monocotyledonous species share a “free-nuclear” type of endosperm development that includes four stages: syncytial, cellularized, differentiated, and dead [45]. Cellularization and differentiation are temporally and spatially regulated [41]. Shortly after fertilization and prior to the first zygotic division, the triploid endosperm nucleus undergoes a series of mitotic divisions that occur without cytokinesis and form a multinucleated cell called the coenocyte. Before cellularization, the nuclei orderly migrate to specific areas of the coenocyte and define three mitotic regions in the endosperm: mycropilar endosperm (MCE), located at the anterior pole close to the embryo; peripheral endosperm (PEN), the largest portion at the center of the endosperm; and chalazal endosperm (CZE), located at the posterior end of the endosperm in *Arabidopsis*. Similar regions are defined in the maize

endosperm: embryo-surrounding region, starchy endosperm, and basal endosperm transfer layer, respectively. This nuclear positioning seems to be essential to determine cell fate and differentiation [50].

Nuclear division occurs synchronically within each domain and asynchronically among the different regions of the endosperm. Endosperm cellularization in *Arabidopsis* begins at the mycropilar endosperm after the eighth mitotic cycle, in correspondence with the late globular stage of the embryo development and continues through the peripheral endosperm [51]. Cellularization involves the formation of radial microtubule systems (RMS) from the membrane of each nucleus and subsequent alveolation or formation of a tube-like wall structure. Each nuclea thus become isolated by the development of cell walls. At completion, most of the endosperm becomes cellularized except at its most chalazal end where a large multinucleate coenocytic cyst persists. It sits atop a specialized area of maternal tissue called the chalazal proliferating tissue, suggested to play a role in transfer of maternal nutrients similar to the transfer cells in cereal endosperm [52]. The central region of the cellularized endosperm specializes in the production of enzymes involved in starch synthesis. At the end of the cellularization stage, which corresponds with the late-heart stage of embryo development, the reserves of the central endosperm are depleted gradually as they are translocated to the cotyledons of the embryo [53]. Endosperm cell differentiation also defines a peripheral layer of cells or aleurone. This cell layer contains storage lipid and proteins and has a role in supplying sugars to the growing seedling. This determinant role makes of the aleurone the sole cells of the endosperm tissue that remain alive in the mature seed.

In cereals, the nuclei are evenly spaced in the entire peripheral cytoplasm of the coenocyte and, in contrast with *Arabidopsis*, the entire endosperm cellularizes. Endosperm differentiation in cereals also defines four types of tissues: embryo-surrounding tissue, aleurone layer, starchy endosperm, and basal endosperm transfer layer. Specification of cell fates in the cereal endosperm appears to occur via positional signaling: cells in the peripheral positions, assume aleurone cell fate; cells over the main vascular tissue become transfer cells and all interior cells become starchy endosperm cells [41]. Neither the precursor cells nor the exact function of the embryo-surrounding tissue have been elucidated. However, roles in embryo nutrition, signaling between the embryo and the endosperm and/or physical barrier between the embryo and the endosperm are possible. The aleurone layer is the outermost and covers the entire endosperm except the region occupied by the transfer cells. Aleurone layer is the site for antocyanin synthesis. As in *Arabidopsis*, cereal aleurone cells are the only cells of the endosperm that remain alive in the mature seed and are responsible for the synthesis of

hydrolytic enzymes that are secreted into the starch endosperm to mobilize reserves during seed germination. At the end of the germination, aleurone cells undergo programmed cell death [54]. The starchy endosperm represents the largest body of cell mass in the endosperm and contains enzymes involved in starch synthesis; after cellularization and differentiation, starchy endosperm cells undergo endoreduplication [55]; at the end of the grain-filling period, the starchy endosperm cells die, involving a process that resembles programmed cell death in animal cells [56]. Transfer cells possess an extensive and complex endomembrane system that between 5 and 10 days after pollination forms cell wall ingrowths that make most effective the transfer of nutrients from maternal tissues into the endosperm.

4c. Formation of the maternal seed coat

Ovule integuments enclose the mature female gametophyte and have shown to play an important role in seed development. In *Arabidopsis*, integument cell proliferation starts in mature ovules prior fertilization and ends 4 days after pollination. As a result of fertilization, integument cells initiate a process of elongation [57] and the integuments start differentiating into the seed coat or testa [58]. A hallmark of integument differentiation is the formation of flavonoids [53], also called prothocyanidins (PCs) which confer the brown color to mature seeds after oxidation [59], participate in seed defense [60], and maintain seed dormancy and longevity [61]. Proper seed development and final seed size is the result of a balanced coordination between maternal seed coat formation and endosperm growth [40].

5. Parent-of-origin effects during early seed formation

Over the last years, several studies have confirmed that the female gametophyte exerts a maternal control over early embryo and endosperm development at several levels. While a number of proteins that play important roles in the nascent endosperm are initially expressed in the female gametes prior to fertilization, the unpollinated female gametophyte contains several types of maternal factors essential for embryo and endosperm development. The following sections discuss the general trends that are progressively emerging from the study of parent-of-origin effects in model systems such as maize and *Arabidopsis*.

5a. Non-equivalency of parental genome activity

Wide evidence showing that parental genomes are differentially expressed following fertilization both in *Arabidopsis* and maize suggests that

early seed formation is mainly under maternal control [62,63]; however, the early presence of paternally-derived transcripts has been shown for a few loci, and equivalent levels of parental expression have been reported under certain experimental conditions [64,65]. Several genetic analyses showing that mutations that affect early embryo development segregate as maternal gametophytic recessive mutations have fueled the perception that requirements for the initiation of parental transcriptional activity differ on a gene-specific basis [21,66,67]. More recently, the transcriptional activation of paternally imprinted genes was shown to be under maternal control [68,69,70,71], a finding subsequently extended to a subset of non-imprinted genes that act during early stages of seed formation [72]. In *Arabidopsis*, a single gene has been found to contribute paternally inherited transcripts in the first 72 hours following fertilization [73]. Although not all paternally inherited alleles are completely inactive during early seed development [65,66], the current evidence suggests that a transcriptional non-equivalence of parental genomes prevails in flowering plants; however, the mechanisms responsible for controlling parental transcriptional activity during early seed formation are unknown. Recent evidence analyzing the activity of *RNA POLYMERASE II* have shown differences in the transcriptional activity of embryo and endosperm; while the embryo is transcriptionally quiescent, the endosperm is active (Pillot, 2010).

5b. Maternal effects

Due to the particularities of the plant life cycle, it is often difficult to distinguish between the effects of maternal factors and genomic imprinting on the regulation of a specific locus. Whereas, a “maternal effect” (caused by a maternal factor) refers strictly to a reciprocal cross resulting in differential phenotypes that are exclusively determined by the genotype of the female parent, genomic imprinting refers to a specific type of genetic regulation that consistently inactivates one of the parental alleles following fertilization. While maternal effects can result from the action of imprinted genes, they can also result from the activity of gene products cytoplasmically stored in the female gametophyte but acting after double fertilization, from dosage sensitive genes acting in the endosperm, or from products encoded by the genome of maternally inherited organelles. Maternal factors fall into 3 categories: those that are caused by genes that are active in the female gametophyte and are passed as either transcripts or proteins into the embryo (gametophytic maternal factors), those that are caused by genes that are active in the sporophytic tissues of the ovule and have an effect on embryogenesis (sporophytic maternal factors), and finally those that depend on genes active in

the endosperm but required for embryo development (endospermic maternal factors). Although numerous examples of gametophytic and sporophytic maternal effects exist in *Arabidopsis* and maize [74,75,76], to this date strict endospermic maternal factors have yet to be discovered.

5c. Genomic imprinting

For some traits, parent-of-origin effects can be the consequence of distinct transcriptional rates of paternally or maternally inherited genes in the embryo and/or the endosperm. Genomic imprinting refers to a specific type of genetic regulation resulting from a mitotically stable epigenetic modification that consistently inactivates one of the parental alleles. Loci that are regulated by genomic imprinting will be differentially expressed in a parent-of-origin dependent manner. If genomic imprinting regulates a gene, transcription will occur exclusively from one of the two parental gene copies.

While genomic imprinting has been extensively studied in mammals [77,78], the first demonstration of a gene regulated by genomic imprinting was obtained for the *r1* locus in maize [79]. More recently, direct evidence of genomic imprinting has been obtained for several *Arabidopsis* genes, including members of the *FERTILIZATION-INDPENDENDENT SEED* class (*FIS*). These three *Arabidopsis* genes were identified on the basis of their gametophytic maternal effect seed abortion mutant phenotype, coupled to their ability to initiate endosperm formation in the absence of fertilization [80]. *MEDEA* (*MEA*) and *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) encode Polycomb group proteins (PcG) known to repress homeobox transcription factors by forming complexes that regulate higher order chromatin structure in animals and plants [81]. The *Arabidopsis FERTILIZATION INDEPENDENT SEED-2* (*FIS2*) gene encodes a Zn-finger protein involved in the formation of the PcG complex [82].

To this date, genes regulated by genomic imprinting have been only identified unambigously in the endosperm. Recent evidence in maize suggests that the *maternally expressed in embryo 1* (*mee1*) could be imprinted in the embryo and endospem; its specific expression is associate to differential allelic methylation (Jahnke 2009). Experiments involving *in situ* hybridization (ISH) to detect nascent RNA transcripts (transcriptional nuclear dots) and reverse-transcriptase polymerase chain reaction (RT-PCR) provided conclusive evidence that following fertilization *MEA* is only transcribed from the maternally inherited allele [83]. Due to the absence of detectable transcriptional dots in the egg cell, the question of whether the paternally inherited *MEA* allele is also transcriptionally inactive during early embryo formation has been difficult to answer. Whereas RT-PCR experiments

suggest that only the maternal allele is expressed in the embryo 54 hours after pollination (HAP), independent experiments showed that *MEA* is expressed from both parental alleles in the embryo at 6 days after pollination [84].

Based on the existing evidence, it currently appears that two distinct classes of mechanisms regulate seed development in flowering plants [85,86]. Delayed activation of the paternal genome indicates that a wide mechanism of repression and reactivation ensures that both parental genomes are not functionally equivalent following fertilization. In addition, specific loci such as members of the *FIS* class of genes could be imprinted until later stages of seed development, as it has been shown for *MEA* [83,84]. In both cases the evidence implies that a mechanism of allele-specific inactivation must be established during gametogenesis. Maintenance of the resulting 'imprint' (probably through methylation) must be ensured through successive haploid mitotic cycles. It is proposed that during female gametogenesis occur a decreasing in DNA methylation and then is compensated by *de novo* methylation during early embryo development (Law 2010, Jullien 2010). For the endosperm, the loss of an epigenetic mark is facultative: in some cases, genes that are required at late stages of development might be eventually transcribed from both parental gene copies; however, the transient nature of the endosperm – not contributing to the sporophytic lineage – allows maintenance of the imprinted nature until seed germination (maize) or complete reabsorbance by the embryo (*Arabidopsis*).

6. Future perspectives

The elucidation of the genetic basis and molecular mechanisms that control female gametogenesis and early embryo formation will remain a key subject of plant developmental biology in the coming years: What are the mechanisms that specify cell identity in the young ovule? How is meiotic and mitotic commitment established and controlled? Is positional information necessary for gamete function? Molecules responsible for gamete recognition and fusion are yet to be identified, and the mechanisms leading to the transition from a maternal to zygotic developmental activity are not determined. Whereas female gamete formation is mediated by *Arabidosis* protein ARGONAUTE 9 (AGO9), which restricts the specification of gametophyte precursor in a dosage-dependent, non-cell-autonomous manner (Olmedo-Monfil et al. 2010), the assymetric distribution of the hormone auxin is responsible for female gametophyte pattering during its syncial development (Pagnussat 2009). The long-standing barriers that impede access to progamic cells are now slowly overcomed by technologies that allow laser-capture microdissection and subsequent analysis of nucleic material [87].

These technologies can now be combined to new procedures that allow in vivo imaging of cell dynamics in the ovule [88]. The possibility of visualizing in detail living cells will provide a completely new perspective of the biological particularities associated with sexual plant reproduction.

While some progress has been made on the elucidation of the genetic basis and molecular mechanisms regulating genomic imprinting, most of the crucial questions remain to be answered**.** What is the overall importance of parental gene activity during early embryo and endosperm development? How many transcripts are produced in the zygote and how many are preferentially transcribed from either a maternally or paternally inherited allele? Over the next years, the discovery of regulatory networks driving the establishment of parental imprinting in *Arabidopsis*, combined with the results of new genetic screens that have been recently undertaken, and the fast emergence of genomic technologies applied to the study of parent-of-origin effects promise to provide important clues to determine the total number of loci that are imprinted, the mechanistic basis of their regulation, and their overall influence on plant development and evolution.

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6. Limb development

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The degree to which the appendages of diverse tetrapod species are adapted to the habitat in which they live is astonishing. In organisms such as birds, bats, crocodiles, whales, horses and humans, appendages show as many different shapes; however, when these appendages are compared at early stages of embryonic development, it is impossible to distinguish among appendages of different tetrapod species. During embryonic development, tetrapod limb buds are always formed in a precise position and in the same number: two anterior and two posterior limbs, always in opposite position from one another, and equidistant from the embryo midline. In spite of the later morphological diversity, three anatomical regions have been recognized from proximal to distal in all tetrapods: the proximal region (stylopod) forms the humerus of the forelimb and the femur of the hindlimb; the middle elements (zeugopod) form the radius/ulna of the forelimb and the tibia/fibula of the hindlimb; finally, the highly segmented distal element (autopod) develops into the metacarpal/carpal/finger bones of the forelimb and the metatarsal/tarsal/toe bones of the hindlimb. The autopod shows the largest morphological diversity among different vertebrate species (Figure 1).

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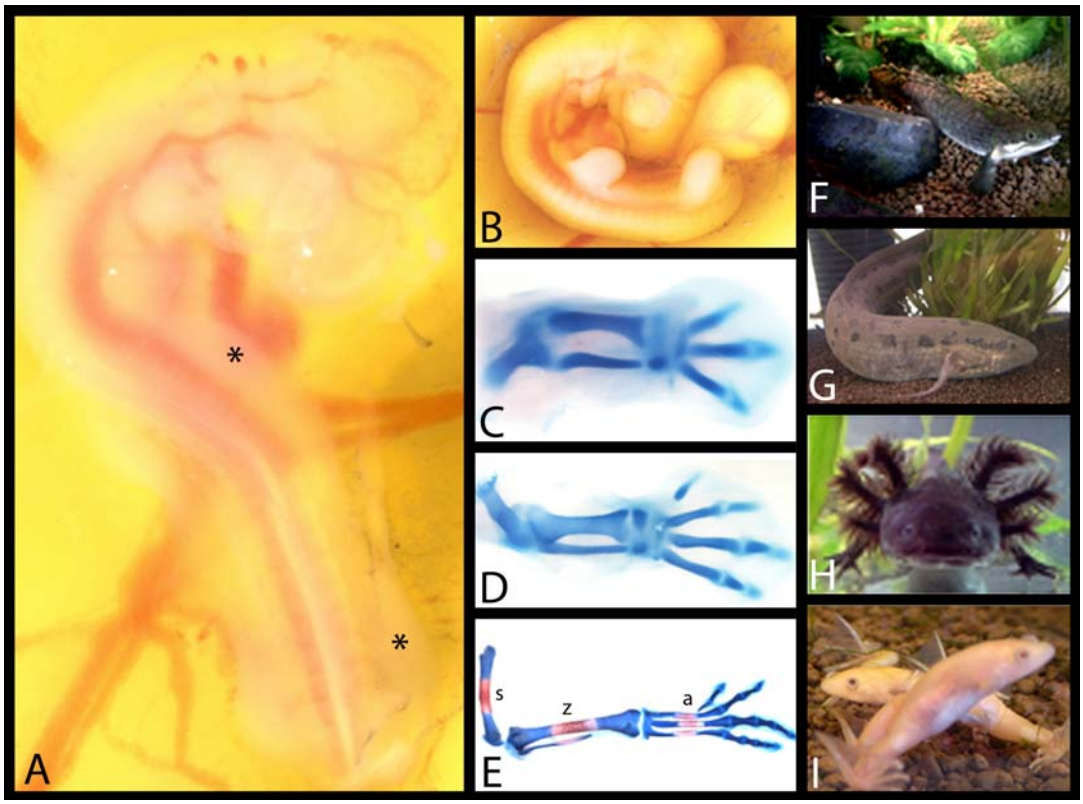

Figure 1. The basic organization of tetrapod limb development. Wing and leg bud (arrows) are developed in specific areas at the embryonic flanks (A). Bud elongates and undergoes detailed changes in shape to form a limb (B). Whole mount chick embryos leg at E28 HH (C), E30 HH (D), and E33 HH (E) stained with Alcian blue showing proximal to distal structures and organization in the three regions of skeletal elements: stylopod (s), zeugopod (z) and autopod (a). Lungfish are models that contribute to understand evolutionary events of tetrapod limbs; *Protopterus annectens* (F) presents fins with only one repeated segment, while *Polypterus palmas* (G) presents radial fins. Models used to understand the molecular process of limb formation given their regenerative limb ability are: *Ambystoma mexicanum* (H) and *Xenopus laevis* (I).

Limb induction

Vertebrate limbs consist of paired appendages which arise from the lateral plate mesoderm (LPM) at defined locations along the rostral-caudal axis of the embryo, (between somites 15-20 for forelimb and somites 26-30 for hindlimb in chick embryo), as small protuberances formed by morphologically homogenous mesenchymal cells enclosed in a layer of ectodermal cells [1-4]. The territories of the LPM give rise to limb fields, when they are grafted to ectopic sites (eg. the embryo flank or head) result in fully developed limbs. Limb induction begins when Fibroblast Growth Factor (FGF) 8 signals from the intermediate mesoderm (IM) to instruct the LPM to

express *Fgf10* specifying the limb forming region and also establishing a positive regulatory loop between FGF10 and FGF8. Mutation of *Fgf10* ablates limb initiation, while FGF-beads implanted into the chick flank are capable to direct the development of ectopic limbs. In addition, Wnt signaling has been involved in limb induction, since *Wnt2b* is expressed in the IM and LPM at the forelimb level, and *Wnt8c* in the LPM at the hindlimb level. Misexpression of *Wnt2b* and *Wnt8c* results in induction of ectopic limbs by inducing *Fgf10* expression in the LPM, whereas blocking Wnt signaling mediated by β-catenin inhibits limb initiation [4a].

Since appendages arise at defined locations along the rostral-caudal axis of the embryo, it is relevant to examine the molecular mechanism for limb identity. T-box transcription factors, *Tbx5* and *Tbx4*, and a paired-like homeodomain factor, *Pitx1*, are involved in limb-type-specific initiation. *Tbx4* and *Pitx1* are expressed in the developing hindlimb bud, whereas *Tbx5* is expressed in the forelimb. Misexpression studies have shown that *Tbx5* is able to induce wing-like morphological changes in the chick leg. In contrast, *Tbx4* induces leg-like changes in the chick wing [5-7]. However, genetic studies in mouse embryos show that *Tbx5* and *Tbx4* are not sufficient to determine limb identity, although a role for both genes in initiating limb outgrowth has been suggested.

Limb patterning

Once the limb primordium is formed, the three-dimensional organization of the limb is directed by three interdependent signaling centers, which establish the spatial coordinates that shape the limb bud. The first signaling center directs growth in the proximal to distal axis (shoulder to hand); the second, in the anterior to posterior axis (thumb to pinky), and the third in the dorsal to ventral axis (back to palm of hand).

Proximodistal patterning

Limb outgrowth is directed by the thickened epithelium rimming the distal tip of the limb called Apical Ectodermal Ridge (AER) [7-9]. Interestingly, removal of the AER at different embryonic stages of chick development causes limb truncation at different levels. If AER is removed at early stages, only proximal elements develop, whereas if this occurs at later stages, elements become progressively more distal [7]. AER expresses four members of the FGF family of signaling molecules *Fgf8, Fgf4, Fgf9* and *Fgf17*, which together are referred to as AER-Fgfs. *Fgf8* is expressed at the earliest stages of limb development before any morphological evidence of AER

formation, and its expression marks the precursors of AER. FGFs are able to rescue the removal of the AER since an exogenous FGF source is able to maintain the limb outgrowth. Genetic removal of FGFs of the AER suggests that AER-Fgfs signaling should be instructive in limb proximal-distal patterning. Mouse limb phenotypes of triple deletion of *Fgf4, Fgf9* and *Fgf17* are normal, suggesting that FGF8 is sufficient to sustain normal limb formation. AER-FGFs control proximodistal patterning and are released into the underlying mesenchyme, at the most distal region of the limb bud, where these cells are maintained in an undifferentiated stage (Figure 2) [10-13]. Three different models illustrate how proximodistal patterning during limb development is established. The "progress zone" (PZ) model hypothesizes that P-D patterning develops progressively; the layer of 300 μm of the most distal mesenchymal cells under AER control constitutes the PZ. The PZ cells

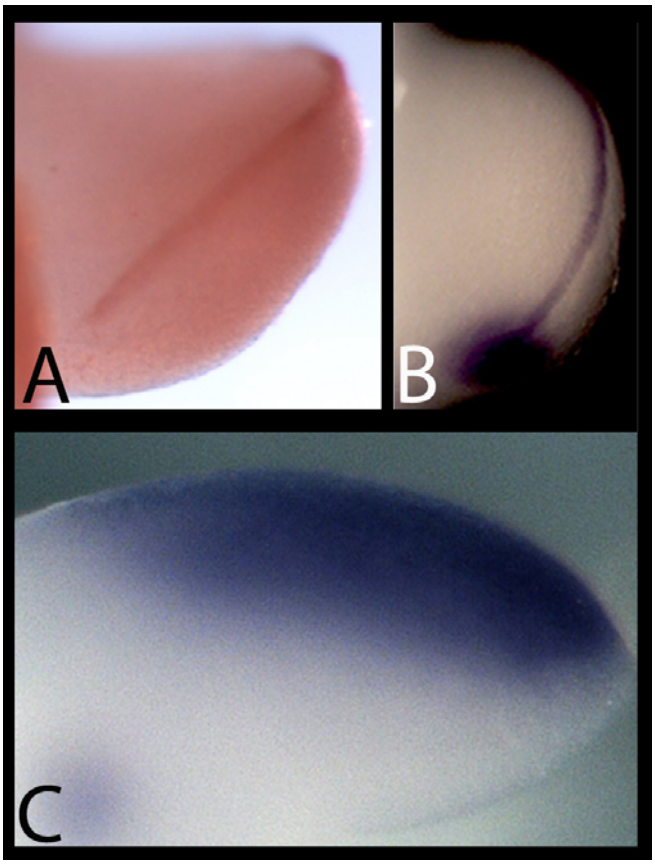

Figure 2. The three organization centers establish the limb pattern. Chick embryo leg at E22 HH stained with neutral red shows the apical ectodermal ridge (AER) oriented towards the ventral side and rimming the limb tip (A). Double in situ hybridization shows *Shh* and *Fgf8* expression in the posterior region of zone polarizing activity (ZPA) and in AER, respectively, in chick leg at E22 HH (B). *Lmx1* is expressed in dorsal mesoderm in chick leg at E22 HH and is involved in dorsal-ventral axis formation (C).

acquire a positional value that changes over time. At early stages of limb development, the first mesenchymal cells that leave PZ originate proximal structures, while mesenchymal cells that are maintained for more time in the PZ give rise to distal structures. In contrast, the "early specification model" hypothesizes that cells at early stages of limb development are specified to make the stylopod, zeugopod and autopod. Finally, the "two-signal dynamic specification" postulates that mesenchymal cells are exposed to a proximal signal that specifies proximal structures and then to distal signals from AER that specify distal structures. The middle structures originate from the interaction between boundaries of proximal regions and distal regions (Reviewed by Ticlke [13a]).

Anteroposterior patterning

Digital patterning along the anteroposterior axis depends on the Zone of Polarizing Activity (ZPA) which is a group of mesodermal cells, located at the posterior region of the limb [14-16]. When ZPA is grafted into the anterior region of the limb bud, it is able to generate mirror-image symmetry of the normal pattern [17]. ZPA produces the morphogen protein Sonic hedgehog (SHH) (Figure 2), which elicits mirror-image symmetry limb duplications when applied in the anterior region of the limb. The function of SHH involves the control of digit number and digit identity [17-20]. Inactivation of Shh in the mouse produces a phenotype in which all posterior digits are lost, presumably only digit one is conserved. Some experiments demonstrate that *Shh* expression is induced by Retinoic Acid (RA) action through *Hoxb8* and *dHand*. SHH signaling occurs when SHH binds directly to its receptor Patched 1 (Ptc1) and then the transmembrane protein Smoothened (Smo) is activated and inhibits PKA allowing that Gli proteins enter the nucleus and act as transcriptional activator. In the absence of SHH, Gli is phosphorylated by PKA functioning as transcriptional repressor. During early stages of limb development, Gli3 activator is mainly observed in the posterior zone mediating SHH polarizing activity. In contrast there is high concentration of Gli3 repressor in the anterior region of limbs.

Three different models illustrate how anteroposterior patterning during limb development is established. In the first, the "classic morphogen model", SHH indeed generates a posterior-anterior gradient. Then cells acquire a positional value that changes over time. *Gli3* processing is a consequence of the gradient generated by the morphogen. *Gli3* mutant mice give rise to polydactyly with unpatterned digits; the double mutant of *Gli3* and *Shh* results is similar phenotype, suggesting that the main role of Shh is to avoid *Gli3* repressor activity in the ZPA, thus ensuring its polarizing activity. The

"Temporal expansion model" proposes that longer exposure and high concentrations of SHH promote more posterior digital fates, whereas low concentrations of SHH only promote anterior fates even if exposure is for longer periods. In the mouse limb, the digits 5 and 4 and the half posterior region of digit 3 are formed progressively by descendents of *Shh*-expressing cells, while digit 2 is formed by nondescendents of *Shh*-expressing cells and requires a spatial gradient of SHH. The third model, the so-called "biphasic model", results from a careful analysis of the temporal *SHH* signaling requirement to pattern the mouse digits. Using an inducible *Hoxb6*CreER line, it has been showed that SHH specifies digits at early stages of limb development; for example, a 3 h pulse of Shh is enough to form digits 1 and 4. The formation of digits 2, 5 and 3 takes place within 9 h of SHH activity. This model states that digit specification occurs at very early stages by a Shh concentration gradient; thereafter, SHH is only required as a mitogen for progressive digit formation (Reviewed by Ticlke [13a]).

SHH also plays a role in evolutionary limb adaptations; for example, natural occurring digit reductions in the Australian lizard of the genus *Hemiergis* correlates with decreased cell proliferation and shortened Shh transcription. In this sense, digit reductions in Axolotl limbs caused by inhibition of SHH signaling resemble the digit loss found in natural variations of urodele species. Also, the *Lmbr1* locus contains a conserved intronic sequence called MFCS1, which functions as a limb-specific *Shh* enhancer that is lost in some limbless species of snakes and newts. Understanding the functions of SHH is crucial to explain the morphological changes of the appendicular skeleton along evolution [21].

Finger identity depends on their length and phalanx number. Each finger has unique morphology in accordance with its antero-posterior position. This particular morphology is controlled by the adjacent posterior interdigit (ID), that is, once Shh is downregulated, the ID acts as a secondary signaling center on the cells of the digital ray. Cell fate experiments have shown that phalanges arise from cells of the phalanx-forming region (PFR). PFR cells are non-condensed mesenchyme cells located between the condensed cartilage of metatarsals and the AER, which express the Sox9, Bmpr1b, *Activin βa* genes and are positive to pSMAD 1/5/8. PFR cells are exposed to signals which stem from the interdigit, and experiments have shown that members of the BMP family, probably GDF5, are the molecules that ultimately control the different levels of SMAD1/5/8 activity that establish digit identity. Digit 1 PFR has lower SMAD1/5/8 activity followed by digit 2 PFR and digit 4 PFR. Digit 3 has the highest PFR activity. Notably, the activity level correlates with the longitude of fingers. By manipulating the activity level it is possible to change finger identity [21a].

Dorsoventral patterning

Dorsoventral patterning is controlled by dorsal and ventral ectoderm. Dorsal ectoderm signals to dorsal mesenchyme to pattern dorsal structures, and ventral ectoderm organizes ventral mesenchyme to form ventral structures. When ectoderm covering the chick limb bud at stage 20 was rotated 180° in such a way that dorsal ectoderm covered the ventral mesenchyme and ventral ectoderm covered the dorsal mesenchyme, results showed distal elements with inverted dorsoventral polarity. Some molecular signals responsible of patterning dorsoventral polarity have been recognized. *Wnt7a* is expressed in the dorsal ectoderm of the limb bud and homozygous *Wnt7a* mutant mice show ventral features in the dorsal region [22]. *Lmx1b* codes for a LIM-homeodomain transcription factor expressed in dorsal mesenchyme of the limb bud (Figure 2). Comparably to the *Wnt7a* mutant, *Lmx1b* mutant mice generate biventral structures at the distal portion of the limb [23]. Since *Wnt7a* mutant limbs downregulate *Lmx1b* expression in dorsal mesenchyme, it has been proposed that *Wnt-7a* signals to dorsal mesenchyme, controlling *Lmx1b* expression to establish the dorsal patterning [24]. Complementarily, *En1,* a homeobox-containing transcription factor, is expressed in ventral ectoderm of the limb bud and mutant mice for this gene show bidorsal limbs (the ventral region is characterized by dorsal structures) [25]. Analysis of these mutants has provided a model of regulation between these signals to establish dorsovental patterning. Conditional mutant mice for BMP receptor type I (*Bmpr1a*) in limb ectoderm [26] and misexpression of BMP antagonist Noggin in chick limb bud [27] inhibited *En1* expression in ventral ectoderm resulting in *wnt7a* expansion and bidorsal limbs. These results along with the expression pattern of *Bmpr1a* localized in ventral ectoderm indicate that BMP signaling regulates *En1* expression positively to establish ventral structures.

Coordination of signaling centers

All studies performed independently to elucidate the molecular mechanisms that control the establishment of the three polarities of the limb indicate that there is coordination and interaction between the three signaling centers to generate correct morphogenesis of the vertebrate limb. *Fgf4* in the posterior portion of AER and *Wnt7a* in the dorsal ectoderm maintains *Shh* expression in ZPA. At the same time, Shh regulates BMP antagonist *Gremlin* expression positively to block the BMP inhibitory action on AER-FGF expression. In addition, *Wnt7a* also helps to maintain *Fgf-8* expression in AER. Thus a deregulation of some of these signals can generate a limb with alterations in three polarities.

Skeletal development

Molecular interactions between the organizer signaling centers regulate limb shape. One of the first events during limb development is skeleton formation. The appendicular skeleton is initially formed when prechondrogenic mesenchyme aggregates forming a cartilage scaffold. Then, cartilage differentiation begins prefiguring the future skeletal bone elements. They are integrated by proliferating chondrocytes which allow their growth, so when the skeletal pattern is established, cells at the middle region of the skeletal elements exit the cell cycle and initiate the joint formation program, delimiting the boundary of each skeletal element. Later in development the segmented skeletal elements continue to grow and again the cells at the middle region exit the cell cycle but now they adopt an alternative fate and become pre-hypertrophic, leading down the path to hypertrophy and eventual ossification instead of becoming pre-joint cells.

Joint formation

Joint formation occurs at specific regions of the continuous precartilaginous condensations and involves loss of chondrogenic markers and apoptosis. The interzone is the first morphological evidence of joint development and is composed by three cellular layers; one central lamina with low cell density flanked by two areas of high cell density. The central layer disappears by cavitation giving rise to the formation of a primary joint cavity. The two areas of high-density differentiate into articular cartilage of adjacent bones. Ligaments, synovial lining, and tendons develop from cells located laterally to the skeletal element [11]. Joint formation may be related to the regulation of the balance between chondrogenic signals and joint inductive signals. The interzone is formed when chondrocyte differentiation is inhibited, presumably by *Wnt9a*; it is expressed in joint-forming regions and has the ability to initiate joint formation [7]. Wnt9a is able to induce cellular and molecular events characteristic of the first steps of interzone formation. Also, it inhibits cartilage differentiation and induces the expression of joint markers such as *Gdf5*, *Autotaxin*, *Chordin* and *CD44*, which are expressed in the interzone. Wnt9a can act at two levels, blocking chondrogenesis, and inducing expression of joint markers. Mice mutant for Wnt9a do not show an abnormal phenotype related with joint formation, although β-catenin deletion suggests that *β-catenin* signaling is necessary and sufficient to induce joint formation [28].

Chondrogenesis

Al later stages of limb development the undifferentiated cells underneath the AER are recruited by chondrogenic signals to give rise to digital rays, while cells not recruited into digital rays turn into mesenchymal cells of interdigital membrane which, in species with free digits, will subsequently die [29-35]. Cartilage differentiation or chondrogenesis starts when the transcription factor with a high-mobility-group (HMG-box) DNA binding domain *Sox9* is expressed in mesenchymal cells, promoting aggregation and expression of cartilage-specific proteins such as type II collagen, aggrecan, and sulfated proteoglycans [36-43]. Cartilage differentiation into digits occurs simultaneously with limb outgrowth; at the most distal region of the limb, undifferentiated cells are recruited to outline mesenchymal condensations for future skeletal elements. The importance of Sox9 at the onset of cartilage differentiation is evident after deletion of Sox9 expression in prechondrogenic mesenchyme. Under these conditions cartilage differentiation is stopped, and this leads to absence of skeletal elements. Interestingly, in these mutant limbs massive cell death is observed, rather than cartilage differentiation. These observations suggest that in the absence of chondrogenic factors mesenchymal cells are responsive to cell death signals [39]. Furthermore, misexpression of *Sox9* induces extra-digit formation in developing limb buds and rescues in hypodactyly caused by a *Hoxa13* mutation [40].

During the early stages of digit development, Activins A and B and Transforming Growth Factor β2 (TGFβ2), all of them members of the TGFβ superfamily, are expressed in digit primordia and act as primary signals which control the onset of chondrogenesis [30,42]. The chondrogenic potential of Activin and TGFβ is evident regarding their capacity to induce ectopic digits in the interdigital membrane [42].

Activin/TGFβ proteins placed in interdigital tissue induce the formation of an ectopic digit as a consequence of triggering a molecular cascade that recapitulates the normal chondrogenesis molecular cascade. *Sox9* is expressed as soon as 30 minutes while 6 hours after the *Bone Morphogenetic Protein Receptor 1b* (*Alk6*) is expressed [44]. Bone Morphogenetic Proteins (BMPs) were originally described in terms of their role in ectopic bone induction, when they were subcutaneously or intramuscularly implanted in adult rats [45]. BMP signaling during condensing prechondrogenic cells promotes “compaction” of mesenchymal cells, that is, a cohesive cell behavior in mesenchymal cells to delineate the boundaries and size of cartilage elements [46]. BMP signaling is mediated by Alk3 and Alk6 receptors. Cartilage differentiation is inhibited if these receptors are

inactivated or if BMP antagonist soaked beads are implanted in chick pre-chondrogenic mesenchyme. Also the KO of a number of BMPs expressed during limb development manifests cartilage differentiation deficiencies [43,47-51]. Accordingly, when BMP signaling is activated in the prechondrogenic mesoderm, cartilage differentiation is observed [45,51-54].

Undifferentiated mesenchymal cells under the AER are able to respond to signals that promote cell differentiation, cell proliferation or cell death. Chondrogenesis is activated by Activin/TGFβ signaling while FGF-AER arrests cells in their progenitor state. WNT signaling from ectoderm, including AER [29] promotes proliferation and inhibits cartilage commitment by inhibiting *Sox9* expression [13,55-56]. During the early stages of limb development, the mesenchymal cells beneath the AER are in progenitor state while cell proliferation and cell differentiation is inhibited as a result of high signaling levels of FGF-AER and WNT. When the limb grows out, some cells lie outside the influence of these factors; now undifferentiated cells are competent to chondrogenic signals giving rise to the Digit Crescent (DC) at the tip of the forming digits leading cells to the cartilage lineage [29,32].

Once morphogenesis of the skeletal elements leads to formation of individual elements they are formed by proliferating chondrocytes. The chondrocytes from the central region of cartilage elements finish proliferating and turn pre-hypertrophic and later hypertrophic by endochondral ossification. Changes in the extracellular matrix (ECM) molecules are very important to allow invasion of blood vessels, bone marrow cells, and osteoblasts, resulting in replacement of cartilage with bone. Later, cartilage grows only at the end of long bones in a narrow area called growth plate, which resembles embryonic chondrocyte differentiation.

Cartilage differentiation from the proliferating stage to the pre-hypertrophic and hypertrophic stages is controlled by several factors [36,41, 43,47-49,57-58]. Control progression of the cell cycle in proliferating chondrocytes requires *Sox5* and *Sox6* genes, [47] which down-regulate chondrocyte pre-hypertrophy by down-regulating *Runx2*, an essential gene that promotes cartilage maturation. When proliferating chondrocytes undergo pre-hypertrophy, *Runx2* [59], *Ppr* (receptor for parathyroid hormone and parathyroid hormone-related peptide) [46] and *Ihh* (Indian hedgehog) [49] expression occurs. Mice mutant for *Runx2* show complete absence of chondrocyte maturation [52], while misexpression of *Runx2* induces development of ectopic hypertrophic cartilage [43]. Down-regulation of *Ppr* and *Ihh* expression is a prerequisite for the onset of hypertrophy, and type X collagen is up-regulated.

Joint formation versus chondrogenesis

The mechanism of control by which proliferating chondrocytes are directed towards a pre-joint or pre-hypertrophic fate is poorly known. Experimental evidence suggests that integrin signaling regulates the decision between these two fates. Integrins are receptors for ECM proteins; in mouse limbs, blockade of α5β1 integrin function by specific antibodies or RGD peptides results in ectopic joint formation between proliferating chondrocytes and hypertrophic chondrocytes with a plane of segmentation perpendicular to the long axis of the bone, concomitantly with expression of Wnt9a, Gdf5, Chordin, Autotaxin, type I collagen and CD44 in the ectopic joints. Also, inhibition of pre-hypertrophic chondrocyte differentiation is evident. In normal development, Wnt9a and α5β1 integrin expression suggests that this integrin is down-regulated during joint formation. Moreover, human α5β1 ntegrin misexpression in the embryonic chick leg autopod is able to inhibit joint formation and to promote pre-hypertrophic differentiation evaluated by IHH expression. The fate of proliferating chondrocytes to become either pre-joint or pre-hypertrophic tissue is regulated by integrin signaling. In the presence of integrin α5β1, proliferating chondrocytes turn into pre-hypertrophic cartilage, while in the absence of integrin α5β1, proliferating chondrocytes enter the pre-joint program [41,43].

Tendon development

The main function of tendon is to transmit the force generated by muscle contraction to the skeleton [60]. Tendons are dynamic and exhibit a number of specialized regions along their length named myotendinose junction and enthesis junctions, which allow force transference from muscle to bone contributing to the overall integrity of the musculoskeletal system [61-63].

The functional integrity of the musculoskeletal system relies on the coordinated development of muscle, tendon and cartilage. Most studies of tendon morphogenesis are focused on the distal autopod tendons. One of the earliest stages of limb tendon development is characterized by the appearance of the mesenchyme lamina, a scaffold for the subsequent condensation of pre-tendinous mesenchyme cells [64-65]. However, this structure has not been detected in the proximal dorsal and ventral tendon-forming regions [62-63]. Another aspect of tendon development is the necessary interaction with muscles; for example, in the absence of muscles, proximal tendons fail to pattern and subsequently degenerate, while distal tendons will continue to form although they require the attachment to muscle bellies for their maturation [66-68]. Tendon progenitors are induced in the mesenchyme

directly under the ectoderm following the proximal to distal outgrowth of the limb [69], and connect the differentiating muscles and corresponding cartilage elements [63].

Recent studies identified *scleraxis* (*Scx*), a bHLH (basic helix-loop-helix) transcription factor, as an early marker of tendon cell fate [70]. Disruption of TGF-ß signaling results in the loss of tendons and ligaments and the phenotype resembles that of scleraxis gene knockout mouse. *Tgfb2*$^{-/-}$;*Tgfb3*$^{-/-}$ and *Tgfbr2*^{Prx1Cre} mutant embryos showed no *Scx* expression, indicating that TGF-ß signaling controls *Scx* expression acting upstream in a molecular cascade that establishes tendon formation. Tendons are originated in a portion of somites called syndetome which express *Scx*. Some studies have shown that in early tendons the molecular cascade is initiated by FGF signalling. This pathway has been described during syndetome formation in which scleraxis is induced by the dermomyotome through FGF8 [66,68,71]. It is also known that ectodermal BMP signaling represses *Scx* expression [66].

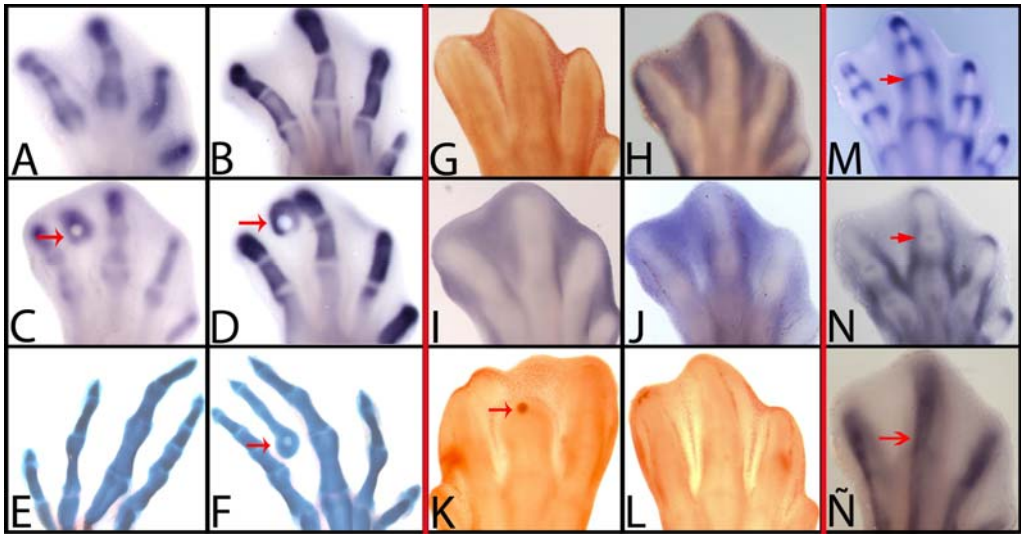

Figure 3. Limb morphogenesis involves the fine-tuning of various processes mediated by molecular interactions. *Sox9* expression in chick leg bud at E29 HH showing cartilaginous condensations (A); *type II Collagen* in chick leg bud at E30 HH in digital rays (B). TGFß soaked bead implantation (arrow), which is a chondrogenic factor, induces *Sox9* 30 minutes after implantation in interdigit (C), and *type II Collagen* at12h (D) and results in ectopic digit formation (E); control (F). Apoptotic cell death takes place in interdigital membranes in organisms with free digits and those areas can be stained with neutral red (G), in addition these areas express *Bmp7* (H), *Bambi* (I) and *Rarß* (J). Implantantion of retinoic acid-soaked beads induces cell death in digit tip (K); control (L). Besides, simultaneous to the joint formation process, chondrocytes lose their condrogenic markers and express *Gdf5* (M) and *Wnt9a* (N), while *Scleraxis* is expressed in tendons during chondrocyte formation (O). A-D, H-J and M-O, whole-mount in situ hybridizations; E and F, Alcian blue staining; G, K and L, neutral red staining.

Programmed cell death

Programmed cell death (PCD) is a key process which occurs during embryogenesis at predictable times and in known spaces, eliminating cells in order to sculpture various embryonic structures [72]. In developing chick embryos, PCD is responsible for sculpturing limb shape. Two zones show evident PCD in the anterior and posterior regions of the limbs, and they are hence called anterior and posterior necrotic zones (ANZ and PNZ, respectively). PCD also occurs between the radius and ulna and in interdigital tissue; these zones are called opaque patch (OP) and interdigital necrotic zone (INZ), respectively [50,73-74].

The ANZ and the PNZ have been associated with the reduction of digit number (three digits in the wing and four in the leg). Indeed talpid^3 chicken mutant lacks ANZ and PNZ resulting in polydactylous limbs, whereas the wingless mutant chick presents massive cell death in the ANZ [75]. At later stages of limb development, when digit morphogenesis is taking place, in species with free digits mesenchymal cell death occurs in the INZ [72-76]. When interdigital membranes are permanent such as in ducks and bats, cell death in the INZ is inhibited [53,73,77]. If cell death is inhibited in interdigital membranes of species with free digits then interdigital membranes are permanent, and this is called syndactyly.

Programmed cell death by apoptosis occurs as a result of two main pathways. In the first, the intrinsic pathway, cytocrome *c* is released from mitochondria by activity of pro-apoptotic proteins Bax and Bak. Activation of Caspase 9 occurs by interaction of cytochrome *c* and dATP and Apaf-1, which in turn will activate executioner caspases such as Caspase-3, leading to the classic features of apoptotic cell death. The second pathway is extrinsic and requires the activation of death receptors located on the cell surface leading to Caspase-8 activation. Furthermore, lysosomal cathepsins have been involved in the extrinsic pathway, triggered by TNF-α [75]. *Bax*$^{-/-}$, *Bak*$^{-/-}$double mutant mice and *Apaf-1*$^{-/-}$ mutant mice lack of interdigital cell death leading to soft-tissue syndactyly [78-79]. On the contrary, mutant mice for any of the caspases that are active in the interdigital membrane (Caspase-2, Caspase-3, Caspase-6, Caspase-7, Caspase-8 or Caspase-9) or when they are suppressed by broad-spectrum caspase inhibitors, such as Z-VAD-FMK in chick limbs, do not exhibit inhibition of interdigital cell death [80-86] suggesting a mechanism of caspase-independent apoptosis. Although the expression of lysosomal cathepsins in mesenchymal cells committed to die has been demonstrated [83], the loss of cathepsin function does not inhibit programmed cell death in interdigital membranes. Even so, if simultaneous experimental inhibition of both cathepsins and caspases is achieved,

inhibition of interdigital cell death occurs, suggesting cooperation between extrinsic and intrinsic pathways mediating interdigital cell death [84]. In addition, cathepsins are able to induce cell death by a mechanism independent of caspases [85-86].

Among molecules involved in cell death control are those related with BMP signaling; the *Bmp2, Bmp4*, *Bmp5* and *Bmp7* expression pattern coincides with interdigital cell death [42,87-91]. When BMP signaling is activated by soaked beads implanted in the INZ or by misexpression of the Alk6 receptor, premature massive cell death ensues, associated with the expression in the INZ of *Fgf receptor 3* (*Fgfr3*), *Snail, Dkk*, *BAMBI*, *Msx2, Smad8* and its inhibitory Smad, *Smad6* [91]. Contrariwise, the blockade of the BMP signaling function by over expression of *Alk3* and *Alk6* dominant negative forms and by BMP antagonists in the interdigital membrane, results in cell death inhibition [48,52-54,73,88]. BMPs signal through Smad1, Smad5 and Smad8; from these only *Smad1* and *Smad8,* but not *Smad5* are expressed in interdigital tissue. The onset of programmed cell death coincides with an increase in phospho-Smad1 levels, suggesting that cell death in the INZ may be mediated by BMP-SMADs [73]. Also BMPs and their receptors are expressed in the AER and have been implicated in the control of cell death in the INZ. Inactivation of *Alk3* in the AER results in syndactyly, suggesting that BMP signaling, through Alk3 in the AER, regulates interdigital cell death [89]. However, double deletion of *Bmp2c/c*; *Bmp4c*/c in mesenchyme and in the AER leads to interdigital cell death inhibition, although when the removal occurs in the AER, expression of the *Fgf8* gene is maintained [54,90]. FGF signaling has been considered as a survival factor, protecting interdigital mesenchyme from cell death by activation of BMPs. When BMP signaling is activated, premature cell death is associated with a decrease in *Fgf8* expression in the AER; oppositely, when BMP signaling is inactivated, up-regulation of *Fgf8* in the AER is evident. However, BMPs are unable to promote cell death in the interdigital membrane when FGF signaling is simultaneously inhibited, suggesting that there is cooperation between BMP and FGF signaling, with respect to interdigital cell death control [91]. Further studies are required in order to clarify the dual role of FGF in this process.

Webbed digits observed in species such as the duck or bat are the consequence of cell death inhibition by different mechanisms. *Gremlin*, a BMP antagonist is expressed in interdigital mesenchyme in the hindlimb of duck embryos, which suggests that it may inhibit cell death promoted by BMP in the INZ[53]. Bat embryos express *Gremlin* in addition to *Fgf8* in forelimb interdigital mesenchyme [73]. These results suggest that the high levels of FGF signal and the inhibition of BMP signaling by *Gremlin* in

interdigital tissue prevents cell death in this species. In addition, SHH signaling has been involved in cell death inhibition, since *Shh* expression and its target *Patched* in bat limbs is evident in the interdigital membrane. It has been hypothesized that FGF8 and SHH reactivate a feedback loop, which contributes to the survival of interdigital tissue [92].

In addition to the role of BMP signaling in the control of interdigital cell death, retinoic acid (RA) signaling plays a pivotal function in this process. RA is a derivate of Retinol (vitamin A) metabolism. Its signaling is mediated by two types of nuclear receptors, RAR and RXR, composed each one by three isoforms (α, β and γ) [83-84]. Compound mutant mice for these nuclear receptors display inhibition of cell death in INZ and, in consequence, syndactyly [91,93]. The *Hammertoe* mutant mouse is characterized by inhibition of cell death and syndactyly in all four limbs. RA treatment of pregnant *Hammertoe* females can rescue the limb phenotype [94]. It is known that interdigital cell death induced by RA is mediated by BMPs, since simultaneous treatment with RA and the BMP antagonist Noggin are unable to induce cell death [95]. Notably, inhibition of RA signaling leads to a block of naturally occurring interdigital cell death which results in ectopic digit formation [95] suggesting that RA signaling activates the molecular cascade that leads to interdigital cell death and concomitantly inhibits cartilage differentiation.

Cell differentiation versus interdigital cell death

Retinoic Acid (RA) metabolism is a key regulator of chondrogenic and apoptotic signals. The onset of RA synthesis occurs when retinol is converted to retinal by reversible oxidation of alcohol dehydrogenases and then by irreversible oxidation of retinal to RA by retinaldehyde dehydrogenase 2 (RALDH2). RA availability in tissue is led by CYP26 enzymes, cytochrome P450 family members, which catalyze RA oxidation into a wide variety of metabolites, such as 4-oxo-RA, 4-OH-RA or 18-OH-RA [96]. Mutant animals for *Raldh2* or *Cyp26* genes provide information concerning the role of RA in early limb development but not on digit formation and cell death [96-97]. In the mutant mouse model for synpolydactyly there is deficiency of *Raldh2* expression, as a consequence of a *HoxD13* mutation, a gene expressed during autopod development. Analysis of mutant mice phenotypes demonstrates ectopic expression of *Sox9* in interdigits, which explains the polydactyly observed in this mutant. Notably this limb phenotype is rescued by intrauterine treatment with RA [98]. Activation of Activin/TGFβ signaling or inhibition of RA signaling into interdigital tissue inhibit cell death and induce formation of an ectopic digit. Deletion of the *Sox9* gen from

undifferentiated mesenchymal cells of limb buds, results in complete absence of digit formation and massive cell death [39]. All these data suggest that the chondrogenic potential of interdigits is repressed and that the absence of chondrogenic signals results in cell death.

Finally, the molecular mechanisms by which BMP signaling promotes cell death or cell chondrogenesis still need to be defined. It is known that GDF5, a member of the BMP family, promotes chondrogenesis when it is implanted in the tip of digits [94]. Likewise, constitutively active forms of *Alk6* and *Alk3* misexpression result in the enlargement of cartilage [52], while only Alk6 is expressed in digit cartilage condensations, so that cell response to chondrogenesis or cell death may be receptor dependent. On the other hand, once the molecular cascade of chondrogenesis is triggered by TGFβ in the interdigit, subsequent implantation of BMP-soaked beads is unable to induce cell death [44]. A possible explanation may be that when *Sox9* is expressed 30 minutes after implantation of TGFβ, mesenchymal cells become insensitive to cell death inducing signals and BMPs are thus unable to promote cell death, as the role they play in the chondrocyte lineage is to potentiate cartilage differentiation. Contrastingly, when *Sox9* is conditionally deleted from undifferentiated mesenchymal cells, prior to signs of condensation, cells become sensitive to cell death inducing signals and, as a result, BMP are able to induce interdigital membrane cell death.

In conclusion, our interpretation of the above results suggest that the digit-interdigit pattern depends on a balance between chondrogenic and apoptotic signals acting on the fate of undifferentiated cells beneath the AER during autopod formation.

Limb evolution

Although the apendicular skeleton in tetrapods evolved from paired fins, any discussion about the transition from fin to limb should begin with the analysis of structures and development of unpaired median fins. The fossil records indicate absence of paired fins but well-developed median fins in the earliest vertebrates. Actually, the mechanisms of fin development were established in medial finfolds before the origin of vertebrates. Cephalochordates and Tunicates together with Vertebrata are subphyla of the phylum Chordata. Features common to these subphyla are expected to be ancestral, as are the median fin fold, notochord and dorsal nerve cord. Cephalochordates are fish-like organisms with a dorsal and a tail fin supported by fin rays composed of connective tissue. They lack paired fins but have paired ventro-lateral longitudinal ridges named metapleural folds. These ridges run from the anterior pharyngeal region to the atriopore.

Cephalochordates are considered the closest living relatives of vertebrates but recent phylogenomic analysis of chordate relationships demonstrate that Urochordates are the closest living relatives of vertebrates. Urochordates possess a free-swimming larval form that undergoes a dramatic metamorphosis. A fin is found running completely along the midline of the length of the tail. The analysis of median fin fold development in Cephalochordates and Urochordates (especially in the neotenic Larvaceans) should be important to understand the developmental mechanisms that led to the evolution of paired fins.

Paired fins are characteristic of Gnathostome vertebrates (jawed vertebrates) and the most primitive paired fins belong to extinct acanthodians and placoderms. The study of primitive fish embryos of Chondrichtyans and Agnatans is important to understand the evolutionary changes from median fins to paired fins. M. J. Cohn and collaborators have shown that shark median fin outgrowth is led by an apical ectodermal ridge [99], and also that median fins express a nested pattern of Hoxd genes as occurs in tetrapod limbs [100]. N. Shubin and his group has shown that retinoic acid treatment in shark embryos generates mirror image duplications of median fins a response observed in the paired appendages of many vertebrates [101-103].

The fin to limb transition took place around 360 million years ago. The origin of the tetrapod limb is the result of important anatomical and functional changes. One of the most important was the appearance of digits on limbs. During limb evolution the first step to digit formation was the appearance of the autopodial field, which represents the distal end where the autopodial structures are formed. The autopod can be divided into more proximal bone elements or mesopodium, composed by ankle and wrist bones and, the distal bone elements or acropodium, composed by the metacarpals and phalanges. The fossil record shows that digits evolved before the full complement of ankle and wrist bones [102]. *Acanthostega* and *Ichthyostega,* two early tetrapods now extinct, had limbs structured with the three recognizable bone elements of extant tetrapods: the stylopod, the zeugopod and the autopod. However, the acropodium of these Late Devonian tetrapods were polydactylous and webbed with reduced or absent mesopodial bones.

Tiktaalik and *Panderichthys,* two extinct tetrapod-like fish, had limbs with intermediate characteristics between those of early tetrapods and sarcopterygian fish. The distal part is an expanded array of rod-like bones similar to the autopodial pattern of basal tetrapods. However, a fan of lepidotrichia rimmed the distal region as occurs in most fish. In fact has been demonstrated that the distal limb portion of *Panderichthys* is more tetrapod-like than that found in *Tiktaalik* [104].

Eusthenopteron is an extinct fish that shares many unique features with early tetrapods. The fin endoskeleton of this sarcopterygian fish had bones showing clear homology with the humerus, ulna and radius. But distally, they had a series of mesomeres and radials with a shape and arrangement that makes the equivalence with the tetrapod bone elements uncertain. However, it has been demonstrated that the *Hoxd13* gene, whose expression is associated with digit development, is expressed in the distal radials of the *Neoceratodus* lungfish fin, a living sarcopterygian fish [105]. Another striking observation is the occurrence of programmed cell death in the inter-radial spaces of developing shark fins [100]. Taken together, these results support the old idea that the distal radials can be interpreted as digit homologues. All these observations imply that the not neomorphic autopod is a structure that evolved in the water before the origin of tetrapods.

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7. Stem cell niches in animal development and adulthood

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Stem cell niches are able to maintain stable populations of stem cells allowing stem cell self-renewal and the production of differentiating progeny. Niches create a permissive environment for self-renewal by providing physical support and signals to resident stem cells. Niche-maintained stem cells support organogenesis, tissue remodelling and tissue homeostasis by replacing cells lost through natural cell death or injury. In this chapter we examine the importance of stem cell niches for the preservation of adult tissues and review the molecular mechanisms involved in niche-dependent stem cell maintenance in animals.

1. Introduction: The importance of stem cell niches

Adult stem cells are able to generate multiple cell types specific to the tissue in which they reside, and are thus the long-term progenitors of tissues. In some instances, this property of stem cells depends upon their capability to undergo asymmetric, self-renewing divisions that result in one stem cell and

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one differentiating sister. The daughter stem cell of each division remains undifferentiated, and may continue to divide during the organism's life span, whereas the non-stem cell daughter enters differentiation, finally giving rise to the different types of mature cells present in the adult tissues. In other cases, homeostasis is achieved through an overall balance between proliferation and differentiation implying that stem cells must be finely regulated in their host tissues. Stem cells are maintained in specific locations where the surrounding cellular microenvironment enables them to reside for an indefinite period of time and to produce progeny cells while self-renewing. These microenvironments are called stem cell niches and always rely on a population of support cells (also known as stromal cells) that provide a physiologically permissive environment for correct stem cell function [1].

The ability of adult stem cells to generate multiple cell types characteristic of the tissue in which they reside has created tremendous excitement about the prospect of using these cells for tissue engineering, an area with enormous potential for regenerative medicine. Therapies based on adult stem cells may allow patient-specific treatments to be performed without risk of immune rejection, and provide an alternative to the controversial use of embryonic stem cells. So far, the only successful adult stem cell therapies reported have utilized stem cells derived from bone marrow (BM) and umbilical cord blood [2]. A deeper understanding of the molecular mechanisms controlling stem cell function *in vivo* is necessary to enable the use of other stem cell sources for regenerative medicine.

In recent years, there have been important advances in our understanding of stem cell establishment and maintenance. However, there are still fundamental questions to be answered regarding the biology of adult stem cells and their interactions with the cellular microenvironments in which they are maintained. The use of different model systems, such as the fruit fly or mouse, will undoubtedly help in this task, especially since the term niche is appropriate to describe stem cells populations in species from *Drosophila* to vertebrates and since the essential mechanisms involved in the maintenance of stem cells are likely to be conserved.

2. Definition of a stem cell niche

Stem cells found within adult tissues often reside within special microenvironments or niches. Schofield [1] was the first author to coin the term "niche" to describe "the physiologically limited microenvironment that influences stem cell behaviour". Since then, the niche hypothesis has

been supported by experiments in which the niche is first manipulated to eliminate the resident stem cells, and later repopulated with exogenous donor cells [3-6]. Thus, “a niche is a subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo*” [7]. However, it is important to take into account that not only environmental regulatory signals, but also intrinsic genetic programs, are required to maintain stem cell properties and to direct stem cell proliferation and differentiation. Therefore, a combination of both extrinsic and intrinsic factors ensures a perfect regulation of stem cells.

2.1. Regulation of niche numbers and activity

Niche architecture plays an essential role in controlling the numbers of stem cells that they can host. Stem cell populations are regulated both spatially and throughout development in response to environmental factors and by mechanisms that depend on positive and negative feedback loops that have been conserved during evolution. For instance, the number of stem cells could be constrained by the amount of available space in the niche. In this case, stem cells generated in excess of the limited space would undergo differentiation [8]. The number of niches is also regulated developmentally. For example, the number of hair follicles in the mouse normally does not increase after birth [9]. However, niches in other tissues multiply from juvenile to adult stages to ensure proper tissue functionality, such as in the adult mouse intestine which contain many more crypts than newborns guts [10].

In addition to the developmental regulation of niche activity and numbers, there are some signals that are able to induce *de novo* production of niches. For example, high levels of Hedgehog signalling in the *Drosophila* ovary increase the size of the follicle stem cell niche and also may form *de novo* niches [11]. Similarly, niches are able to change their regulatory mechanisms in response to different physiological situations. For instance, germline stem cells in the *Drosophila* ovary are susceptible to respond to different nutritional levels [12]. Hormones can also regulate the proliferation of stem cells such as in the breast tissue of mammals [13]. Finally, the association of stem cells with their niches is also dynamic. The same type of stem cell can use different niches at different times or under different physiological conditions. As an example, stem cells in the hair follicle bulge can move out of their niche and populate the niche associated with the dermal papilla at the base of the hair follicle [14].

2.2. Recruitment and maintenance of stem cells in their niche

During development and adulthood, niches are able to function, at least transiently, as signalling centres to attract stem cells, a process called ``homing´´ in the case of the haematopoietic stem cell (HSC) system [15]. Studies in *Drosophila* reveal that when germline stem cell (GSC) niches are experimentally depleted, niches still persist, and somatic stem cells (SSCs) are able to occupy the empty niche, maintaining some of their stem cell features [16]. The phenomenon of stem cell recruitment is due, not only to long range extracellular signalling molecules from the niche, but also to interactions with the extracellular matrix mediated by integrins (as in HSCs in mammals [17]) and to cell-cell adhesion molecules dependent on cadherins and catenins (as in GSCs [18]).

Once niches are formed, stem cells can reside within them for an indefinite period of time through interactions with their support cells [19]. What retains stem cells within their niche? Studies in *Drosophila* have shown that direct, physical interactions between stem cells and the stromal complement are needed to maintain the stem cells in the niche. One of the molecular components that anchor GSCs to their niches is *D*E-Cadherin and its intracellular partner Armadillo (β-catenin in vertebrates), which are concentrated at high levels in the region of contact between GSCs and their support cells of the ovarian niche [18] (see box 2). Cadherins and catenins participate in the formation of intercellular junctions called adherens junctions. The importance of adherens junctions in GSC retention by the stromal cells has been revealed through genetic studies, which have shown that mutations in either *D*E-*cadherin* or *armadillo* result in a failure of the niche cells to maintain GSCs [20]. Other players known to participate in stem cell retention are the integrins, receptor molecules that mediate cell to extracellualar matrix adhesion, that are often found at elevated levels in stem cells. Integrins can assist stem cells to remain in their niches, while the loss of integrin expression in mice can result in stem cell lost by differentiation or apoptosis [21]. Therefore, both integrins and adherens junctions play critical roles in the maintenance of stem cells within tissues and in regulating their proliferative capacity.

2.3. Stem cell maintenance is vital for adult homeostasis

During development, embryonic stem cells give rise to all of the different cell types contained within a particular tissue. After birth, cell differentiation is nearly complete and most adult stem cells, including both germline and somatic stem cells, already reside in their niches. Recent reports have proven

that adult stem cells reside in almost every tissue, including brain, bone marrow, peripheral blood, kidney, epithelia of the digestive tract, skin, retina, muscle, pancreas and liver [22].

The location of stem cells within their niches is essential for tissue homeostasis, as their proliferative capacity can support ongoing tissue regeneration, replacing cells lost due to natural cell turnover, injury or tissue remodelling. Although almost every tissue is able to renew, they do not do so at the same rate. For instance, tissues such as blood, skin, gut, respiratory tract and testis are renewing constantly. However, under normal conditions other tissues such as heart or brain have a low turnover [23-26].

Stem cells are able to sustain adult homeostasis due to their capacity to self-renew and to give rise to different types of mature cells. To achieve this, a delicate balance between self-renewal and differentiation must be maintained within the niche.

3. Regulatory mechanisms of stem cell self-renewal versus differentiation

3.1. Intrinsic genetic programs control stemness

Upon division, adult stem cells in specific tissues have the capacity to self-renew and to generate committed daughter cells. In order to ensure tissue integrity, this process must be very tightly regulated. Studies on stem cells using diverse systems have now shown that stem cell behaviour is controlled not only by external cues provided by the niches but also by the specific intrinsic genetic programs of stem cells themselves [27]. Thus, specific stem cell genes are modulated in response to niche signalling and as a consequence epigenetic mechanisms start to operate inside the cell. Therefore, in order to ensure an appropriate balance between self-renewal and differentiation it is necessary that the specific genetic programs of stem cells are subjected to environmental regulation.

In recent years, a series of intrinsic factors involved in the maintenance of the stem cell characteristics have been described [28]. Factors such as chromatin structure, micro RNA metabolism, translational repression and cytoskeletal organization play key roles in the specification of the stem cell fate. Various lines of evidence suggest that adult stem cells are maintained by repressing key differentiation genes. For instance, Polycomb family proteins have been shown to function as repressors of differentiating genes in adult stem cells by regulating chromatin structure [29]. In adult *Drosophila* females, GSCs express very few differentiation genes [30] and

these GSCs require a chromatin-remodelling factor, 'imitation SWI' (ISWI) to repress the differentiating gene *bam* [31] perhaps by interacting with Polycomb proteins. Moreover, the overexpression of telomerase activity can stimulate stem cell proliferation [32], as it has been shown that telomeres of sufficient length are also essential for stem cell proliferation [33].

3.2. The balance between self-renewal and differentiation in niches

Spindle orientation is an important intrinsic factor that determines stem cell fate. In this regard, the orientation of the mitotic spindle perpendicular to the stromal cells in the *Drosophila* germline stem cells can lead to asymmetric division, giving rise to a progenitor daughter and a stem cell daughter. Thus, these niches participate in the precise orientation of the mitotic spindle and therefore regulate the balance between the stem cell population and the committed progeny. Daughter stem cells must retain self-renewal instructions and inhibitors of differentiation, while daughter cells destined to differentiate must inherit proliferation and differentiation cues. In order to maintain proper stem cell number within the germline niches, some stem cells can also divide symmetrically, with the spindle orientated parallel to the support cells, giving rise to two daughter stem cells. The mechanisms involved in the establishment of stem cell polarity and spindle orientation are very important and include factors such as *adenomatous polyposis coli (APC)*, dynein-dynactin, PKC, actin and myosins [34-36]. For instance, *APC* gene in male *Drosophila* GSCs disrupts the normal orientation of the spindle perpendicular to the support cells impairing the asymmetric division [37]. The adhesive milieu of the niches also plays an important role in the regulation of self-renewal versus differentiation, retaining stem cell daughters within the niche, but forcing differentiating daughters to leave. Thus, niches display specific physical features and mechanisms designed to facilitate appropriate daughter cell movement. Once as a committed daughter first reaches a location where one or more critical signalling factors are no longer present, it often proliferates, giving rise to a "transit amplifying" population. In this case the progeny of stem cells do not specialize immediately after leaving the niche. Gene profiling studies are currently being used to identify candidate genes expressed differentially in stem cells and their progeny, with the aim of identifying key factors involved in controlling the transitions between self-renewal, proliferation and differentiation.

Under certain conditions, niches appear able to recover lost stem cells by dedifferentiation of transit amplifying cells. This probably occurs because

daughter stem cells do not immediately lose the capacity to function as stem cells and respond to niche signals. Thus, when a newly committed daughter cell finds an empty niche it can sometimes revert to a stem cell fate and re-populate the niche. For instance, it has been shown that differentiating cells in the *Drosophila* germline can revert to functional stem cells with very high efficiency when the appropriate factors are expressed [38,39]. Thus, daughter cells that do not lose the capacity to function as stem cells immediately after mitosis might be a source for therapeutic tissue repair in the future. However, since most stem cells daughters are prevented from reverting to a stem cell fate once they have entered differentiation, further experimentation is needed before this knowledge can be clinically applied.

3.3. Niche classification

As mentioned earlier, the orientation of the mitotic spindle during stem cell mitosis can often determine the fate of the two daughter cells. Depending of the behaviour of dividing stem cells, niches can be classified in three types [7]:

- Linage niches are characterized by the asymmetric division of their resident stem cells. This asymmetry might depend on the orientation of the mitotic spindle and implies specific positioning for daughter cells within the niche. Thus, the daughter self-renewing cell remains in contact with the basement membrane of the stromal compartment and continues to be maintained as a stem cell, while the committed progeny is expelled from the niche and its signals and enters differentiation.
- Population niches are based on the symmetric division of their stem cells. Thus, both daughter cells can remain within the microenvironment and become stem cells or both daughters can commit to differentiation. Linage niches and population niches can be distinguished by labelling experiments. In linage niches, genetically marked single stem cells remain within the niches over time and their progeny can be followed. In contrast, population niches homogenise their complement of stem cells and eventually they all present the same genotype.
- Finally, both mechanisms can be operating in a given niche. Thus, stem cells might orientate their mitotic spindle so that both daughter cells are either kept in the area of influence of the support cells, or both are removed from it. On other occasions, the spindle aligns so that after asymmetric division, both daughters follow different fates.

3.4. Niches use diverse regulatory pathways to regulate stem cell self-renewal

As mentioned previously, specific external signals that come from the support cells help maintaining stem cell populations within niches. Genetic studies on stem cell regulation have revealed that there are multiple regulatory pathways involved in the maintenance of this balance in many stem cell niches and, in most cases, they are conserved throughout evolution.

One of these pathways is the Notch signalling pathway**,** a highly conserved cascade with a pre-eminent role in cell-cell communication. The Notch protein is a transmembrane receptor that normally binds to a ligand that is expressed in neighbouring cells. Upon ligand binding, Notch is cleaved and the released intracellular domain enters the nucleus where it converts a transcriptional repressor into a transcriptional activator, thus driving expression of target genes. In many stem cell systems, Notch is produced in stem cells and is downregulated in progeny cells, whereas the ligand is present in the niche cells. For instance, Notch is expressed, and plays and important role, in haematopoietic stem cells (box 4) and intestinal stem cells in mammals (box 5) [40].

Another key family of signalling molecules involved in stem cell maintenance is the bone morphogenetic protein (BMP)/transforming growth factor-β (TGF-β) superfamily. Members of this family are involved in tissue remodelling and regeneration processes, where the regulation of stem cell behaviour is important. BMPs, which function through receptor-mediated intracellular signalling to modulate the transcription of target genes, play an important role in stem cell regulation in many different niches. However, their specific functions are different. For instance, in the *Drosophila* ovary, Dpp (the *Drosophila* homologue of BMP2/4) is essential for the maintenance of germline and somatic stem cells (box 2) [41,42]; in mouse intestinal stem cells, BMP signalling inhibits stem cell activation and expansion (box 5) [43]. Finally, BMP signalling via Bmpr1a controls haematopoietic stem cell numbers by regulating niche size (box 4) [44].

Jak/Stat (Janus Kinase/Signal transducer and activator of transcription) signalling is also required for the balance of stem cell maintenance versus differentiation in vertebrates and in *Drosophila*. The role of this pathway has been studied in great detail in the *Drosophila* testis [45,46]. Somatic hub cells express *unpaired* (*upd*), the ligand that activates the Jak receptor in GSCs. Recently, it has been described that this pathway also plays a critical role in the *Drosophila* ovarian niche, in a group of somatic stem cells in the ovary called escort stem cells which require Jak/Stat signalling to maintain the wild type population of germline stem cells [47] (box 2).

Another player involved in stem cell maintenance is Wnt, which acts through the β-catenin molecule. Wnts play an important role in cell fate specification during embryogenesis and recently has been involved in cell proliferation and lineage specification of somatic stem cells in the adult mouse [48,49]. Diverse studies implicate the canonical Wnt pathway in regulating SSCs in the skin epithelium (box 1) [50], the fly ovary (box 2) [51], the intestinal crypt (box 5) [52,53], and the brain (box 3) [54].

4. Common properties

In spite of their apparent diversity, niches seem to share some basic characteristics [7,27]:

- Many niches contain groups of cells that are involved mainly in the maintenance of stem cell populations. For instance, cap cells in the *Drosophila* ovary or osteoblastic cells in the bone marrow in mice.
- Niches often make use of molecules such as cadherins and integrins to mediate cell adhesion between stem cells and support cells or between stem cells and the extracellular matrix. For instance, E-cadherin is required for anchoring GSCs and a type of somatic stem cells in the *Drosophila* ovary.
- Stem cell behaviour and maintenance are controlled by extrinsic signals sent by support cells and received by stem cells. Signalling molecules that have been shown to be involved in the regulation of stem cell behaviour include Wnts, BMPs, Notch and the JAK/Stat pathway.
- Most niches modulate stem cell self-renewal and at the same time control the production of progeny cells that will support different cell lineages. This property relies on the physical structure of the niche and requires intrinsic characteristics of stem cells.

5. Perspectives

Niches have emerged as major tools for stem cell regulation in organisms. Adult stem cell populations are established in niches or specific locations that regulate how they participate in tissue generation, maintenance and repair. Niches prevent stem cells from depletion and at the same time avoid stem-cell overproliferation. Thereby, niches are important structures responsible for proper tissue physiology, integrating signals that mediate the balance between self-renewal and differentiation, in response to environmental cues. Although there have been important advances in our understanding of the communication between stem cells and their niches, many of the molecular mechanisms that control stem cell maintenance

remain unknown. Considering the therapeutic potential of adult stem cells and their ethical and clinical advantages over embryonic stem cells, there is a clear need for a deeper understanding of the mechanisms utilized to control adult stem cell biology. Until now, successful adult stem cell-based transplantation therapies have made use of bone marrow (BM) stem cells and stem cells derived from umbilical cord blood (UCB). Examples of such therapies include the treatment of myocardial infarcts with BM-derived stem cells and hematotherapy using UCB [2,55,56]. However, the scientific community still faces serious challenges before the widespread use of adult stem cells in stem cells therapies can be implemented.

Recent research has unveiled that stem cell niches harbour another important function that has been previously underestimated. Niches prevent tumourigenesis by controlling stem cell proliferation, as the misregulation of niche activity may give rise to uncontrolled proliferation of stem cells, resulting in stem cell-based tumourigenesis or cancer [57]. The concept of cancer stem cells was first shown by the work of Dick and others in the early 1990s [58]. Since then, many laboratories have demonstrated the existence of cancer stem cells that are able to perpetuate cancer in organs such as the breast or brain [59-61]

These findings have profound implications for cell-therapies and shed a serious warning on the clinical use of stem cells. Adult stem cells have the potential to enable patient-specific treatments for a host of diseases, but their use presents significant biological hazards, because of the potential for stem cell tumourigenesis.

Box 1

Epidermal stem cell niches in the skin

There are two well characterised skin stem cell niches, one located within the hair follicle and within interfollicular regions.

Skin or interfollicular niche

Proliferation in the interfollicular regions of the skin takes place in the basal layer of keratinocytes, which are typically attached to a basement membrane. Three types of keratinocytes can be distinguished in the basal layer of the epidermis: Stem cells, transit amplifying (TA) cells and committed cells [62]. Stem cells retain high capacity for self-renewal throughout adult life and are responsible for epidermal repair and maintenance. TA cells enter differentiation after a few rounds of division.

These committed cells detach from the basement membrane and move through the suprabasal layers until they finally die as cornified squames on the tissue surface [63]. The movement of committed cells from the basement membrane implicates the inactivation of the β_1 integrin. Integrins are known to play key roles in skin morphogenesis and homeostasis, as they mediate keratinocyte adhesion to the basement membrane and regulate the initiation of keratinocyte differentiation [59-61].

In addition to integrins, keratinocytes also express the classical E- and P-Cadherin [64]. Perturbation of cadherin function in cultured keratinocytes affects integrin expression and results in decreased proliferation and in an increase in terminal differentiation [65]. c-Myc is a member of the basic helix-loop-helix/leucine zipper family of DNA-binding proteins and regulates transcription in a variety of cell types to induce proliferation and to inhibit differentiation [66]. However, in the epidermis and in culture, c-Myc is expressed by keratinocytes in the basal layer and is downregulated during terminal differentiation [67]. In addition, it has been published that constitutive expression of c-Myc results in a reduction in keratinocyte proliferation [68]. Thus, c-Myc in the skin seems to stimulate differentiation into TA cells [69].

Hair follicle niche

The hair follicle or bulb is a complex structure composed by multiple layers such as the outer root sheath (ORS), the inner root sheath (IRS) and the hair shaft. The ORS is contiguous and biochemically similar to the basal layer (BL) of the epidermis. The dermal papilla (DP) is a signalling centre formed by specialized mesenchymal cells that maintain contact with transient-ampliying (TA) cells until they differentiate to form the IRS and hair shaft. The keratinocytes located in the lowest part of the hair bulb, known as germinative cells, have been considered stem cells [70] but there is now strong evidence that stem cells reside in a region of the follicle called the “bulge”, located below the sebaceous gland and at the level of the arrector pili muscle [14,71].

In the hair follicle, Wnt signalling has been shown to affect all phases of stem cell regulation, from quiescence and identity to proliferation and terminal differentiation [50,72]. In addition, two independent laboratories have identified, by expression profiling, 97-157 genes (with a concordance of 80-90%) differentially expressed in bulge stem cells and differentiated keratinocytes. These genes include fibroblast growth factor-1 (FGF1) and its receptor, TGF-β, and BMP and Wnt pathway inhibitors, all of which are known to be involved in the regulation of epidermal stem-cell proliferation and differentiation [73,74].

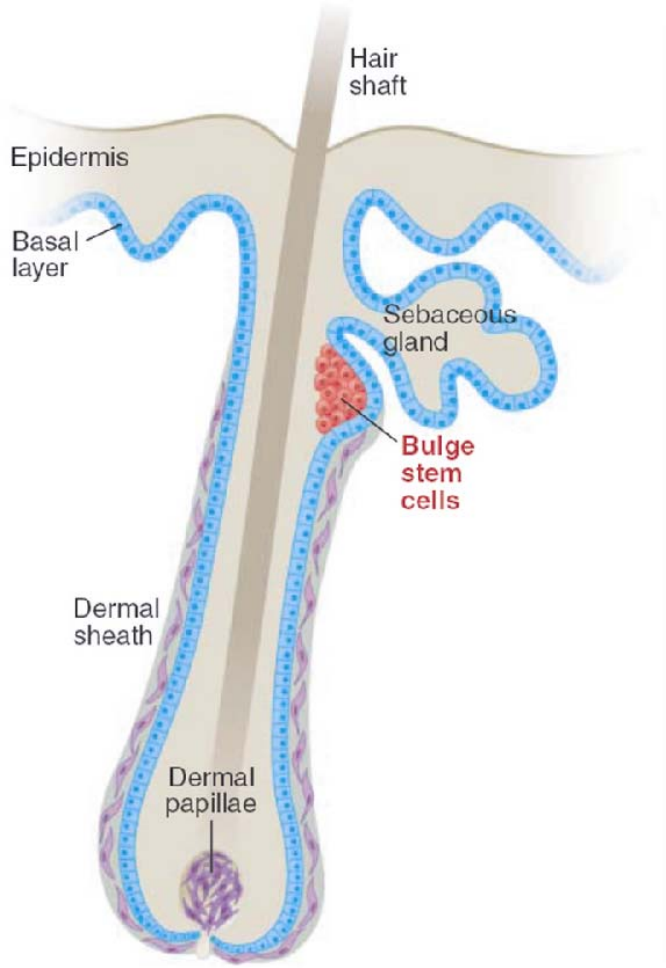

Figure 1. Diagram of a hair follicle niche showing the main cell types that make up the hair bulb. (Reproduced with permission from Moore and Lemischka, Science 311:1880-1885, 2006).

Box 2

Germline stem cell niches: The case of *Drosophila*

The *Drosophila* male and female germline have emerged as one the best models to study the biology of adult stem cells *in vivo*. These models have two main advantages: i) germline stem cells (GSCs) can be distinguished from early differentiated progeny and ii) the existence of multiple genetic techniques in *Drosophila* allows the genetic dissection of stem cell niches.

Drosophila ovary

The *Drosophila* female possesses two ovaries, each composed of approximately 16-20 egg-producing tubes called ovarioles. At the apical part

of each ovariole 2-3 GSCs reside in a conical structure termed the germarium. Each germarium has three subpopulations of adult stem cells, one population of GSCs and two populations of somatic stem cells (SSCs) essential for egg chamber production during oogenesis. In the germarium, GSCs are associated to three types of somatic cells that form the ovarian niche: terminal filament (TF) cells, cap cells (CCs) and escort stem cells (ESCs) [75].

GSCs can be unambiguously identified by the presence of a cytoplasmic organelle called the spherical fusome or spectrosome, and by their interaction with CCs through *D*E-cadherin-mediated adhesion [20]. At interphase, the spectrosome, an organelle rich in membrane skeletal proteins such as spectrins and Hu-li tai-shao (Hts), is located at the apical side of the GSC cytoplasm [76,77]. When the GSC undergoes asymmetric cell division, one daughter cell remains in contact with the CC and retains stem cell identity while the other daughter cell moves away from the niche to differentiate into a cystoblast. The cystoblast also contains a spectrosome but it is smaller in size and does not keep an apical localisation. The cystoblast undergoes four rounds of synchronous division with incomplete cytokinesis to form a 16-cell cyst. All cystocytes are interconnected through branched spectrosomes, called fusomes [76].

The most relevant extrinsic signal that controls GSC maintenance in the germarium is the activation of BMP/TGF-β signalling pathway. The BMPs known as Decapentaplegic (Dpp) and Glass-bottomed boat (Gbb) are produced by the CCs [75] and act as short-range signals to activate the type I (Thickveins (Tkv) and Saxophone (Sax)) and type II (Punt) receptors in the GSCs to mediate their survival [41]. The downstream mediators of this pathway are Mad and Med, which act to repress *bag of marbles* (*bam*) transcription [78,79]. The expression of *bam*, with the cooperation of *benign gonial cell neoplasm* (*bgcn*), is necessary and sufficient for GSC differentiation [80].

A second extrinsic signal in this niche involves the Yb, PIWI and Hedgehog (Hh) proteins. Loss of *Yb* or *piwi* results in a decrease in the number of GSCs, while the ectopic expression of these genes induces extra GSCs [77,81-85]. Yb is a novel intracellular protein with RNA binding domains that is generated in TF and regulates *piwi* and *hedgehog* (*hh*) expression [84,85]. Piwi is a nucleoplasmic protein expressed in TF, CC and germ cells and Hh is a signalling molecule whose expression is located in TF and CC [86]. GSC maintenance defects can be reverted by Hh overexpression in both *Yb* and *piwi* mutant ovaries [84]. However, Hh plays a small, redundant role in the maintenance of GSCs [84] and it is unknown how Piwi acts in GSC maintenance. Finally, the activity of the JAK-STAT

signalling pathway in somatic Escort cells is also essential for GSC maintenance [47].

Intrinsic factors are those that act within the stem cell to control its behaviour. Two essential target genes of the TGF-β pathway, *bam* and *bgcn*, are known to play a role in GSC maintenance and have already been discussed. The expression of *bam* is repressed within GSCs by an ATP-dependent chromatin remodelling protein termed Imitation SWI (ISWI) but it is independent of TGF-β signalling [31]. Pumilio (Pum) and Nanos (Nos) are RNA binding proteins considered intrinsic self-renewal factors because loss of function of either of them results in GSC loss [81,87-89].

Drosophila testis

The *Drosophila* male possesses two testes with a tubular structure and a distinct polarity. Each testis contains at the apical tip a group of support cells called “hub cells” that serve as a niche for seven to nine GSCs [37,90]. The hub cells contact GSCs directly through high levels of DE-cadherin and β-catenin, which form adherent junctions between the hub cells and the GSCs [37].

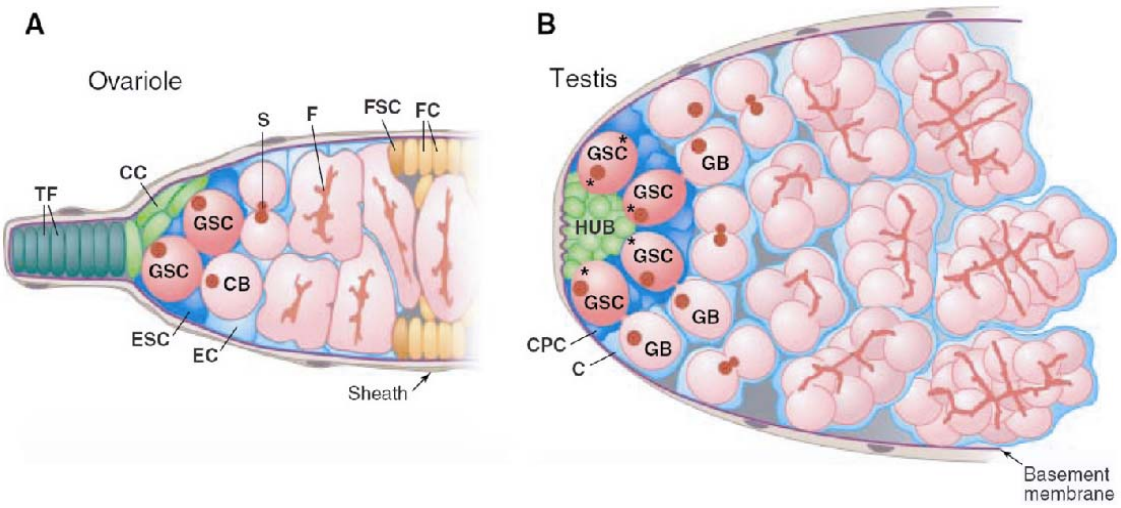

Figure 2. *Drosophila* female (A) and male (B) germline stem cell niches. (A) Anterior part of a germarium where the female germline stem cells (GSCs) are located. GSCs are in close contact with cap cells (CCs) and possess an apical spectrosome (S). Terminal Filament cells (TF), Escort Stem Cells (ESC) and Escort Cells (EC) are somatic components of the niche. Cystoblasts (CB) are committed daughters of GSCs. Fusomes (F) are an organelle characteristic of differentiating cysts. (B) Anterior part of a testis where male GSCs are found in close contact with support hub cells. Somatic cyst progenitor cell (CPC) and cyst cells (C) associate with GSCs and their committed daughters, called gonialblasts (GB). (Reproduced with permission from Fuller MT and Spradling AC, Science 316:402-404, 2007).

Two main signalling pathways, JAK-STAT and BMP pathways, regulate the maintenance of male GSCs. One of the ligands of the JAK-STAT pathway, a short-range signal known as Unpaired (Upd), is secreted by hub cells and received by GSCs to promote their self-renewal [46,91]. Thus, the role of the JAK-STAT pathway in male GSC maintenance is similar to that of Dpp/BMP in the ovary. In the testis, BMP signalling plays an important role in GSC maintenance, as removal of BMP downstream genes from somatic cells causes GSC differentiation [92].

As in the female germarium, *bam* expression in the testis is controlled by TGF-β signalling but the function of *bam* appears to be somewhat different in the male GSC niche. As in the female, male GSCs divide asymmetrically to self-renewal and produce differentiating daughter cells called gonialblasts (GBs). In this system, *bam* expression is dispensable for GB differentiation [93] but its repression is required to maintain GSC self-renewal [92]. It has been demonstrated that the asymmetric division of male GSCs requires APC2 to orientate the mitotic spindle perpendicular to the hub cells and this orientation is essential to preserve GSC numbers [37]. In addition, it has been suggested that the adherens junctions between the hub cells and the GSCs are needed for the binding of the APC2 to the GSC cortex [37].

Box 3

Neural stem cell niches

The adult mammalian brain hosts populations of neural stem cells (NSCs) that support neurogenesis in two regions: the subventricular zone (SVZ) of the lateral ventricules and the subgranular zone (SGZ) of the dentate gyri in the hippocampus [70,94-96]. The ability to generate new neurons over prolonged periods of time in these zones suggests the existence of specific microenvironments populated by NSCs.

NSCs in both regions exhibit some features of differentiated astrocytes like the expression of the glial fibrillary acidic protein (GFAP) [97] but, outside of these regions, astrocytes do not appear to harbour neurogenic capacity. Thus, the neurogenic behaviour of SVZ and SGZ astrocytes appears to be determined by signals restricted to their niches [98,99].

Three main cell types can be distinguished in SVZ and SGZ: astrocytes, inmature precursors and neuroblasts. In the case of the SVZ, astrocytes behave as stem cells (B cells) that divide to give rise to immature precursors (C cells). C cells differentiate into neuroblasts (type A cells) that migrate away from the SVZ through glial cells to the olfactory bulb, where they differentiate into interneurons [100]. In the SGZ, astrocytes (As cells) also act

as NSCs to give rise to progenitors (D cells), which in turn mature into new granule cells (G cells). Finally, G cells migrate a short distance to integrate into the dentate gyrus granule cell layer [101].

In addition to the above similarities, both neurogenic niches integrate several common elements: signalling molecules, ECM and basal lamina, the vasculature, and cell-cell interactions.

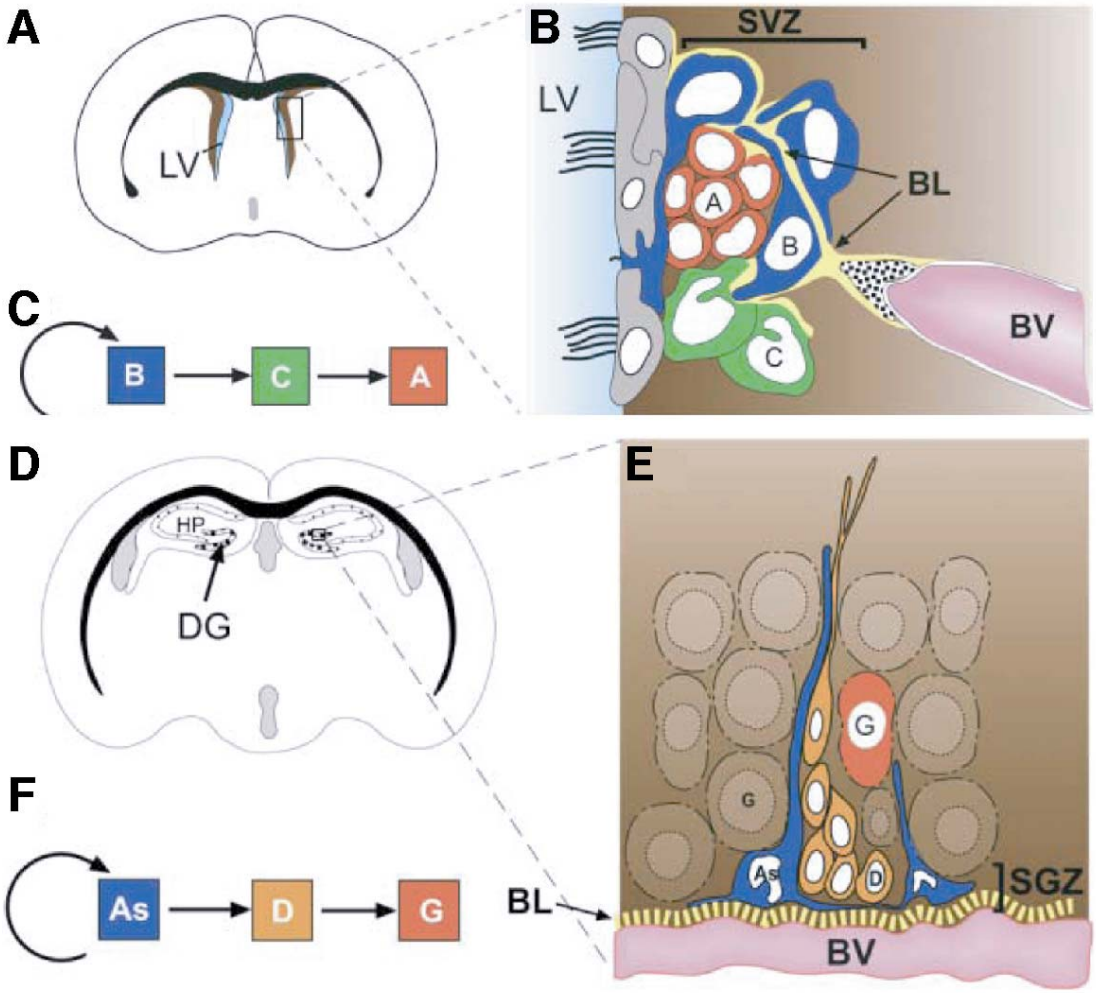

Figure 3. Neural stem cell niches in the mammalian brain. (**A**) Coronal section of an adult mouse brain to show the lateral ventricles (LV). (**B**) Representation of the Subventricular zone (SVZ) where the position of different cell types (A, B and C cells, and ependymal cells), the basal lamina (BL) and the blood vessels (BV) are illustrated. (**C**) Cell lineages of the SVZ. (**D**) Coronal section of an adult mouse brain at the level of the dentate gyrus (DG) of the hippocampus (HP). (**E**) Representation of the Subgranular zone (SGZ) where the different cell types (As, D and G cells), the basal lamina (BL) and the blood vessels (BV) are ilustrated. (**F**) Cell lineages of the SGZ. (Modified with permission from Alvarez-Buylla A and Lim DA, Neuron 41: 683-686, 2004).

Signalling molecules

Growth factors, neurotransmitters and hormones are the most characterized molecules acting in neurogenic niches. Growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF1), transforming growth factor-α (TGF-α) and vascular endothelial growth factor (VEGF) [102] control cell proliferation in adult neurogenic regions, including NSCs and their descendants. In cell culture experiments, NSCs can be expanded with EGF and bFGF [103]. Moreover, it has been demonstrated that EGF and bFGF stimulation of SGZ in damaged brains results in cell replacement and recovery of the hippocampus function [104]. Other molecules such as Notch1 and ciliary neurotrophic factor (CNTF) are crucial regulators of NSC maintenance and self-renewal within the neurogenic SVZ niche [105]. *In vivo*, CNTF induces neurogenesis in the adult mouse brain, probably by the CNTF α-receptor that is expressed in NSCs [106].

In the SVZ, proliferation occurs predominantly in regions innervated by dopaminergic projections from the midbrain (dopaminergic regions). Elimination of dopaminergic regions reduces proliferation in the SVZ, thus highlighting the importance of neurotransmitters in SVZ niche activity [107-109]. 5-hydroxytryptamine or serotonin projections converge with the dopaminergic projections in the SVZ [110] and with the noradrenergic projections over the SGZ, and stimulate neurogenesis in both niches. Other neurotransmitters have been also reported to contribute in SGZ, such as acetylcholine (Ach) [111,112] and glutamate [113,114]. Finally, hormones also have an important role in adult neurogenesis. Thyroid hormone increases NSC proliferation in the SVZ through α-receptors and transcriptional regulation of c-myc [115] and prolactin induces SVZ proliferation and increase neurogenesis during pregnancy [116].

Extracellular matrix (ECM) and basal lamina

The ECM is composed of proteins such as proteoglycans and collagens while the basal lamina is a specific type of ECM that permits the anchorage of factors and cells present in the SVZ. This niche, in which all cell types are interconnected by the basal lamina, is enriched in several ECM components such as tenascin-C, collagen-1, chondroitin sulfate proteoglycans and heparan sulfate proteoglycans [117-121]. Many factors involved in neurogenesis such as morphogens and mitogens (BMP-2, -4, Shh and Wnts), components of the ECM (collagens, laminins and tenascin), growth factors (EGFs, FGFs, IGF-1, PDGF and VEGF), chemokines and cytokines [117-119,122-124] bind to

heparan sulfate proteoglycans, highlighting the importance of extracellular components in the regulation of neurogenic niches.

The vasculature

In both neurogenic regions, SVZ and SGZ, endothelial cells form blood vessels and a specialized basal lamina. In addition, these endothelial cells secrete known mitogens and specific factors involved in both neuron differentiation and survival [125-127]. Since the disruption by irradiation of the intimate association of endothelial cells and SGZ precursors results in the impairment of neurogenesis [128], the interaction between endothelial cells and the ECM and/or the basal lamina is key to SVZ and SGZ niche regulation.

Cell-cell interactions

Astrocytes or NSCs project processes to connect to all cell types of the SVZ through gap junctions. In addition, they also contact with the basal lamina via end-feet connection [129]. This complex, spatial branching of NSCs may be used to detect alterations in cell numbers and to translate signals from the blood vessels and other cells to the neurogenic niche. In addition, NSCs are also in contact with the lateral ventricle, facilitating the perception of secreted factors in the cerebrospinal fluid.

Box 4

The haematopoietic stem cell niche

Blood cell production is sustained by haematopoietic stem cells (HSCs) during an organism's lifetime. HSCs are a subset of BM cells capable of self-renewal and of producing all types of blood cells. In addition to the haematopoietic cell population, the BM also includes mesenchymal stem cells (MSCs) which give rise to a variety of cell types including myocytes (muscle), adipocytes (fat), fibroblasts (connective), endothelial cells, osteoblasts (bone) and macrophages [130-133]. HSCs have been identified and isolated with the help of different markers: i) they express low levels of the differentiation surface antigen Thy-1, ii) they are negative for the lineage differentiation surface markers Lin^-, and iii) they are positive for the stem-cell antigen-1 (Sca-1). Thus, HSCs were identified like $Thy\text{-}1^{lo}Lin^-Sca\text{-}1^+$ cells [134]. HSC behaviour (including their maintenance, proliferation, differentiation, mobilization and homing) is regulated by the molecular and

cellular properties of the two types of niche in which they reside, namely the osteoblastic niche and the vascular niche.

The **osteoblastic niche** is mainly composed of the endosteum, the inner surface of the bone that provides an ideal location for HSC maintenance and mobilization. The endosteum is covered with osteoblasts (bone-generating cells) and osteoclasts (bone degrading cells), which secrete and/or activate a variety of factors that regulate HSC maintenance in the BM [135]. Recent data point towards the osteoblastic cells as important components of the HSC niche because of their location and expression of several haematopoietic growth factors [130,136]. Moreover, it has been demonstrated that an increase of osteoblast number in mice results in an increase of HSC number in the BM [44,137]. However, since osteoblast ablation from the BM increases HSC frequency over time and differentiating cells disappear from the BM [138], it has been suggested that HSC maintenance may be less dependent upon osteoblasts than differentiating cells. Finally, osteoclasts seem to have an important role in mobilizing HSC into circulation and in HSC survival, as the high amounts of calcium generated by osteoclast activity are involved in HSC maintenance [139,140].

An alternative niche, called the **vascular niche**, also contributes to the BM population of HSCs. A fraction of HSCs is adjacent to specialised blood vessels termed sinusoids [141], which consist of a single layer of endothelial cells where HSCs reside. It has been demonstrated that a stromal cell type called reticular cells surrounds these blood vessels, and that these cells secrete high levels of a chemokine known as stromal-cell-derived factor1 (SDF-1) that is required for HSC maintenance [142]. Moreover, CXCR4, the main receptor of SDF-1, is expressed in human HSCs and is required for HSC engraftment [143].

A variety of cytokines, growth factors, ligands, adhesion molecules and multiple development signalling pathways play important roles in HSC regulation. The Wnt-, BMP-, Notch-, Hh- and FGF-signalling pathways contribute to the modulation of HSC- niche activity [44,144-147]. The signalling of angiopoetin-1 (Ang-1), a secreted protein, from osteoblastic cells to HSCs through the cell cycle regulator receptor tyrosine kinase Tie-2 is essential for HSC maintenance [135]. In addition, the fact that a target gene of the Ang-1 pathway is N-cadherin [135] suggests that a link between adhesion molecules and cell cycle regulators exists in the HSC niche. Other adhesion molecules such as $\alpha_4\beta_1$ integrins [148], involved in HSC mobilisation and migration, have long been shown to be critical for HSC maintenance [149-151]. Lastly, Fibroblast growth factors are involved in haematopoietic progenitor cell recruitment and adhesion to the vascular niche and in HSC proliferation *in vitro* [152].

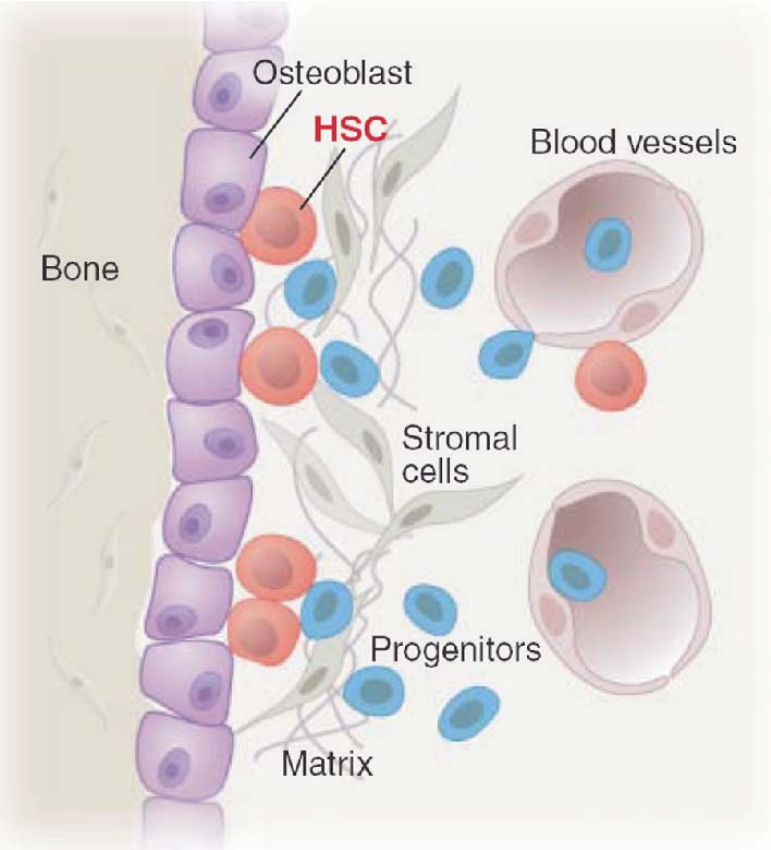

Figure 4. Schematic diagram of a haematopoietic stem cell niche. Two types of haematopoietic niches, the osteoblastic niche (defined by the osteoblastic cell-HSC interaction) and the vascular niche (defined by the endothelial cell-HSC interaction) are represented. HSC: Haematopoietic Stem Cell. (Reproduced with permission from Alvarez-Buylla A and Lim DA, Neuron 41: 683-686, 2004).

Box 5

Intestinal stem cell niche

The intestine is composed of a simple columnar epithelium with glandular invaginations called crypts. Four main differentiated cell lineages exist in the intestinal epithelium: columnar cells, mucin-secreting cells or ``goblet´´ cells, endocrine cells, and, in the small intestine, Paneth cells. This epithelium is supported by a structure termed lamina propria that contains numerous cells including fibroblasts, fibrocytes, vascular endothelial and smooth muscle cells, various blood cell lineages and one main type of myofibroblast called the intestinal subepithelial myofibroblasts (ISEMFs).

The intestine has a rapid epithelial turnover during adult life and it is one of the most common sites of cancer formation due to carcinogen exposure and high mitotic rate. The epithelial turnover depends on a population of

multipotent stem cells located near the base of the crypt in the small intestine, in the middle of the crypt in the ascending colon and at the crypt base in the descending colon [153]. Intestinal stem cells (ISCs) have the capacity to generate all cell lineages of the intestinal epithelium [154] and the balance between proliferation, differentiation and apoptosis in this system is essential to avoid cancer [155]. Two ISC markers have been identified allowing the identification of these stem cells: i) Mushasi-1 (Msh-1), an RNA-binding protein, and ii) the transcriptional repressor molecule Hes-1.

It has been proposed that ISEMFs [156] and vascular endothelial cells [157] of the lamina propria constitute the intestinal niche. ISEMFs are closely related to the epithelium and they exist as a syncytium, which extends throughout the lamina propria and merges with the endothelial cells. The ISEMFs secrete cytokines and growth factors, such as hepatocyte growth factor (HGF), keratinocyte growth factor (KGF) and transforming growth factor beta 2 (TGFβ2), that are essential for the regulation of epithelial cell differentiation and proliferation [158].

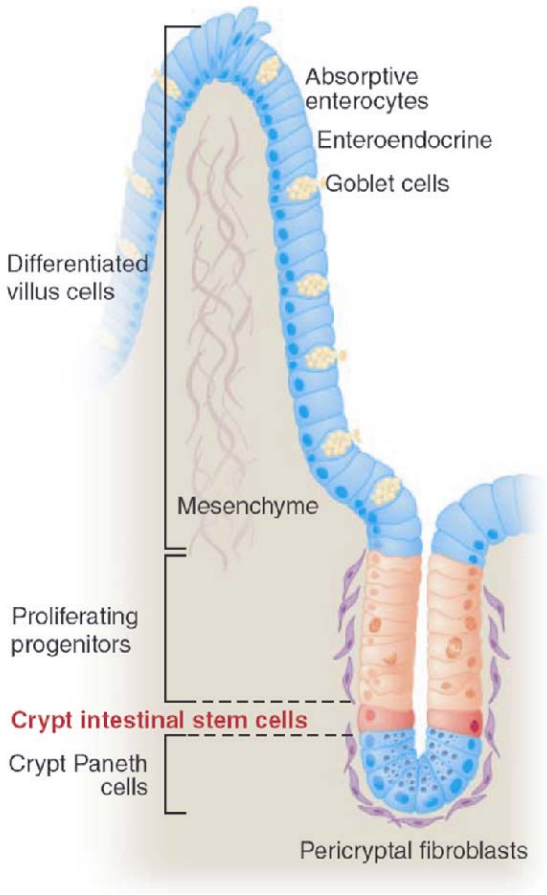

Figure 5. Schematic diagram of the intestinal stem cell niche. An intestinal crypt and the main cell types that compose it are shown. (Reproduced with permission from Moore and Lemischka, Science 311:1880-1885, 2006).

Two signalling pathways have been involved in the maintenance of these adult stem cells. The Wnt/β-catenin pathway acts via Tcf-4, a cell proliferation factor of the T-cell factor/lymphocyte enhancer factor family (Tcf/LEF). Upon activation of the pathway, β-catenin binds to Tcf-4 to form a protein complex to regulate target gene expression and cell proliferation [159]. The APC protein competes with Tcf-4 to bind β-catenin and suppresses cell proliferation [160]. Importantly, it has been demonstrated that mutations in APC in ISCs result in migration of these cells to the intercryptal zone between crypt orifices. These mutations are responsible for 80% of sporadic colon cancers [161].

The other signalling pathway, the Notch/Delta pathway, is involved in the differentiation of ISCs into the four intestinal epithelium cell lineages. For instance, the deletion of Math 1, a basic loop-helix transcription factor and a downstream component of the Notch/Delta pathway, results in the depletion of Goblet, Paneth and enteroendocrine cell lineages in the small intestine [162].

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8. Stem cells: Basic aspects and possible therapeutic applications

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Stem cells' abilities to self-renew and differentiate have captured the attention of both developmental biologist and medical practitioners. In this chapter, characteristics that define a stem cell are discussed, followed by description of different types of stem cells. In particular, embryonic stem cells are cited as an example of pluripotent cells that can be used to study human development, and also have possible future therapeutic applications. Two of the best characterized somatic stem cells, hematopoietic and neural, are then described. Hematopoiesis is dependent on stem cells throughout lifespan. Umbilical cord hematopoietic stem cells and those found in adults are critically discussed. The clinical applications of hematopoietic stem cells are recapitulated, since this cell type is already used in the treatment of a few specific hematological diseases. Fetal neural stem cells are essential to central nervous system assembly, but adult neurogenesis is restricted to specific areas in the adult brain. Even though anatomical evidence indicates integration of newborn neurons in the mature brain, the functional significance of this phenomenon has not been clarified. Throughout these pages, we analyze published evidence to speculate on manipulation of stem cells that might make them competent in the treatment of human diseases.

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Introduction

As in many scientific disciplines, several concepts in developmental biology have changed in a profound manner during the last decade. This has been due to the implementation of new experimental techniques that have contributed to our understanding of complex biological mechanisms, the discovery of molecular elements that play key roles in the formation and development of cells and tissues, and to the characterization of a rare population of cells that act as "managing" cells, directing the organization and dynamics of the early embryo, as well as that of adult tissues. These so called *stem cells*, have caught the interest of the lay public, governments, biotechnology industry and scientists, to a point in which they have been included in forums and debates regarding not only science, but also economy, politics, ethics and religion.

In this chapter, we will present a brief overview on stem cell biology, including some general and basic concepts; and then will analyze in more detail particular stem cell types, such as those developed during early embryogenesis (embryonic stem cells), and those giving rise to blood cells (hematopoietic) and cells of the central nervous system (neural).

Definition and basic concepts

Stem cells (SC) are undifferentiated cells with a high capacity for self-renewal, that can give rise to one or more specialized cell types with specific functions in the body [1-3]. In other words, SC can give rise to daughter cells identical to their mother (self-renewal) and to progeny with a more restricted potential (differentiated cells). Proliferation is required for self-renewal of SC, but it is important to clearly state that not all proliferating cells are potential SC. Functional assays, ideally combined with phenotypic characterization, to demonstrate that daughter cells retain SC properties are therefore very important.

Throughout mammalian development, different types of SC are generated (Fig. 1). The fertilized egg, or zygote, is capable of producing both embryonic and extraembryonic tissues. Thus, it is referred to as a totipotent cell [4]. As development proceeds, the embryo reaches the blastocyst stage, where 2 types of cells are present. The outer layer is called trophoblast and will give rise to extraembryonic structures. The second cell type present in blastocysts is contained in the inner cell mass; these cells will differentiate into tissues that constitute the embryo itself, but do not contribute to extraembryonic tissue. If isolated and grown in culture, cells from the inner mass generate cell lines called embryonic stem cells (ESC). As expected from

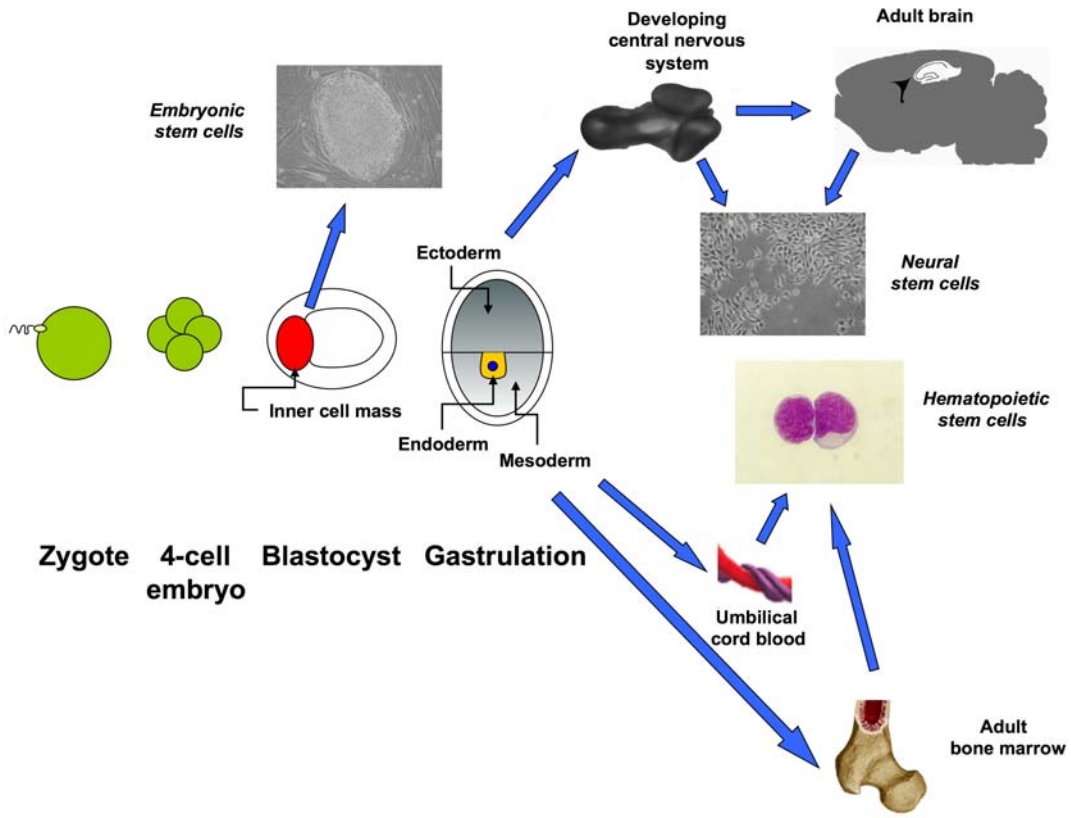

Figure 1. Origin of embryonic, hematopoietic and neural stem cells. The fertilized mammalian egg (zygote) starts dividing to generate embryos constituted by blastomeres (2- to 8-cells embryos). Embryonic development continues to the pre-implantation stage called blastocyst. From its inner cell mass, pluripotent embryonic stem cells (ESC) can be isolated and kept in culture. Top left picture shows a colony of ESC growing on a feeder layer of fibroblasts. Later on, the embryo gastrulates, which means that 3 germinal layers are formed: endo, meso and ectoderm. The central nervous system is ectoderm-derived and in its fetal state is a source of multipotent neural stem cells (NSC). The adult brain also contains NSC but only in two regions: i) the subventricular zone (black), that produce neuroblasts migrating to the olfactory bulb; ii) the subgranular zone in the hippocampus (white). NSC from fetal or adult brain can be grown in culture and differentiate to neurons, astrocytes and oligodendrocytes. Hematopoietic stem cells (HSC) can be isolated from the umbilical cord blood at birth, and also from the bone marrow of adult organisms. Multipotent HSC can differentiate to all lineages present on circulating blood. Schemes and pictures are not to scale.

its early origin, these cells have the potential to form any fully differentiated cell of the body, and therefore they are referred to as pluripotent SC [5, 6]. Interestingly, under specific culture conditions, they can be induced to unlimited proliferation without differentiation [7]. Cells of blastocyst's inner mass will produce different tissue-specific multipotent somatic SC as development proceeds, including those that give rise to central nervous

system, peripheral nerves, blood, liver, pancreas, muscle, etc. Yet, a different type of non-somatic SC is produced during development: the germline SC, which migrate to the developing gonads (genital ridges) and eventually give rise to the gametes [8].

Stem cells possess no morphological features that could be used for their identification. Thus, current ways to recognize these cells involve both immunophenotypic analysis and functional *in vivo* and *in vitro* assays. As we shall see later in this chapter, these observations are true for both embryonic and somatic SC. In any given somatic tissue, the frequency of SC is very low (0.01% - 5% of the cells present in the tissue; 3). This fact, evidently, gives studies on SC biology a particular degree of difficulty and complexity.

According to their definition, SC are capable of giving rise to different cell types. There are two ways by which stem cells generate differentiated progeny. On the one hand, stem cells may undergo asymmetric divisions, in which two different daughter cells are produced every time a stem cell divides, i.e., one is a stem cell and the other one a progenitor cell, capable of differentiating into mature cells; examples of this mechanism abound in invertebrates [9]. Asymmetry may result from the differential distribution, in the two daughter cells, of particular molecules, either cytoplasmic or integrated into the cell membrane (divisional asymmetry); alternatively, asymmetry may be due to the differential influence of particular elements from the surrounding microenvironment (environmental asymmetry).

On the other hand, stem cells may give rise to two similar daughter cells (symmetric division) that have a finite probability of being either stem cells or committed progenitors. At steady state, each stem cell division gives rise, on average, to one stem and one committed daughter, but asymmetry is achieved on a population basis rather than at the level of individual cell divisions [10].

Stem cell viability, self-renewal, proliferation, commitment and differentiation depend on both intrinsic and extrinsic elements. The former include a variety of regulatory molecules present in a cell, according to the specific tissue or lineage to which the cell belongs; the latter, on the other hand, include all the different cell types and cell products that form part of the microenvironment in which the cell develops. In other words, stem cell function ultimately depends on intrinsic cell regulators which are modulated by external signals [1].

Embryonic stem cells

ESC are the best studied pluripotent cells [11]. Although Embryonic Carcinoma (EC) cells were described before [12] than ESC, their neoplasic

origin has precluded its use. The third type of pluripotent cells are Embryonic Germ (EG) cells, which are derived from the Primordial Germ cells in the developing gonad [13, 14]. These cells are diploid and chromosomally stable, but there are not many available cell lines. We therefore will focus on ESC. The derivation of mouse cells was reported independently by 2 groups more than 25 years ago [15, 16]. The gold standard to prove pluripotency is the formation of chimeric rodents. Mouse ESC can be injected into the inner cell mass of same-species blastocysts; if these mice are allowed to proceed, ESC will contribute to both somatic and germline lineages. This remarkable property has been used to perform gene targeting of ESC, introduce modified ESC to early embryos and obtain mice that preserve such genetic manipulation in their gametes, allowing the generation of Knock-out technology [17]. These experiments are difficult to carry out and consequently, some simpler alternatives have been devised to test pluripotency *in vivo*: subcutaneous implantation of ESC in immuno-deficient adult mice results in teratoma formation. Teratomas are non-invasive tumors that contain differentiated cells derived from the three embryonic layers (endoderm, mesoderm and ectoderm). The third test for pluripotency is *in vitro* differentiation of ESC. Theoretically, ESC in culture can produce all differentiated cell type in the body. As we will discuss later, many cell types have been produced from ESC under culture conditions, opening the possibility of therapeutic use of their differentiated progeny in the long term.

Human ES cells have been derived from supernumerary frozen embryos donated from parents that received fertility aid and agreed to the terms of a consent donor form [18]. Currently, derivation of ESC implicates the interruption of embryo's development, but this could change in the next few years. Attempts have been made to separate one blastomere of a 8-cell mouse embryo and allow the remaining 7 cells to develop normally, whereas the single blastomere will produce ESC [19]; a similar approach has been reported for human 8-cell blastulas, but using more than one blastomere per embryo and without further embryo development, with 2 human ESC lines established [20]. Other possibilities to produce human ESC are oocyte parthenogenesis to produce female embryos, a procedure already achieved in non-human primate cells [21], and somatic cell nuclear transfer, which has been possible in various species including non-human primate species [22-24]. Two other procedures already working in human tissue are i) cell fusion of somatic cells with ESC to produce heterokaryons (tetraploid cells with 4 sets of chromosomes instead of the normal number of 2) that preserve pluripotent properties [25] with the obvious complication of having extra genetic information, and ii) reprogramming of somatic cells to an embryonic state by expressing central genes for this process [26-28]. These cells are

called induced pluripotent stem (iPS) cells and are so far indistinguishable from ESC. Human ESC are similar in many aspects to mouse ESC, but also present differences that could be relevant for their function. Common characteristics of murine and human ESC include the presence of transcription factors that regulate pluripotency, principally Oct-4, Nanog and Sox-2. In fact, these genes constitute a core regulatory circuit for maintaining pluripotency in human ESC [29]. Among the most prominent differences, are the growth factors required to preserve pluripotency in cultured ESC: mouse cells require Leukemia Inhibitory Factor and human cells depend on Fibroblast Growth Factor 2. Human ESC pluripotency has been tested only *in vitro* and in teratoma formation, due to the ethical barriers inherent to form early chimeras of human cells with experimental species.

In vitro differentiation of mouse or human ESC reveals the same plasticity to differentiate to a wide variety of cells types including neuronal [30-33], hematopoietic [34, 35], pancreatic [36], cardiac [37] and germline lineages [38, 39]. Differentiation of human ESC to produce high amounts of specific cell phenotypes can be used for *in vitro* drug testing at large scale to promote differentiation or to prevent cell death. Furthermore, if reprogramming of somatic cells to embryonic state can proceed routinely in human cells, it would be interesting to use biopsies of patients suffering diseases where the pathogenesis mechanisms are unknown (Parkinson disease for example). Such cells would then differentiate to the affected cell type (in this example dopaminergic neurons) to study in detail what are the factors contributing to neurodegeneration of human dopamine neurons.

The production of terminally differentiated cells from ESC has opened the possibility of using this progeny for cell therapy treatments. So far, only experimental species have received ESC-derived cells. Experimental models that resemble human diseases have been employed to establish proof of principle that *in vitro*-generated cells can indeed be used to treat deficits in a particular tissue. Both mouse and human ESC have been used for this purpose. For example, rodents treated with streptozotocin develop hyperglycemia due to death of beta cells in the pancreas, a model for type I diabetes. Transplantation of insulin-producing cells caused normoglycemia [40-42]. In other series of studies, heart infarcts were induced and ESC-derived cardiomyocytes were grafted; functional recovery was observed by independent groups [43-45]. Among the most complex diseases are those affecting the central nervous system. Parkinson disease is due to the selective degeneration of dopaminergic neurons in the substantia nigra. Dopaminergic neurons produced by ESC differentiation were tested in animal models of this disease. So far, mouse [46-49], monkey [50] and human ESC [51] have been effective in alleviating motor signs in experimental animals. Furthermore,

even mouse ESC derived from somatic cell nuclear transfer (cloning) promoted recovery in parkinsonian mice [52]. One cause of paralysis of lower limbs is degeneration of motor neurons in the spinal cord. Using a model of motor neuron damage by viral infection, it has been shown that ESC-derived motor neurons caused recovery from paralysis in rats [53]. Finally, production of hematopoietic precursors from mouse nuclear transfer ESC that received gene therapy, were transplanted to cause recovery in immunodeficient mice [54].

Hematopoietic stem cells

Hematopoietic SC (HSC) have been defined as primitive, undifferentiated cells capable of both, self-renewal and differentiation into all blood cell types [55, 56]. The vast majority of them reside in the bone marrow (Fig. 1), where they represent 0.005% of the total cells in that tissue. HSC possess an extremely high proliferation potential. It is estimated that in normal humans there are approximately 50 million HSC, some of which can generate up to 10^{13} mature blood cells over a normal life span [55]. These cells can be identified and quantified by using *in vivo* assays in which their capacity to repopulate the hematopoietic system of immunodeficient, Non-Obese Diabetic (NOD) – Severe Combined ImmunoDeficiency (SCID) mice is assessed; accordingly, they are also known as SCID-repopulating cells (SRC; [55]). It has been shown that a single HSC can regenerate and maintain the entire hematopoietic system following transplantation into an immunodeficient host [55]. Their immediate progeny, referred to as hematopoietic progenitor cells (HPC), comprise cells with a limited capacity to self-renew, and the ability to form hematopoietic colonies in semisolid cultures (thus, they are also known as colony-forming cells or CFC; [55, 56]). HPC represent 0.1% of the total cells in the marrow, and include cells with multilineage potential, as well as cells committed to individual lineages.

Most HSC and HPC express the CD34 antigen, an integral membrane glycoprotein of 90 – 120 kDa that functions as a regulator of hematopoietic cell adhesion to stromal cells of the hematopoietic microenvironment [57, 58]. Antigens such as CD90, CD117 and CD133 are also expressed by HSC [59, 60]. In keeping with their immaturity, HSC do not express CD38, CD45RA, CD71, HLA-DR or any other lineage-specific antigen; thus, they are referred to as lineage-negative cells (Lin^- cells; [59, 60]). Interestingly, some reports indicate that a small subpopulation of HSC does not express the CD34 antigen, that is to say, they are $CD34^-$ $CD38^-$ Lin^- cells, and there is evidence that these latter cells give rise to HSC expressing CD34 [61-63].

The functioning of hematopoietic stem and progenitor cells depends on intrinsic regulators (including nuclear transcription factors, as well as molecules involved in signal transduction and cell cycle; [64, 65]) which are modulated by external signals. The latter are provided by molecules (cytokines and extracellular matrix) produced by stromal and accessory cells. Together, stromal and accessory cells, and their products, constitute an intricate structural and functional network known as the hematopoietic microenvironment (HM; [66, 67]). In postnatal life, more than 90% of the hematopoietic activity takes place in the bone marrow, where HSC and HPC develop under the influence of the different elements of the HM. Such a microenvironment is crucial in hematopoiesis, and alterations in the structure and/or function of some of its components may contribute to the development of hematological disorders [68, 69].

To date, more than 20 hematopoietic cytokines have been identified. These molecules regulate, both in a positive and a negative manner, stem cell survival, proliferation and differentiation [70, 71]. Cytokines can be presented to their target cells as soluble or as membrane-bound molecules, and exert their effects via specific receptors on the cell membrane [72]. In some cases, cell-to-cell interactions between cytokine-producing and cytokine receptor-bearing cells must take place, so the specific cytokine can act efficiently on its target.

It is clear that cell death and cell division are processes controlled by cytokines [10]; in contrast, the role of cytokines in lineage commitment is still a controversial issue [73-75]. Indeed, some studies suggest that cytokines play an inductive role on HSC, directing them into a particular lineage of differentiation; others suggest that cytokines play a permissive role, allowing the progression of a particular cell lineage, without influencing the decision of a stem cell to commit into such a lineage.

The interactions of hematopoietic cells with microenvironment cells, as well as with extracellular matrix molecules, are mediated by cell adhesion molecules (CAMs; [76, 77]). Three groups of CAMs have been recognized: The Immunoglobulin superfamily of adhesion receptors (that includes CD2, CD54, CD58, VCAM-2, etc); Integrins (LFA-1, Mac1, VLA-1, VLA-2, VLA-4, VLA-5, etc) and the Selectin/LEC CAMs (including L-selectins, E-selectins and P-selectins). Together, CAMs play a crucial role in homing, attachment and localization of HSC and HPC within the medullary cavity.

It is interesting the fact that the distribution of HSC and HPC within the medullary cavity is not random. Most HSC are located within the endosteal region, whereas lineage-committed progenitors and mature cells are distributed away from this region, predominantly in the central marrow area, in close proximity to the central marrow vessels [78-80]. Thus, it seems

evident that there is a spatial organization of the hematopoietic system within the marrow that allows the controlled egress of hematopoietic cells from the bone marrow to the blood.

HSC from umbilical cord blood

Although the vast majority of HSC and HPC are localized in bone marrow, a small proportion of such cells are present in circulation [56]. This occurs not only in adult subjects, but also during fetal development and at the moment of birth. Accordingly, HSC and HPC are also found in umbilical cord blood (UCB; Fig. 1). This was first reported by Knudtzon in 1974, who described the presence of relatively mature myeloid progenitors in UCB [81]. About ten years later, Ogawa and colleagues documented the presence of more primitive hematopoietic cells [82], and in the late 1980s, Broxmeyer *et al*. showed that UCB contains vast amounts of both primitive and mature hematopoietic cells [83]. To date, UCB is recognized as a major source of HSC and HPC both for research and clinical application [84, 85].

When comparing the relative levels of stem and progenitor cells in UCB and bone marrow, it has been found that no significant differences exist in the values of total progenitors; however, important differences in the frequency of particular HPC subpopulations have been observed. That is to say, whereas the levels of relatively mature progenitors are similar in both sources, the frequency of primitive progenitors, including multipotent, erythroid and bipotent granulo-monocytic, is significantly higher in UCB than in marrow [86-91]. The frequency of stem cells also seems to be significantly higher in UCB than in adult bone marrow [92, 93].

Important functional differences between adult and neonatal HSC/HPC have been described [56]. Both proliferation and expansion potentials from UCB cells are significantly higher than those from adult subjects [94, 95]. The reason for this is not totally clear, however, some studies indicate that such functional differences are the result of differences in telomere length and biology, cell cycle regulators and expression of particular “master” genes and signal pathways [96-98].

HSC plasticity

A general concept in somatic stem cell biology has been that such cells are restricted in their differentiation potential to an individual organ system. Accordingly, HSC would produce blood cells only; neural stem cells would give rise solely to neurons, astrocytes and olygodendrocytes; satellite cells of muscle to muscle cells only, and so on. However, during the last decade, a

great deal of evidence has been generated from *in vivo* studies, mainly in mice, indicating that this concept may not be true. Although it is still a controversial issue and the evidence is not conclusive, it seems that somatic stem cell differentiation plasticity is actually wider than previously envisioned [99]. Indeed, growing evidence has emerged indicating that HSC can also differentiate into non-hematopoietic cells [100]. The actual mechanisms for plasticity are not completely understood; however, some possible mechanisms have already been suggested: One possibility is that a stem cell "de-differentiates" into a more primitive and plastic state and then "re-differentiates". A second possibility is that a stem cell "trans-differentiates", that is to say, a stem cell from a particular tissue directly takes on another differentiation path, without going into intermediate stages. Any of these processes could involve or not cell division [1]. In spite of the mounting evidence, stem cell plasticity, strictly defined, has yet to be rigorously proven.

Neural stem cells

The neuroepithelium has an ectodermal origin and is responsible of neural tube formation to generate the central nervous system. Somatic SC, isolated from developing or adult central nervous system, are called neural SC (NSC; Fig. 1) [4, 101]. These multipotent cells normally differentiate to different types of neurons, and glial cells (astrocytes and oligodendrocytes). Neurons are responsible for the transmission of information in the brain, whereas astrocytes were believed for a long time to provide mechanical support and trophic factors to neurons, but as we will see, this view radically changed in recent years. Oligodendrocytes produce myelin that isolate the electrical impulse traveling along the neuronal axon. One of the most used markers to identify NSC in vitro and in vivo is the filamentous protein Nestin [102]. NSC, similar to HSC, can be grown in culture for restricted periods of time. There are 2 forms of keeping them in vitro: 1) neurospheres are floating aggregates of neural cells [103]. 2) monolayers [104] with the aid of extracellular matrix proteins such as fibronectin or laminin to promote attachment to the culture dish [105]. In both cases, NSC respond proliferating to application of either Fibroblast Growth Factor-2 or Epidermal Growth Factor. With neurospheres is easy to test for self-renewal, because the amount of secondary or tertiary neurospheres can be quantified upon mechanical desegregation of primary spheres. If dissociated cells contain SC, new neurospheres will form. Differentiation is normally achieved with growth factor withdrawal and cells will start expressing proteins characteristic of neuronal, astrocytic or oligodendrocytic phenotypes.

NSC during development of the central nervous system

During development, all central nervous system regions contain abundant SC in early stages and later on, precursor cells are the dominating cell population. These two cell types can be isolated and grown in culture from cerebral cortex [106], midbrain [107], hippocampus [105] and the spinal cord [108]. In vivo, NSC originate neurons first, followed by astrocytogenesis and finally oligodendrocyte differentiation. This temporal program is recapitulated by cultured NSC, because early passage cells are neurogenic and older NSC are gliogenic [109, 110]. Also, NSC respond to specific growth factors by differentiating to neurons, astrocytes or oligodendrocytes [105].

Neurogenesis in the developing cerebral cortex

The development of cerebral cortex is one of the best *in vivo* studied regions of the brain. Cortices are formed from multipotent NSC that divide initially in the ventricular zone (VZ) and later in the subventricular zone (SVZ), a region that is though to be preserved in the adult brain as we shall discuss later. The cerebrocortical epithelium is constituted by asynchronously dividing NSC that produce migrating neuroblasts that radially migrate to the cortical plate, where they differentiate in 6 distinct layers of neurons. Along the cortical plate there are glial fibrillary acidic protein (GFAP)-positive cells called radial glia (RG), that span the thickness of this structure from the VZ to the cortical plate, and serve as scaffolds for dorsal migration of neurons to the corresponding cortical layer [111]. Recently, a portion of RG cells have been identified as the neural stem cells in cerebral cortex [112]. NSC were though to be immature and therefore devoid of receptors for neurotransmitters. This turned out to be false, since neuroactive substances such as glutamate [113], gamma-amino-butyric acid [114] can regulate proliferation and differentiation of these multipotent cells.

NSC in the adult brain

One of the central dogmas in Neurobiology during the 20th century was that the number of neurons in the adult brain was fixed and no neurons were generated after the newborn period. This view started to change slowly after the seminal work of Joseph Altman, who reported some neurons labeled with radioactive thymidine given in adult life, suggesting adult neurogenesis (reviewed by Kempermann [115]). This dogma is no longer valid in this new

century, since newborn neurons are produced in discrete regions of the adult brain in several species, including humans. As the reader might realize, adult neurogenesis is a phenomenon that stimulates the idea of brain plasticity (the ability to modify existing neural circuits), and the possibility for brain repair in diseased or aged central nervous system. We next review some data on characterization of the two main neurogenic regions in adult mammalian forebrain.

Subventricular zone

Brain lateral ventricles are used for cerebrospinal fluid circulation. Very close to ventricles is located the SVZ, which is the region that contains the largest population of SC in adult rodent brain [116, 117]. This area contains GFAP-expressing multipotent NSC [118], though to originate from the embryonic ventricular zone [119]. Structural and functional studies have provided a detailed picture of the SVZ. Neural stem cells (called B cells) are in close apposition with ependymal cells (ciliated cells facing directly the ventricles). These B cells divide and generate transit amplifying (C) cells that differentiate into migratory neuroblasts (A cells) that reach olfactory bulbs through the rostral migratory stream; once arriving to this target region, neuroblasts differentiate to GABAergic and dopaminergic interneurons. Recently, a similar germinal region has been described in humans [120]. The functional role, if any, of SVZ neurogenesis remains to be conclusively established.

Subgranular zone in the hippocampus

The hippocampus is a cerebral structure closely related to learning and memory tasks. Hippocampal SC express GFAP and Nestin [121, 122]. They are found in the SGZ of the dentate gyrus, at the hilus / granule cell layer interphase. NSC are also called type-1 cells and divide slowly to generate a highly dividing population (type-2 cells, D cells) that migrate a short distance to integrate as neurons into the granule cell layer of the dentate gyrus. Hippocampal neurogenesis in humans was reported ten years ago [123]. In rats, newborn neurons decline with age; however, lowering of corticosteroid levels can reverse this process [124]. There are many other factors positively regulating this phenomenon, such as living in an enriched environment [125], running [126], stroke [127], and non-coding double-stranded RNA [128]. The possibility that adult hippocampal neurogenesis could play a physiological role during learning and/or remembering has been suggested [129, 130].

Concluding remarks

Stem cell biology has emerged as a scientific field with a two-fold relevance. On one hand, it has helped to our understanding of complex cellular processes, such as proliferation and differentiation, as well as tissue development, renewal and repair. On the other hand, it may have significant impact on the treatment of a variety of human diseases, such as cancer, diabetes, cardiac, and neural disorders. There is still a long way to go in the characterization of these "master" cells, and genomics and proteomics will surely play key roles in our understanding of the way SC function.

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9. A MADS view of plant development and evolution

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MADS-box genes are important transcriptional regulators of plants, animals and fungi during multiple developmental processes. At least an ancestral duplication that occurred before the divergence of plants and animals gave rise to two lineages of MADS-box genes represented in these three groups of eukaryotes: Type I and Type II. The similarity of the MADS-box sequences within each lineage suggests strong functional conservation. The first and best characterized MADS-box genes in plants were those of Type II, which encode modular proteins with I, K and COOH domains in the 3' region of the MADS domain. Of these, those involved in the determination of floral organs were first characterized: the so-called ABC genes of flower development that are necessary for the specification of sepals, petals, stamens and carpels, characteristic of most angiosperms. MADS-box genes have been found to be also key integrators of signal transduction pathways in response to

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external (light and temperature) or internal (hormones) cues, to which plants respond during their transition from vegetative to reproductive growth. More recently, MADS-box genes implied in gametophytic development or in the regulation of processes of vegetative structures have been characterized. In contrast to the first studies, recent studies are suggesting that most plant MADS-box genes are expressed in multiple tissues and stages. Thus their function could be regulated at different levels (e.g., miRNA´s) and/or depend upon the composition of protein complexes of MADS proteins with members of the same or other families, that are specific to different tissues and/or stages. This family will continue to be a useful system to understand the complexity of the logic of transcriptional regulation underlying developmental decisions and how these integrate multiple signal transduction pathways, as well as the relationship between molecular and morphological evolution in plants. The ease of *in vivo* studies in plant systems will likely contribute to novel insights for understanding their plastic, and at the same time, robust developmental responses. The conservation of some of the molecular components underlying such processes and the existence of generic characteristics of these will make plant studies useful for unraveling animal and fungal systems, too.

Why plants and MADS-box genes: Evolutionary context within eukaryotes and functional conservation with respect to animal genes

Plants are the source of key products and the base of the planet's ecosystem equilibrium, and it is intrinsically important to understand their development and physiology for the well being of humans. But plants also pose clear advantages in comparison to animals as research systems of the *in vivo* interplay of genetic, epigenetic and environmental factors that cause normal or aberrant morphogenesis. Plant growth and development depends largely on the cellular processes occurring in the meristems (exposed pools of undifferentiated and actively dividing cells), that contain the niches of stem cells, which remain active along the plant's whole life-cycle and adjust their response according to environmental conditions. Adult plant development, growth and form depend on equilibrium between cell proliferation and differentiation in such niches of mother cells within the meristems. Therefore, studies of the interplay of cell division and differentiation under the action of environmental factors can be readily and quantitatively studied in plant meristems *in vivo*.

Additionally, plant cellular organization is simpler than that of animals and it will be more feasible to propose computable plant models than animal ones. Such models may be used for *in silico* simulations that may help to think about the concerted action of multiple genetic and non-genetic factors during development, and the role of particular components or sets of them in cell patterning and morphogenesis *in vivo*. Finally, key molecular aspects of Gene Regulatory Networks (GRNs) and signal transduction pathways are conserved between animals and plants [1,2], thus plant research is becoming also a source of novel basic molecular and genetic knowledge that may be generally relevant for understanding development of animal, as well as plant systems, or even yield biomedical applications. Molecular genetics of plants, in particular of the experimental system *Arabidopsis thaliana*, has paved the way to key discoveries in the biomedical and biological sciences and these should now be routinely considered in the "portfolio" for the search of the basis and cure of important human diseases [3].

Recent advances in evolutionary biology have recognized that deciphering developmental processes and the mechanisms that underlie cell patterning and morphogenesis at the molecular level are necessary in order to understand morphological diversification [4]. Indeed, development determines how genetic variation is mapped into morphological or phenotypic variation [5]. MADS-box genes encode transcriptional factors that are key in plant and animal development [6,7]. Hence, phylogenetic studies of the MADS-box genes contribute to bridging the gap between molecular evolution and phenotypic evolution in a macroevolutionary scale. We uncovered two MADS lineages (Type I and II) in plant, fungi and animals [8,9], and although there is still contention on whether Type I are monophyletic or not *e.g.*, [10], our analyses suggested that at least one gene duplication occurred before the divergence of plants and animals, after which, strong functional constraints yielded widely conserved genes within most Type I and Type II MADS-box genes. Important animal genes belong to these MADS lineages [11], *e.g.* proto-oncogene, Serum Response Factor (SRF) in the Type I and Myocyte Enhancer Factors (MEF) in the Type II (see Figure 1a) together with plant MADS.

Molecular analyses of MADS-box genes in plants should, therefore, yield important insights to understanding the role of these key transcriptional regulators in animal development and human disease. This is particularly true at least concerning the role of the MADS DNA recognition domain that seems to have been conserved after plant-animal divergence within the MADS lineages.

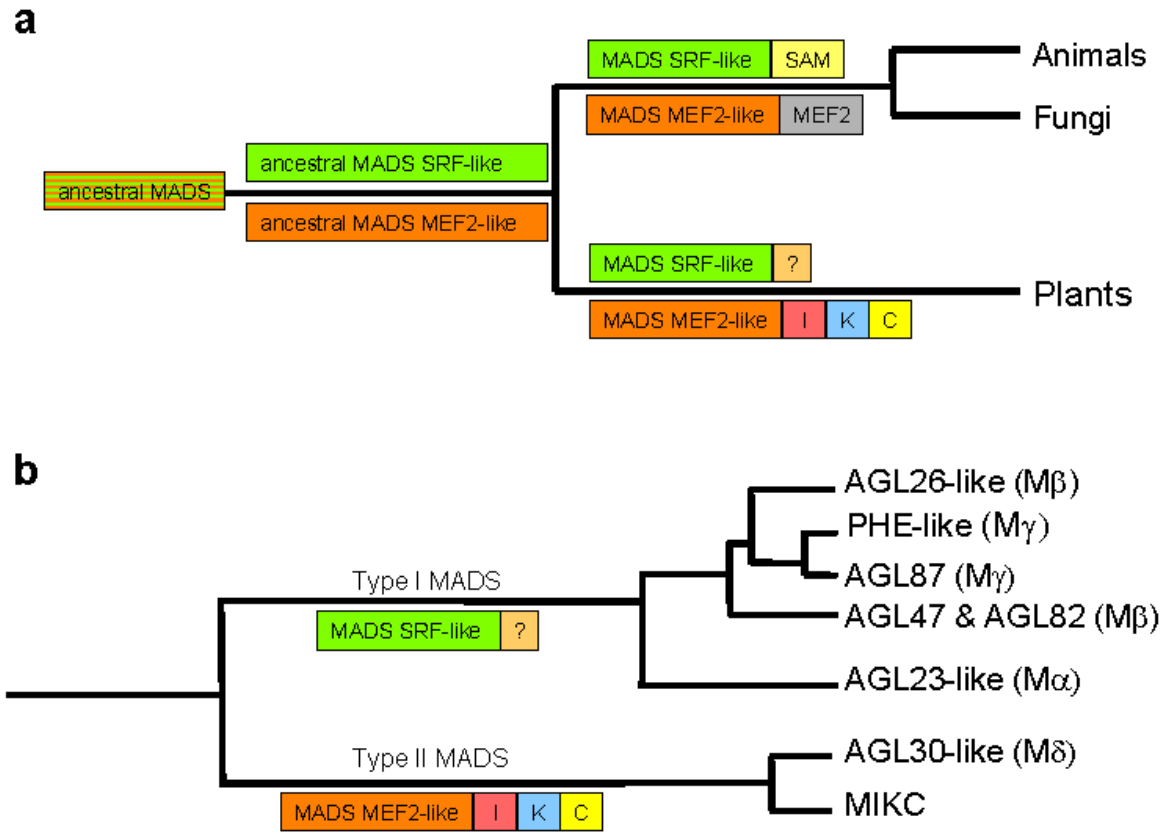

Figure 1. a) Schematic representation of the domains (boxes) of the MADS proteins from different taxa and their phylogenetic relations. At least one duplication of an ancestral MADS-box gene must have occurred before the divergence of plants and animals giving rise to the Type I (SRF-like) and the Type II (MEF2-like) lineages present in plants, animals and fungi [11,9]. After the divergence, each group acquired different accompanying domains. It has also been described that bacteria may have MADS-domain-like proteins [12], but their phylogenetic relationships with respect to those from other organisms has not been determined. b) Schematic representation of the tree classification made by Martínez-Castilla & Álvarez-Buylla (2003), [9]. In boxes are the MADS-domains of the proteins from angiosperms and in parenthesis the classification made by Parenicová & collaborators (2003) [10]. Phylogenetic relationships among Type I genes result in the separation of different clades whose monophyletic origin is still controversial [13]. According to Parenicová et al. (2003) both Mα and Mγ lineages are present in monocots and dicots, but there might be an absence of Mβ sequences in monocots. Type II proteins clearly are divided in to two clades that include the same genes in both classifications. In plants, the K-domain appears as part of the Type II lineage proteins, forming the basic MIKC structure. However, a small group of 6 genes (out of 45) do not have a clear K-domain (AGL30-like or Mδ clade). Other classifications describe variability among MIKC proteins and distinguish two groups having either a classic MIKC or a non-classic MIKC structure [14]. MADS-box genes have also been found in other terrestrial plants (i.e. gymnosperms and ferns [14,15]) as well as algae [3].

MADS-box genes

MADS-domain proteins regulate different aspects of development or cell differentiation in a variety of organisms [16,17,18]. The MADS acronym was derived from the initials of the first genes of this kind that were cloned: ***M****CM1* from *Saccharomyces cerevisiae* [19], ***A****GAMOUS* from *Arabidopsis thaliana* [20], ***D****EFICIENS* from *Antirrhinum majus* [21] and **S**RF from mammals [22].

Plant Type II MADS-box genes encoded proteins share a conserved structural organization, the so called MIKC structure including, from the amino to the carboxi-terminal part of the protein: a MADS (M) conserved DNA-binding domain, a more divergent intervening (I) region, a conserved (K) domain which may participate in protein interactions, and a divergent COOH (see below and Figure 1b) [23,24,25,26,27].

The 60 amino acid MADS-domain is at the N-terminus of the proteins. It has been shown that this domain is involved in specific binding to DNA sequences (CArG boxes) conforming the consensus sequence $CC(A/T)_6GG$ in both animals and plants [28,24,25]. This is a characterized conserved motif in the promoter of different MADS target genes [29,30,31,32,33]. However, an *Arabidopsis* genome study demonstrated that, during early flower development where MADS box proteins are fundamental, the occurrence of regulated genes with CArG boxes in the promoter was not significantly different to that of their genome distribution. Therefore, these transcription factors might either be able to recognize another element (*e.g.,* a less conserved CArG box) or they might have a limited number of target genes in this particular developmental stage [34].

The formation of dimers that are capable of DNA binding requires the I-region (60–86 aa long); this domain is at the 3' of the MADS and is a key determinant for the specificity of DNA-binding dimer formation [24,25]. After the I, a second conserved domain, the K (87–150 aa long), is postulated to form three α-helices referred to as K1, K2, and K3 that potentially form coiled-coils structural motifs, with K1 and K2 helices located entirely within the K domain, while K3 helix spans the boundary between the K and the C domains [35]. The K-domain is assumed to generate a three-dimensional structure important for protein–protein interactions [36,26]. Finally, the C-terminal domain is a length-variable amino-acid stretch that may have several functions. For example, it is thought to participate in higher-order MADS interactions [37], it is required for functional specificity [38], it may be involved in transcriptional activation [39], and can also enhance/stabilize interactions that are mediated by the K-domain [26,40].

All MADS-domain proteins studied until now bind to the CArG sequence in the DNA as dimers, either as homo- or heterodimers [29,24,25]. For example, *AG* can bind to DNA either as a homodimer or a heterodimer with *SEP1* [41]. On the contrary, *AP3* and *PI* can only bind to DNA as heterodimers in *Arabidopsis*. Moreover, It has been shown that these genes are only able to enter the nucleus as heterodimers in this species [42].

Plant MADS evolution and diversification: Duplications and natural selection

Representatives of at least two lineages of MADS-box genes are found in most plant lineages. However, while in animals MADS-domain transcription factors and the plants Type I proteins, the box that codifies for the MADS DNA-binding domain is only followed by a few 3´amino acids, in the plant Type II proteins, after the MADS-box, we can find, as shown above, three other boxes (I-, K- and C-regions; Figure 1a). Another trend of plant MADS-box genes is that there are many more types of these genes in plants than in animals. Previous studies suppor the hypothesis that the diversification of these genes could have been important during the evolution of plant form [43,44]. Indeed the more complex combinatory code underlying MADS-protein function could contribute to the robustness and plasticity of plant development.

We recovered 107 MADS sequences from the study plant, *Arabidopsis thaliana* [9]; see also [10,14]; and Figure 1b. Type II genes have been more extensively studied than Type I [10], although a few studies on the latter have started to emerge. These studies shows that although they had faster evolutionary and birth/death rates and some seem to be pseudogenes, several other Type I MADS-box genes are also functional [13]. Extensive homology search studies found that there are 64 presumed functional Type I genes, while there are 43 presumed functional Type II genes in *Arabidopsis*. Genomic studies on MADS-box genes have also started to appear on other angiosperm study systems, such as rice. These studies confirm the evolutionary tendencies of these two types of MADS-box genes (24 functional and 6 nonfunctional Type I; and 47 functional and 1 nonfunctional Type II genes in rice; [45,13]). The first MADS-box genes that were functionally characterized were those involved in cell type determination during flower development, whose loss of function yielded flower organ homeotic phenotypes [46]. It seems that alterations in the floral ABC MADS-box gene expression patterns (see below) could have contributed to the origin and structural evolution of flowers [47,48].

Floral organ identity MADS-box genes are overall conserved across angiosperms and to some extent even in gymnosperms [49,50,51,52,53,54,55,56]. These genes have been shown to be important and overall conserved in monocot and dicot flower development [57]. A recent network dynamic model has uncovered the developmental module of ABC and interacting non-ABC proteins that are necessary and sufficient for floral organ determination [58]. This model robustly converges only to gene activation profiles that correspond to those observed during early flower development in primordial cells of: the inflorescence meristem, and the sepal, petal, stamen and carpel primordia within the flower meristem. This model and its perturbation analyses have been used to propose an explanation for the robustness of the floral genetic developmental program and its observed evolutionary conservation among core eudicots (See: [58,59,60,61] for details on Network Model approaches).

At least ten Type II MADS-box genes are found in all angiosperms (including the flower ABC ones), suggesting that all of these originated before the radiation of the flowering plants and since then have been conserved. These data suggest that MADS-box genes could have been important for the origin of flowers and the establishment of the bauplan of flowers that is overall quite conserved: whorled structure with sepals, petals, stamens and carpels from the outermost to the inner of the flower [62]. This implies that further floral morphological diversification (*e.g.*, meristic structure, symmetry, color, size) among flowering plant species could have been regulated by genes of other families; or additional MADS-box genes with distinct functions yet to be characterized.

In addition to all the flower ABC organ identity MADS-box genes: *APETALA1* (*AP1*), *PISTILLATA / APETALA3* (*PI / AP3*), *AGAMOUS (AG*) and the *SEPALLATA* (*SEP1-4*); the common ancestor of all flowering plant species also had representatives of the *AGL15*-, *AGL17- AGL20,* and B-sister genes. On the other hand, the common ancestor of all seed plants (ca. 300 million years ago) already had MADS-box members of at least six families of MADS-box genes: *AGAMOUS*-, *AGL2*-, *AGL6*-, *AP3/PI*, GGM13- and TM3-like [63].

Available data suggest that the common ancestor of seed plants and ferns (400 million years-old), had at least two genes that are related to the flower MADS-box genes. But it is interesting that in ferns there were independent duplication events and 15 MADS members of different families to those of seed plants, have been identified in one fern species [63]. Recent studies in the moss (Bryophyte), *Physcomitrella patens* and in several algae have also demonstrated that the common ancestor of all land plants already had MADS-box genes [63,44].

Gene duplication is a fundamental substrate for evolution [64]. The question concerning the role of natural selection on the persistence and diversification of duplicates is therefore crucial [65]. After duplication, one duplicate may become a pseudogene, functional divergence (subfunctionalization or neofunctionalization) between the two may operate, or they may remain with overlapping functions. It is hence important to elucidate the role of evolutionary forces in molecular change and the fate of duplicates for clarifying mechanisms of genetic redundancy, as well as the link between gene family diversification and phenotypic evolution [64,65]. Indeed, the evolutionary forces that play during functional divergence of duplicates are still under debate [66,67]. However, the presence of large gene families as that of the MADS-box in plants, suggests that positive diversifying selection might have been important in preserving duplicates for longer periods of time than expected by classical stochastic neutral models [68,69,70].

Recently, we have documented the role of positive selection (PS) in the evolution of the MADS-box gene family [9,71]. We have achieved this for the complete MADS Arabidopsis family and the B-type angiosperm families addressing if positive selection has been important during MADS-box gene evolution among paralogous and orthologous genes (within the B-MADS-box gene class), respectively. Adaptive evolution in developmental regulatory loci, such as the MADS [72] is likely to act along particular lineages and at specific amino acid sites. Interestingly, our analyses have yielded statistically significant results for residues within the different domains of MADS proteins during the diversification of genes that control transition to flowering; a stage that is clearly linked to plant fitness. These findings suggest that changes in coding sequences of transcriptional regulators, rather than only alterations in their regulatory regions, may have been important during phenotypic evolution [9].

Plant form diversification

Probably the major evolutionary event during plant evolution was the relatively abrupt and extensive diversification of angiosperms shortly after they appeared in the fossil record [73,74]. This species radiation has been linked to the origin of flowers. As will be described below, a wealth of molecular genetic information is now available to understand the regulation of flower development using experimental plant species (*Arabidopsis thaliana* and *Antirrhinum majus*). However, it is from Arabidopsis that the most detailed and complete perspective has been obtained and it is going to be used as the model plant in this chapter. Nearly all of the floral organ

identity regulators belong to the Type II MADS-box genes [11,8,75,76,37,77] and available functional studies (see below) strongly suggest that these genes and their regulatory networks are key for flower development, and most probably played important roles during flower evolution, as well [47,48,5,78,79,80,81].

B genes specify petal formation, while when co-expressed with C function gene, they specify stamen formation [82,46,83]. AP3 and PI proteins interact to form a heterodimer that indirectly regulates *PI* expression and directly binds to the *AP3* promoter in a self activating regulatory loop that maintains the B function in the second and third whorls of the meristem during flower development [23,30,31]. Obligate heterodimerization of PI and AP3-like proteins of core eudicots [36,24,25] evolved from the homodimerization typical of gymnosperms, perhaps via a transitory state of facultative homo-heterodimerization [84]. Such heterodimerization evolution in B-class proteins could have been fundamental during the origin and diversification of flowers. Hence we addressed whether or not adaptive evolution was important during the origin of obligate B-protein heterodimerization, particularly following the critical AP3-PI duplication towards the base of angiosperms. Then a main duplication occurred in the AP3 lineage, leading to two AP3-like sublineages distinguished by characteristic motifs in their C-terminal region [85]: paleoAP3 lineage in basal angiosperms, monocots, magnoliids and basal eudicots (with a *paleoAP3* motif in their C-terminal) [86]; and euAP3 lineage of core eudicots B-class genes (*euAP3* C-terminal motif). These two seem to have functionally diverged [38,87]. Interestingly the expression pattern of B genes in core eudicots, which have a conserved floral plan (sepals, petals, stamens and carpels), is preserved [88,49,51], while these genes have divergent expression patterns in non-core eudictos. Coincidently, the flowers of the later do not have a well differentiated calyx and corolla [51,54].

The phylogenies of A, B, C, and *SEP1/2/4* floral MADS-box genes show a duplication close to the base of the core eudicot clade [89,90], coinciding with the moment at which the B genes split into euAP3 and paleoAP3-like lineages [89]. It has been suggested that this duplication played an important role in the origin of clearly differentiated petals in core eudicots [85,38]. Interestingly, our results strongly suggest that shortly after the duplication that led to the *AP3*-like and *PI*-like genes, functional diversification driven by PS acting on different sites within the K domain, which is key for heterodimerization, occurred along both duplicated gene lineages [71, 91].

On the other hand, our studies have suggested a possible functional divergence of *AP3* duplicates that might have been important for the

evolution of the core eudicot floral developmental genetic program. We found three major things associated with this divergence: a) an early origin for heterodimerization of B MADS-box proteins in angiosperms shortly after they diverged from gymnosperms, b) the *euAP3-TM6* duplication coincides with the origin of the core eudicots lineage, and c) a strong signal for positive selection along the *euAP3* branch lineage [71]. The B genes constitute a clear example in which duplicates evolved towards completely different functions.

A second possibility is that each duplicate may evolve a subset of all the functions originally performed by the ancestral gene (called subfunctionalization; [69]); the MADS-box genes that are expressed in carpel and stamen primordia are an example of this. While in *Arabidopsis* there is a single such gene (*AG*) important for the development of both organs, in rice and maize there are two such genes, each expressed in a single whorl (*e.g.,* [57,92]; see review of MADS subfunctionalization in: [93]). A third possibility is that one of the duplicates evolves a new function. Fewer examples of this have been documented and an outstanding one concerns the MADS-box gene that underlies *Physalis* encapsulating fruit structure [94]. The molecular basis of the novel or sub- functions of MADS-box duplicated genes has been documented in very few cases still.

After duplication, redundant genes with overlapping function could also remain for some time as is the case of some MADS-box genes [95,77]. In fact, purifying selection could keep redundant genes that may guard against deleterious mutations [96], or positive selection could favor duplicated copies if there is dose-dependence. *AP1* and *CAULIFLOWER* (*CAL*) are two recently duplicated MADS-box genes that in *Arabidopsis* underlie flower meristem identity, as well as sepal and petal identity [97,98]. While *AP1* can substitute *CAL* for both functions and single *cal1* loss of function mutants lack any visible phenotype, *CAL* cannot substitute for *AP1* functions and hence loss of function mutants of the former have clear phenotypes in flower determinancy and sepal/petal identity. The enhanced phenotype of the double mutant suggests that these genes overlap in meristem identity determinancy [81].

By expressing all the possible chimaeric proteins that result from combining the cDNA boxes that encode the four domains typical of plant Type II MADS-box genes (MADS, I, K and COOH) from *AP1* and *CAL,* under the *AP1* promoter, on an *ap1-1* loss of function background, we were able to map the unique and redundant functions of these two genes. Interestingly, the K and COOH domains, that are important for the formation of high-order multimers characteristic of MADS transcriptional

complexes, seem to be key for the unique and indispensable functions of *AP1* [81].

We have exemplified with some studies that MADS-box genes and plant evolution are clearly interconnected. Thus the study of these genes function in a comparative framework is likely to continue providing important clues to understanding development and phenotypical evolution beyond flowers.

Role of different MADS-box gene lineages in plant development

Plant MADS-box gene function during transition to flowering [99] and flower patterning [100] has been extensively studied ([101]; and updated reviews in sections below). The first characterizations stressed MADS-box gene role during specific stages of development as organ- [20,76], cell- [102] or meristem-identity genes [97,103]. More recent studies are starting to focus on the role of MADS-box genes also in vegetative development and are uncovering novel functions for these genes [104]. In the following sections, we review the studies that up to our knowledge have uncovered the function of MADS-box genes during *Arabidopsis thaliana* development.

Arabidopsis thaliana as a plant model system

Arabidopsis thaliana (Figure 2) has been the most successful experimental study system among plants because of its relatively short life-cycle (six weeks), its high seed output that enables classical genetic studies, its low incidence of outcrossing under laboratory conditions, its relatively small genome size and the availability of large molecular data sets and resources for molecular genetic studies [105]. In addition, it has a typical sporophyte that shows with the eudicot stereotypical eudicot flower structure and development.

As all higher plants, *Arabidopsis* has alternating haploid (gametophyte) and diploid (sporophyte) life-history stages, with the former depending on the latter. The mature female gametophyte is the embryo sac that is formed before fertilization and is found inside the carpel of the flowering sporophyte (see Chapter by García-Campayo and collaborators in this volume), while the male gametophyte is the germinated pollen grain that is produced within the stamen anther. In this review, we focus mainly on the sporophyte development that starts with embryo development within carpels and seeds (Table 1). But we must mention that a few MADS-box factors have been identified as important regulators of gametophyte development. For example, the *fem111* mutant is affected in central cell development and function. It

corresponds to a T-DNA insertion in *AGAMOUS-LIKE 80* (*AGL80*) [106]. Furthermore, *AGL80* is required for the expression of central cell-expressed genes such as: *DEMETER* and *DD46*, but does not affect *FERTILIZATION-INDEPENDENT SEED2* [106].

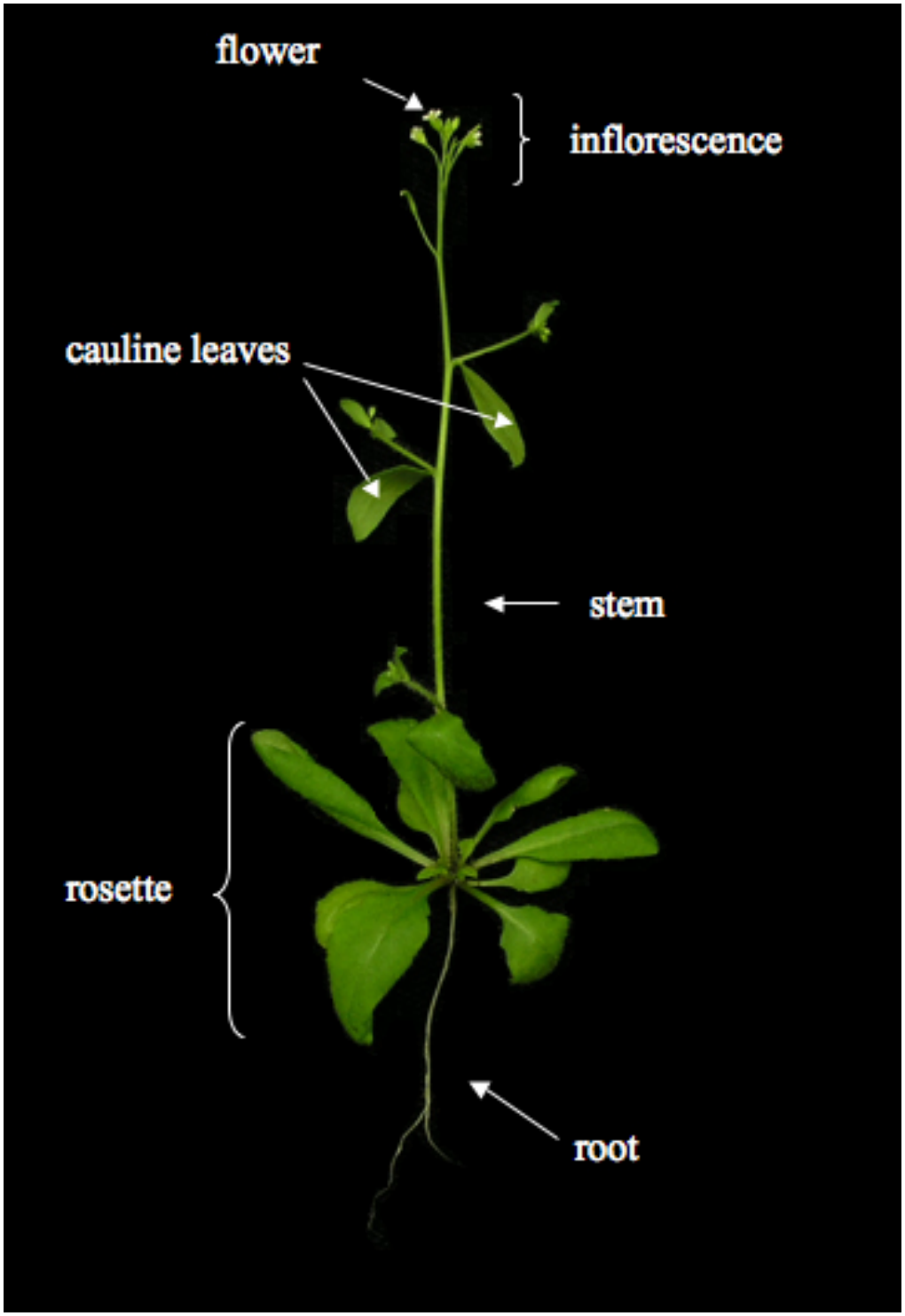

Figure 2. *Arabidopsis thaliana* plant.

Table 1. MADS-box genes function in development.

ORGAN/STAGE & Genes	Protein function	Reference
ROOT		
A NO3(-)-INDUCIBLE MADS-BOX GENE (*ANR1*/*AGL44*)	Lateral root growth upon nitrogen deficiency.	[107]
XAANTAL1 (*XAL1*/*AGL12*)	Pivotal gene for both root and flower development implicated in the photoperiod pathway downstream of CONSTANS (CO) action. Also expressed in embryos.	[104,108]
EMBRYO		
AGAMOUS LIKE 80 (AGL80)	Required for central cell development and function.	[106]
PHERES1 (*PHE1*/*AGL37*)	May be involved in pattern formation of the endosperm.	[109]
AGAMOUS LIKE 62 (*AGL62*)	Regulates cellularization during endosperm development.	[110]
AGAMOUS LIKE 15 (AGL15)	Essential for embryo development. Repressor of *FT* during transition to flowering. Fruit maturation.	[111,112,113]
AGAMOUS LIKE 18 (AGL18)	Essential for embryo development. Repressor of *FT* during transition to flowering. Transcriptional repressor of immature pollen genes downstream of AGL65, AGL66 and AGL104.	[112,113,114]
SEPALLATA1 (*SEP1/AGL2*)	Expresses during the embryogenesis.	[115]
AGAMOUS LIKE 21 (*AGL21*)	Expressed during embryogenesis, unknown function.	[108]
TRANSITION TO FLOWERING		
AGAMOUS LIKE 17 (*AGL17*)	Promoter of flowering and positively regulated by the photoperiod pathway regulator CONSTANS.	[116]
AGAMOUS LIKE 24 (AGL24)	Activator of flowering. Activator of *SOC1* transcription.	[117,118]
FLOWERING LOCUS C (*FLC/AGL25*)	Repressor of flowering transition. Central role in regulating the response to vernalization. Functional Integrator of flowering transition pathways.	[119,120]

Table 1. Continued.

FLOWERING LOCUS M (*FLM*)/ *MADS AFFECTING FLOWERING 1* (*MAF1*/ *AGL27)*	Transition from vegetative to reproductive development. Also expressed in the embryo.	[121]
MADS AFFECTING FLOWERING 2-4 (*MAF2*/*AGL31*), (*MAF3*/*AGL70*), (*MAF4*/*AGL69*)	Floral repressors. May be important for maintenance of a vernalization requirement.	[122,123]
MADS AFFECTING FLOWERING 5 (*MAF5*/*AGL68*)	Putative floral promoter.	[123]
SHORT VEGETATIVE PHASE (*SVP*/*AGL22*)	Repressor of flowering transition in opposition to *AGL24* and *SOC1*.	[124]
SUPPESSOR OF OVEREXPRESSION OF CONSTANS1(*SOC1*/*AGL20*)	Integrator of flowering transition pathways as a positive regulator of flowering. Activator of *AGL24*.	[125,126,127,118]
AGAMOUS LIKE 19 (*AGL19*)	Expressed after vernalization. Induces flowering transition by up-regulating *LFY.*	[128]
MERISTEM IDENTITY		
CAULIFLOWER (*CAL1*/*AGL10*)	Meristem identity.	[129,98]
APETALA1 (AP1/AGL7)	Meristem identity.	[132,97,133]
FLORAL ORGAN INDENTITY		
APETALA 1 (*AP1*/*AGL7*)	Class A homeotic gene.	[132,97,133]
APETALA 3 (*AP3*)	Class B homeotic gene.	[134]
PISTILLATA (*PI*)	Class B homeotic gene.	[135]
AGAMOUS (*AG*)	Class C homeotic gene. Important for stamen, carpel, ovule and fruit development, and floral meristem development.	[82,46]
SEPALLATA1/2/3/4 (*SEP1/AGL2*), (*SEP2/AGL4*), *(SEP3/AGL9,)* *(SEP4/AGL3),*	Regulate activities of B- and C-class homeotic genes.	[76,40,136]
OVULE AND FRUIT		
SEEDSTICK (*STK*/*AGL11*) *FRUITFUL (FUL/AGL8)*	Ovule identity. Fruit development, control of flowering time. Required for normal development of the funiculus.	[137]
SHATTERPROOF (*SHP*/*AGL1*), (*SHP2*/*AGL5*)	AG-independent carpel development. Ovule, and fruit development dehiscence.	[102,129,137]

Table 1. Continued.

POLLEN		
AGAMOUS LIKE 30 (*AGL30*)	Apparently, a crucial component during pollen maturation. Pollen tube competitive ability.	[114]
AGAMOUS LIKE 65 (*AGL65*), *AGAMOUS LIKE 66* (*AGL66*), *AGAMOUS LIKE 104* (*AGL104*)	Pollen maturation Pollen tube competitive ability.	[114]
AGAMOUS LIKE 29 (*AGL29*)	Transcriptional repressor of immature pollen genes downstream of AGL65, AGL66 and AGL104.	[114]

Embryo development

Zygotic embryogenesis in higher plants begins with a double fertilization. The zygote is formed when the egg cell within the embryo sac joins a sperm nuclei, and a second sperm nuclei fuses with the central cell to give rise to the endosperm [138]. The zygote then starts to divide asymmetrically yielding a small apical cell and a large basal cell. The basal region gives rise to the suspensor, which is a structure that supports the embryo, and the apical cell gives rise to the pro-embryo [139]. The pro-embryo goes through diverse cellular stages: globular, heart, torpedo and bent cotyledon or mature stage. During the last stage of embryogenesis, reserves accumulate. Finally, the seed loses water and a quiescent state is established until optimal conditions occur and seeds germinate [140,141].

In animals, embryos at late stages resemble the adult organism, whereas in plants mature embryos prior to germination are very different to adult plants, that grow and complete morphogenesis from the shoot and root meristems. In plant embryos only two different zones can be distinguished: the basal one gives rise to the root and the more apical one to the shoot. Several molecular components of the networks controlling embryo development have been uncovered [142,138,141,139]. Some MADS-box gene factors have been identified as important regulators of this developmental stage (see Table 1).

The MADS-box Type I gene *PHERES1* (*PHE1*), also named *AGL37,* is expressed transiently after fertilization in the embryo and the endosperm. The Polycomb-group (PcG) proteins MEDEA, FERTILIZATION INDEPENDENT ENDOSPERM and FERTILIZATION INDEPENDENT SEED2 regulate seed development in *Arabidopsis* by controlling embryo and endosperm proliferation. These proteins are subunits of a multiprotein PcG complex,

which regulates *PHE1* epigenetically [109] by histone trimethylation on H3K27 residues [143]. It was shown that *medea* mutant plants have a seed-abortion phenotype due to *PHE1* upregulation; *medea* plants that also had a *PHE1* low-expression level rescued the *medea* phenotype. It has been speculated that *PHE1* may be involved in pattern formation of the endosperm [109].

AGL62 encodes another Type I MADS domain protein that has been functionally characterized. It has a strong expression during the syncytial phase of embryo development, in which the endosperm nuclei undergo many rounds of mitosis without cytokinesis, and then is later expressed during the cellularized phase, in which cell walls form around the endosperm nuclei, and finally its expression declines abruptly just before cellularization. Thus, AGL62 is thought to be a component of the mechanisms underlying cellularization during endosperm development [110].

AGL15 is the best characterized MADS-box gene during embryo development. This gene was identified and isolated initially in *Brassica napus* by differential display technique [144]. RNA gel blot analyses and *in situ* hybridization demonstrated that *AGL15* mRNA is accumulated mainly in the developing embryo during all stages. *AGL15* mRNA expression levels were at least 10-fold higher in embryos than in inflorescence apices, young floral buds, young seedlings or vegetative apices [144].

Interestingly, AGL15 protein accumulates in the cell cytoplasm of the egg apparatus and it is translocated into the nuclei during early zygotic embryogenesis [145]. This pattern of accumulation is the same in different types of asexual embryogenesis: apomitic embryogenesis of *Taraxacum officinale* (dandelion), the microspore embryogenesis in *Brassica napus* and the somatic embryogenesis in *Medicago sativa* (alfalfa) [140]. Overexpression of *AGL15* with the constitutive CaMV35S promoter yielded secondary embryonic tissue from cultured zygotic embryos and led to long-term maintenance of the embryonic phase [146]. Also, the longevity of sepals and petals was increased and a delay in the transition to flowering and fruit maturation was observed in these plants [111].

Chromatin immunoprecipitation (ChIP) was used to identify genes that were regulated by AGL15. Those genes were named as *Downstream Targets of AGL15* (*DTA1* and *DTA2*). *DTA1* (*AtGA2ox6*) is a direct downstream target of *AGL15* and encodes a protein with high similarity to gibberellin (GA) 2-oxidases and it was shown to catalyze gibberellins 2β-hydroxylation. Molecular studies showed that the expression of *AtGA2ox6* oxidase is down-regulated in an *agl15* null mutant [147]. On the other hand, *DTA2* encodes a novel protein that is repressed by AGL15 [148].

AGL15 and *AGL18* are sister genes with very similar mRNA spatio-temporal patterns of expression. *AGL18* was detected in the endosperm and embryos by *in situ* hybridization [8] and by RT-PCR and translational fusions with GUS [112]. These assays led Lehti-Shiu et al. (2005) to propose that both genes play an essential role during embryo development. However, no defects in embryo development were observed in single and double mutants of *agl15* and *agl18*, which suggests functional redundancy with other genes [112].

SEP1 (*AGL2*, [76]) mRNA is accumulated in embryos after fertilization but it is also expressed at similar or higher levels in other tissues and stages of development, as ovules [115,76]. There are other members of this family, *FLM* and *MAF1* [122], as well as *XAL1* (*AGL12*) and *AGL21* [108] that have been detected in embryos, but their functions are unknown.

After germination, all aerial *Arabidopsis* structures form from the shoot apical meristem (SAM), while the adult root apical meristem (RAM) develops from the meristem at the tip of this structure after seed germination. In the following sections we focus on the role of MADS-box genes during sporophyte development after germination. The role of these genes during seedling and vegetative development is largely unexplored. We therefore focus on the transition to flowering and flower morphogenesis.

MADS-box genes are key components of flowering transition networks

In wild and cultivated annual plant species, flowering time is an important life-history trait that coordinates life cycle with environmental conditions [149]. Plants initially undergo a period of vegetative development, characterized mainly by the production of rossette leaves from the shoot meristem. Later in development, the meristem undergoes a change in fate and enters a reproductive stage producing flowers and differentiating the germ line. Plant species exhibit variability in flowering time, and the timing of this floral switch is controlled by multiple environmental and endogenous cues [150]. Four different flowering control pathways have been described in *Arabidopsis thaliana* based on genetic data, however it is important to note that molecular data is clearly showing that these pathways crosstalk and are integrated by a complex module of feedback interactions [151] (see Figure 3).

The photoperiod pathway perceives light and responds to it [154,155]. Mutant plants in this pathway are late flowering under long day conditions [156]. The vernalization pathway comprehends genes involved in the response to long periods of cold exposure which accelerates flowering transition [119,157,158]. The gibberellin (GA) pathway, promotes flowering

by the induction and action of this plant hormone [159]. Finally, the autonomous pathway responds to developmental signals independently of the external signals and the GA action, and mutant plants in this pathway are late flowering under long- and short-day photoperiods, but can be rescued by vernalization [160,119,161].

Details on the molecular components of all these pathways have been extensively reviewed (see for example: [162,163,164,127,165]; among others). In this Chapter we will focus on the role of MADS-box genes in such pathways and integrating module. There are two key MADS-box functional integrators of these pathways that have been most thoroughly characterized: *FLOWERING LOCUS C (FLC / AGL25*), which acts as a repressor of flowering [119,120], and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1 / AGL20),* that promotes this developmental process [125,126,166].

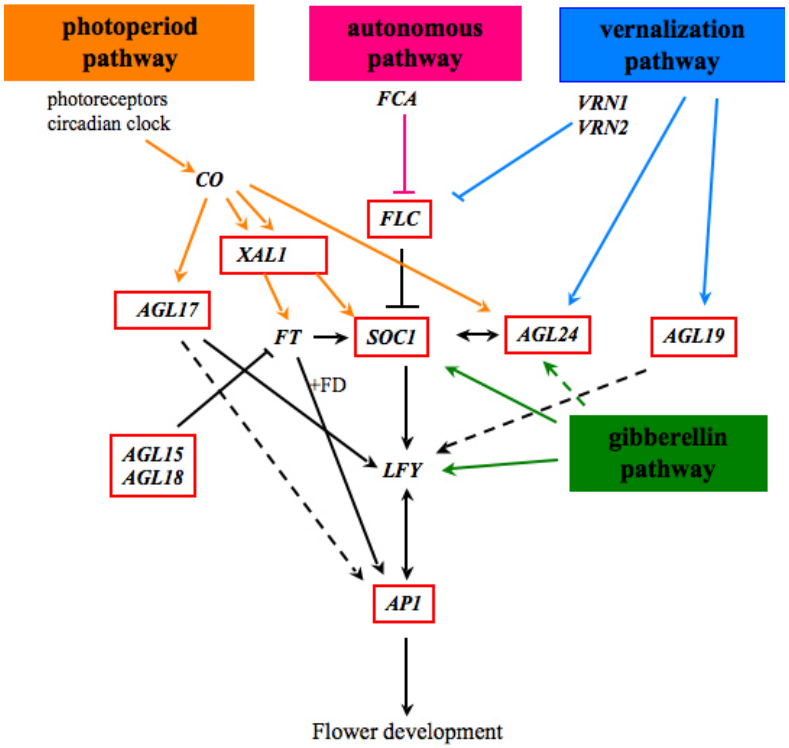

Figure 3. Simplified scheme of the four different flowering control pathways described for *Arabidopsis thaliana* and the integrator module starting to be characterized and in which MADS-box genes are key components. MADS-box genes are framed in red. Dotted lines represent hypothetical interactions. Genetic tests still have to be done to demonstrate XAL1's precise position in the network (Modified from [152] and [153]; for specific protein interactions see also references from text).

FLC is a central repressor of flowering

Plants have different ways to repress flowering until the appropriate seasonal and developmental cues overcome this repression. A central player in this process is *FLC,* which blocks flowering by inhibiting genes such as *FLOWERING LOCUS T* (*FT)* and *SOC1* that are required to induce genes which turn the vegetative meristem into a reproductive one [119,167]. Regulation of the expression of *FLC* has become a key system in plant molecular genetics to uncover transcriptional and epigenetic regulatory mechanisms.

Natural winter-annual *Arabidopsis* accessions need to pass through a long period of cold temperature or vernalization in order to be able to flower during the following spring–summer season. Genetic studies have demonstrated that a single dominant gene *FRIGIDA* (*FRI)* is able to increase the levels of *FLC* expression in these accessions, and only vernalization overcomes this effect by epigenetic repression of *FLC* [168]. Under vernalization, *FLC* expression is progressively reduced by the action of VERNALIZATION INSENSITIVE 3 (VIN3), VERNALIZATION1 and 2 (VRN1/2) and the *Polycomb* Repressive Complex 2 (VRN-PRC2) [169,54]. After this, it remains stably low during subsequent growth in warm conditions by the action of a heterochromatin protein1-like (LHP1) enabling the plant to flower [119,120,165]. It is vital that this memory is lost in the next generation so that the vernalization requirement is re-established [168]. On the other hand, summer-annual *Arabidopsis* accessions have a recessive *fri* allele often produced by a loss-of-function mutation in this gene and these plants do not need vernalization to flower [170].

In the absence of *FRI*, the autonomous pathway negatively regulates *FLC* expression. However, autonomous late-flowering mutants are overcome by vernalization, meaning that both pathways function in parallel [171]. Seven genes have been implicated in the autonomous pathway. Interestingly, three of them (*FCA*, *FPA* and *FLOWERING LATE KH MOTIF* or *FLK*) codify for RNA-binding proteins, and a fourth one, *FY*, codifies for a polyadenylation factor which collaborates with FCA in RNA processing [172]. Two other members identified in this pathway have been implicated in histone deacetylation complexes, FVE and FLOWERING LOCUS D (FLD) [173,174]. Finally, *LUMINIDEPENDENS* (*LD*) encodes a homeodomain protein with an unknown function [175]. It seems plausible to think that the autonomous pathway is conformed by functional redundant genes all targeting *FLC* [165,171].

In order to maintain flowering repression under the vegetative state, several activators of *FLC*, other than FRI, are also part of chromatin-remodeling complexes. For example, *EARLY FLOWERING IN SHORT DAYS* (*EFS*) and *ARABIDOPSIS TRITHORAX1* (*ATX1*) are necessary for the H3K4 trimethylation at *FLC* chromatin domains, which activates this gene's expression. As expected, mutations in *EFS* or *ATX1* produce early-flowering plants due to low transcriptional levels of *FLC* [176,165,177].

SOC1 / AGL20 an integrator and activator of flowering transition

The *soc1* mutant was independently isolated after a screening for suppressors of the early phenotype induced by *CONSTANS* (*CO*) overexpression [178,166], and from an activation screening in the *FRI/FLC* background [126], and using reverse genetics [125]. *soc1* mutants flower late in both long and short day conditions [125,126,127].

It has been demonstrated that FLC binds to the CArG-box in the promoter region of *SOC1* inhibiting its expression, and therefore repressing flowering [32,179]. Vernalization and the autonomous pathways allow the up-regulation of *SOC1*, after lowering the levels of *FLC* [126,180]. However, null mutations in *FLC* are not sufficient to induce high transcript levels and other signals are required to up-regulate *SOC1* during vegetative growth, particularly in the shoot apical meristem [125,126,166,18]. These signals come from the photoperiod and/or the gibberellin pathways [125,181]. In the first one, CO induces *SOC1* expression, partially through FT [166,18,182] as the input pathway. No GA regulatory elements have been found in the *SOC1* promoter [18] even though GA has been demonstrated to be crucial to promote flowering by regulating *SOC1* and *LEAFY* (*LFY*), particularly in short day conditions [183,151,181]. SOC1 induces the expression of *LFY* [184], another flowering integrator that is also a key flower meristem identity gene that activates flower development and the key floral organ identity MADS-box ABC genes that are reviewed below.

AGL24 and *SVP* participate as activator and repressors of flowering, respectively

AGL24 shares many characteristics with SOC1 as an important protein that promotes flowering [117,185]. Like *soc1*, *agl24* mutant is late flowering in both long and short day conditions. This gene is also regulated by GA, and the double mutant *soc1-2 agl24-1* is capable of flowering only after the addition of this hormone [118]. Its overexpression leads to early flowering,

indicating that *AGL24* is another important flowering regulator. Contrary to *SOC1*, *AGL24* is induced by vernalization and by the autonomous pathways, in a *FLC* independent way [117]. During the photoperiod pathway, CO also induces *AGL24* expression but not through FT, whereas *SOC1* is mainly regulated by the FT protein, as mentioned before [185].

It was recently established that *AGL24* and *SOC1* directly regulate each other at the transcriptional level by binding each other's promoters (but not their own), thus establishing a positive-feedback regulation that probably forms part of a larger and more complex module which integrates all the signals known to promote flowering transition [118]. It is also important to note, that Liu and colleagues (2008) demonstrated that SOC1, but not AGL24, binds directly to the *LFY* promoter [118].

SHORT VEGETATIVE PHASE (*SVP / AGL22*) is the sister MADS-box gene and closest homolog of *AGL24*, however it acts as a repressor of flowering transition in opposition to *AGL24* and *SOC1* [124]. Concordantly, the *svp* loss-of-function mutant is early flowering, and *SVP* overexpression causes a late flowering phenotype. Interestingly, both mutants are insensitive to cold acclimation, a phenomenon different from vernalization in which plants become tolerant to freezing temperatures by being previously exposed to short periods of cold (16 ^{o}C). *SVP* mediates the temperature-dependent functions of *FCA* and *FVE* within the "thermo-sensory pathway" and negatively regulates *FT* by directly binding this gene's promoter at its CArG motifs [186]. Interestingly, the flowering time regulators *AGL24*, *SVP* and *SOC1* have shown to be down-regulated and kept away from the floral meristems by the floral identity genes *LFY* and *AP1* that are in turn key flower meristem identity genes [187,188].

Other MADS-box genes implicated in flowering transition

Additional MADS-box genes have been recently implicated in the flowering transition regulatory network. Among the positive flowering regulators, *XAANTAL1* (*XAL1* / *AGL12*) [104] and *AGL17* [116] have been implicated in the photoperiod pathway downstream of CO action. Also, *AGL19* is normally repressed by the polycomb complex, but after vernalization its repression marks are reduced allowing *AGL19* to be highly expressed, which in turn induces flowering transition by up-regulating *LFY* [128]. These three genes are also strongly expressed in the roots. On the other hand, FLOWERING LOCUS M (FLM) / MADS AFFECTING FLOWERING (MAF1) is thought to act as a co-regulator with SVP inhibiting flowering transition [189]. Interestingly, temperature might

suppress the repressive effect of *FLM* on flowering, or temperature might act downstream of FLM to bypass its repressive effect. This has been described in a new pathway that involves the thermal induction of flowering when plants are shifted from 23 to 27^{o}C under short day conditions [190].

Other flowering repressors are AGL15 and AGL18 that act redundantly to down regulate *FT* expression [113]. It is very likely that the number and complexity of MADS-box interactions involved in the regulation of flowering transition is still much larger and rapid progress in understanding their specific functions is expected in the coming years in this exciting and important field for plant development.

The MADS story of flower development

Arabidopsis thaliana is a self-fertilizing plant that has a simple flower structure typical of the Brassicaceae and with a basic floral plan shared by highest eudicots. The flower has two external sterile organs (whorls 1 and 2) surrounding the reproductive ones (whorls 3 and 4): a calyx of four sepals (whorl 1) and a corolla of four petals (whorl 2) whose positions are alternate and interior to those of the sepals. The androecium (whorl 3) consists of four medial, long stamens, and two lateral short stamens with a superior sessile gynoecium (whorl 4) in the center of the flower that consists of two fused carpels [105]; Figure 4.

Based on morphological evidence, flower development can be divided into several stages. Flower meristems arise from the flanks of the inflorescence meristem in a phyllotactic spiral (stage 1), and soon become isolated from the inflorescence meristem by tissue that will later become the flower pedicel (stage 2). Then, the sepals begin to arise from the outermost cells of the flower meristem (stage 3) and elongate (stage 4). The next stage is characterized by sepal growth and emergence of primordia of petals and stamens (stage 5). Subsequently, sepals cover the flower bud (stage 6) and at this stage stamens and carpels become clearly differentiated (stage 7). Stamen primordia elongate (stage 8) and carpels differentiate (stage 9) [105]; for a review see [194]).

Studies at the molecular level suggest that there is an overall conservation among key regulators of floral organ identity and arrangement [195,196,18]. The flowering genes reviewed in the previous section induce the meristem identity genes and these, in turn, regulate the floral organ specification genes among which, MADS-box genes are also key players. Upon induction to flowering, the inflorescence meristem identity genes (such as *TFL1*), that specify the inflorescence shoot as indeterminate and non-floral [197,198] are repressed, while the floral meristem identity genes (*AP1*,

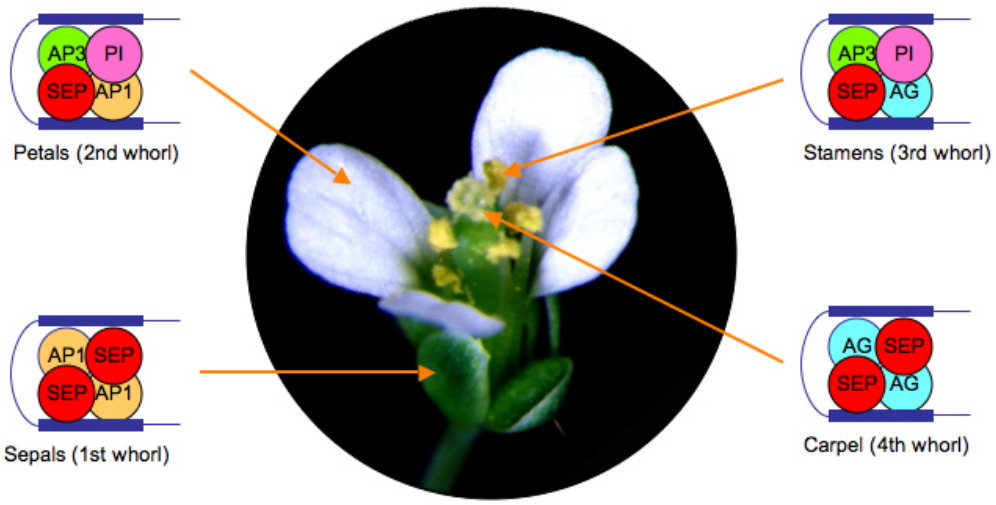

Figure 4. *Arabidopsis thaliana*'s flower structure is determined by the combinatorial action of different MADS-box proteins. The "quartet model" [191] proposes that the transcription factors complex binds DNA at the promoter regions of their target genes. According to the model, two dimers of each tetramer recognize two conserved DNA sites termed CArG-boxes on the same strand of DNA (with specific separation between them), which are brought into close proximity by DNA bending. Binding of the first dimer is thought to facilitate the binding of the second one. Proteins in the tetramer interact through their different motifs: The MADS-domain binds to the DNA, the I and K domains are involved in dimer formation. The C-domains are supposed to be the transactivation sites, but some of the MADS proteins seem to lack these activity [18]). The exact structures of the MADS-box protein tetramers that control the identity of flower organs are still hypothetical, though several studies on MADS-box protein interactions have been done (e.g., [192,50,37]). CArG boxes represented by blue boxes in bent DNA (blue line), MADS-domain proteins are represented as circles. A-function protein: AP1, APETALA1; B-function proteins: AP3, APETALA3; PI, PISTILLATA; C-function protein: AG, AGAMOUS, E-function proteins: SEP, SEPALLATA. (Figure adapted from [193]; Arabidopsis picture by ER Alvarez-Buylla).

AP2, *CAL*, *LFY*), [129,199,18] are turned on [198]. Mutations in the floral meristem identity genes cause primordia that would develop into flowers to acquire inflorescence meristem identity thus becoming indeterminate. Indeed, *TFL1* is a repressor of the expression of at least two of the floral meristem identity genes, *LFY* and *AP1*. The flower meristem identity genes activate downstream floral homeotic ABC genes (*AP1*, *AP2*, *AP3*, *PI* and *AG*), which are transcription factors necessary for floral organ identity [46,200,77,201]. All of these, except *AP2,* are Type II MADS-box genes.

In the classical ABC model, three different types of homeotic genes of overlapping activities have been proposed to control the development of wild type flowers as follow: sepal (A), petal (A+B), stamen (B+C) and carpel

identity (C) [46]. The A and C function genes negatively regulate each other and the B function is restricted to the second and third whorls independently of A and C functions [82,202]. Originally, the function of these genes was inferred by the characterization of their homeotic mutants, which have altered floral organ positions: A class mutants have flowers consisting of carpel-stamen-stamen-carpel; B class mutant flowers bear sepals-sepals-carpel-carpel and the C class mutant flowers have sepals-petals-petals-sepals [46]. Finally, mutations in all three functions lead to the transformation of all floral organs into leaf-like organs, suggesting that flowers are transformed leaves (reviewed in [203]).

Arabidopsis A function genes are: *AP1* and *APETALA2* (*AP2*). *AP1* is a MADS-box gene expressed in the two outer whorls of the floral meristem [97]. Strong *ap1* alleles (*ap1-1*) often lack the second whorl while weaker alleles of this gene do not have a full homeotic conversion of floral organs [132]. This gene is transcriptionally regulated by the B class genes *AP3/PI* [33] and by LFY [201]**.** On the other hand, *AP2* encodes a putative transcription factor that is a member of a plant specific gene family of genes (AP2/EREBP family) with diverse functions [204,205]. Mutants in the *AP2* gene rarely develop petals and, additionally, their sepals are transformed into carpelloid structures due to ectopic *AG* expression which is negatively regulated by AP2 itself [202].

The B class genes are also MADS-box (*AP3* and *PI*). These two genes are expressed in the second and third whorls and mutant flowers for any of these two genes are identical lacking petals and stamens as predicted by the ABC model [18]. It has also been shown that the proteins encoded by these two genes form heterodimers to exert their function and both are required to activate each other and perform the B function during petal and stamen determination [72,135,206]. Furthermore, these proteins move to the nucleus to function as transcriptional regulators only after they form a heterodimer [42].

Another MADS-box gene is the only C-type gene discovered up to now: *AG*. It has been shown that there was an ancestral *AG*-like MADS-box gene that duplicated before the angiosperm radiation, producing two paralogous lineages [134]: C and D. Even though these two functions are not mutually exclusive, the D class function is primarily involved in ovule identity [207]. Mutant *ag* flowers lack stamens and carpels, and also bear indeterminate flowers with reiterating sepals and petals, suggesting that *AG* is important for floral meristem determinancy, besides stamen and carpel identity [208].

Interestingly, all MADS-box genes, have expression patterns that correlate with the site where these are necessary. In contrast, *AP2* mRNA is expressed in all four whorls throughout flower development but mutations in *AP2* only

affect identity of whorls 1 and 2. Recent data has shown that AP2 is repressed at the translational level by microRNA, which is active only in whorls 3 and 4 [199], thus explaining that its role is delimited to the two first flower organs.

MADS-box genes are also crucial during ovule development. In *Arabidopsis*, ovules develop inside two fused carpels and the MADS-box genes *AG*, *SHATTERPROOF1/2* (*SHP1* and *SHP2*), and *SEEDSTICK* (*STK*) promote the identity of this organ [137,209]. It has been shown that the double mutant *shp1* and *shp2* does not affect ovule development but, as the name of the genes suggest, affect the dehiscence zone inhibiting the carpels shattering [102]. However, the triple mutant *shp1 shp2 stk* shows clear alterations in ovule development with these converted into carpel-like structures [137].

Stamen development is also under the control of the overlapping activities of B and C MADS-box genes. Little is known, however, about additional molecular components that participate in this developmental process. Recent transcriptomic analyses are starting to uncover additional components of this developmental process [210,211,114]. Verelst and collaborators (2007) compared the pollen grain transcriptome of an *agl65/agl66/agl104* triple mutant (which is altered in pollen tube competitiveness, but shows normal pollen grain morphology) against the transcriptome of wild type plants, and found that these MADS-box genes are important regulators of pollen maturation. They also compared their results against those reported by Honys and Twell (2003) on different stages of wild type pollen development and found that the absence of these MADS-box proteins and the complexes they usually form, alters gene expression during several stages of pollen maturation. Verelst and collaborators (2007) also analyzed the role of some double mutants in pollen transcription profiles, and inferred some interactions of complex regulatory network controlling pollen development. Importantly, they found that AGL65/66, AGL65/104, AGL30/66, AGL94/66 and AGL30/104 protein complexes repress immature pollen-specific transcription factor genes and activate mature pollen-specific transcription factors such as the MADS-box genes, *AGL18* and *AGL29*. The latter MADS box genes, in turn, are transcriptional repressors that are highly expressed during immature tricellular pollen grain stages. In addition, these complexes also repress *AGL30* and *AGL65* in a negative feedback loop, whereas AGL18 acts to fine tune the expression level of *AGL29*. This study suggest that different combinations of MADS proteins have distinct roles during pollen grain development, that seems to be a nice system to uncover the complexity of MADS-protein complexes and their role during cell differentiation processes. Indeed, it has been postulated that MADS proteins exert their regulatory function as multimeric complexes.

Higher-order MADS-domain protein complexes

Using *Antirrhinum majus,* in which the ABC model was also discovered [46], a ternary complex between A and B function proteins that binds DNA more efficiently in comparison to single proteins, was described. A complex of SQUAMOSA (SQUA, the AP1 ortholog) and DEFICIENS/GLOBOSA (DEF/GLO; the AP3/PI orthologs) bound DNA more efficiently compared with DEF/GLO or SQUA alone [50]. Thus suggesting that transcriptional complexes that combine A and B function proteins are more stable than those formed with any of these functions alone.

Using a yeast three-hybrid experiment, it was shown that *SEP3* (another MADS-box gene) and *AP1* are able to interact with the heterodimer *AP3*/*PI* but not with *AP3* or *PI* alone [37]. Moreover, they described that this interaction was essential for the function of the MADS proteins because the heterodimer *AP3*/*PI* lacks the activation domain necessary for a transcription factor to function and that both *SEP3* and *AP1*, possess it [37]. These findings suggest that the inclusion of *SEP3* or *AP1* together with *AP3*/*PI* could result in an active tetrameric transcriptional complex. Concomitantly, our laboratory in collaboration with M. Yanofsky [40] demonstrated that the ABC proteins on their own or combined (A, AB, BC or C) were not sufficient to determine floral organs when expressed in leaves under the action of the 35S constitutive promoter. However, floral organs could indeed be recovered combining ABC and *SEP* genes were expressed in leaves [40,37]**.**

Another example in which the SEP proteins are necessary for the formation of a ternary (or quaternary) complex is during ovule development. AG, SHP1, SHP2 and STK form ternary complexes among them only when SEP proteins are present [209]. Interestingly, the *SEP* genes, *SEP1*, *SEP2* and *SEP3*, received their names because the floral organs that develop in any of the four whorls in triple *sep* mutant plants resemble sepals, and the flowers become indeterminate [76]. This *sep1 sep2 sep3* triple mutant phenotype is markedly similar to that of double mutants that lack both B and C class activity, such as *pi ag* and *ap3 ag* [212,76]. Single or double mutants for these genes yielded flowers undistinguishable from wild type, thus suggesting that the three *SEP* genes are functionally redundant and are important for the determination of three of the four floral organs: petals, stamens and carpels [37,40,203]. Since the triple *sep1 sep2 sep3* mutant does not show alterations in sepal identity, an additional MADS-box gene could be also involved during specification and development of these floral organs. Indeed, another *SEP*-like MADS-box gene (*SEP4* previously *AGL3*) was characterized [136]. The quadruple *sep1 sep2 sep3 sep4* mutant plants

produce flowers that have leaf-like organs in all whorls, thus validating the contributions of *SEP* genes to flower organ identity in all floral organs. Coincidently, *SEP* genes are expressed in the whole floral meristem during flower development, they regulate B and C genes at the transcriptional level and encode proteins that interact with all the ABC proteins [213].

Based on the *SEP* results, it was proposed that MADS proteins form tetrameric complexes during floral organ determination ([18,191,193,195]; Figure 4). The model suggests that within each transcriptional complex, there would be two MADS dimers, each one of them would bind a single CArG binding site causing the promoter DNA region to bend and enable the MADS dimers to act cooperatively. For example, binding of one dimer of the tetramer to DNA could increase affinity for local binding of the second dimer in the tetramer. Besides, one of the dimers could function as an activation domain of the tetramer allowing an efficient transcriptional activation [37]. In a recent study of the complete MADS-domain protein family provided two-yeast hybrid data to document the complete MADS protein-protein interactome for *Arabidopsis* [192]. Several dimers and potential tetramers can be formed from this database and it will be interesting to test which of them are functional and what is their role during *Arabidopsis* development.

There are few examples of MADS proteins that interact or form complexes with members outside this family. To our knowledge only four examples of complexes that involve MADS-box proteins and unrelated polypeptides have been reported in the literature. Our laboratory was the first group to report non-MADS proteins as interactors of a MADS-domain protein (AG): a phosphatase (VSP1) and a Leucine rich protein called FLOR1 [214]; see also [215]. A second report documented an interaction of histone fold protein NF-YB with OsMADS18 from rice [216]. A more recent report showed that AP1 and SEP3 could form a complex with the transcription co-repressors LEUNIG (LUG) and SEUSS (SEU) [217]. Finally, AGL15 was shown to interact with a protein that forms part of the SWI-independent 3-histone deacetylase (SIN3/HDAC19) complex (SAP18) and with HDAC19 itself [218].

An integrative model of the gene regulatory network underlying floral organ determination

Analytical molecular approaches have been, and will continue to be, successful in producing a wealth of data on specific genes, their most immediate interactors and some cell functions. However, understanding the concerted action of many interacting molecular components, the resulting

behavior of complex and integrated biological systems, as well as the consequences of intervening in them, presents serious challenges to contemporary biologists. We are meeting these challenges by combining experimental molecular approaches with dynamic mathematical/computational models [219,58,59,60,220,61].

We have put forward a dynamic gene regulatory network model which steady states or attractors correspond to the multi-gene expression configurations characteristic of each of the four types of primordial cells during early flower development, those of: sepals, petals, stamens and carpel primordia [58]. Interestingly, simulations of loss of function mutations of the nodes corresponding to the ABC genes recovered observed results. For example, when the activation state of *AP1* is set to "0" at all interactions, in order to simulate a homeotic mutant, the steady states configuration that corresponds to the combinations of gene activation typical of primordial sepal and petal cells is not recovered any more. Instead, all the initial states of the network that used to lead to that steady-state configuration now go to the configuration characteristic of stamen and carpel primordial cells. Thus, the model recovers the profile characteristic of the observed homeotic flower lacking sepals and petals. The same was true for all mutations that have been characterized experimentally in *Arabidopsis*, thus verifying the proposed model [58].

Finally, such type of dynamic computational models are also useful to evaluate how robust are the gene activation combinations that characterize each studied primordial cell-type. Indeed, the basic floral plan consisting of whorled sepals, petals, stamens and carpels, which sequentially appear from the outermost to the inner of the flower during development, is quite conserved among angiosperms (specially among higher eudicot species). This pattern suggests that the mechanisms underlying the determination of such primordial cell types should be robust. Concordantly, our simulations of the proposed model confirmed that the network´s steady states are robust to initial states (all of the more than 130,000 initial conditions converge to the gene expression configurations observed in primordial cells during early flower development), but also to small alterations in the rules of interaction among genes that could correspond to genetic alterations [58,59,60].

Perspectives on the role of MADS-box genes in plant development

As reviewed here, plant MIKC genes have been mostly characterized as regulators of the transition to flowering [99] and flower, fruit or seed

development [82,130,129,221,137]. They are fairly specific meristem- [97,103], cell- [102] or organ-identity [20,76] genes. The first studies suggested that the function of these genes was specific to certain cell types, tissues or stages of development at which these genes were expressed at the transcriptional level.

However, genome-wide studies are suggesting that most MADS-box genes are expressed at several stages of the plant's life cycle and in a variety of organs, tissues and cell types ([14]; for a review see [93]), suggesting that these genes may have developmental roles that affect multiple stages of development and plant organs. Such recent studies are challenging previous phylogenetic analyses, that had suggested that the genes clustered within each clade shared structure, expression pattern and gene function (*e.g.,* [11]). For example, three groups of genes belonging to the clades of *AG, SOC1* and *ANR1* seemed to be specific to roots [8,108], but we now know that they are expressed in several other organs and may have diverse developmental roles or the same function at different tissues and developmental stages [104]. Interestingly, a transcriptional regulation map for *Arabidopsis* development has revealed that at least one of the ABC MADS-box genes (*PI*), that was supposed to be specific to flowers, could be expressed in the roots as well [192,222].

Indeed, in *Arabidopsis, XAANTAL1* (*XAL1; AGL12*), the sister gene of the group where the first cloned plant MADS-box gene was found (*AG*), is a pivotal gene for both root and flower development [104]. This was unexpected because all the genes in this clade are reported to be specifically expressed and functional only in reproductive tissues [137].

Given the high sequence conservation of MADS domains among plant and animal proteins within each lineage (I and II), we have hypothesized that some of their functions exerted in various plant organs and at different life-stages, may have been also conserved. Such conserved functions may be, for example, related to the animal MEF-related MADS proteins roles, which have been implicated in the regulation of cellular homeostasis and linked to cell-cycle control [223]. Indeed, *XAL1* seems to be an important modulator of cell proliferation versus differentiation decisions.

The analyses of MADS-box gene function in the root, that is a transparent organ with a relatively simple cellular structure, may enable quantitative analyses of cell dynamics of mutants of these genes [224,225]. Indeed, the root has become a very useful system for unraveling general features of multicellular developmental mechanisms [226,227,228], and specifically for understanding the links between cellular dynamics and cell type specification during normal morphogenesis of a complex organ *in vivo*

[229,228,230]. Some components of the molecular mechanisms involved in stem-cell niche patterning and behavior [231,232], as well as in the patterns of cell proliferation along morphogenetic gradients, that in the root are importantly determined by auxins, have been characterized as well [233,234,235]. It will be very interesting to unravel the role of other MADS-box genes in such networks.

Our data strongly suggest that *XAL1* is an important regulator of cell proliferation in the root. *XAL1* mutant alleles have short roots with an altered cell production rate, meristem size and cell-cycle duration. Thus *XAL1* is the first MADS-box gene that is shown to be involved in cell-cycle regulation [104]. Auxins have been implicated in cell-cycle regulation [236,237] and our data interestingly also show that *XAL1* is induced by auxins. On the other hand, as it was reviewed above, *xal1* alleles are also late flowering and our data suggest that *XAL1* could be an important promoter of the flowering transition through up-regulation of *SOC*, *FT* and *LFY* [104].

Finally, several studies indicate that MADS-box genes are able to integrate environmental and internal signals and, consequently, are very likely important components of the mechanisms underlying the plastic developmental responses of plants to environmental conditions [119,181,162,187]. For example, recent results appear to indicate that MADS-box genes are the targets of both GA signaling [187] and biosynthesis [148]. It remains to be determined whether the regulation of hormone homeostasis is also one of the many MADS-box gene functions. If such were the case, at least some aspects of the MADS-box phenotypes would be mediated by hormone activity and thus mimic phenotypes of lines with altered hormone activities. Additional recent data has demonstrated that *AG*, *PI*, *AP3* and *AGL15* [148,187] are direct targets of GA signaling (*AG*, *PI* and *AP3*) and biosynthesis (*ALG15*). Our recent studies suggest that *XAL1* may be an important component of the regulatory networks that respond to light in the flowering transition control and is a pivotal element of the developmental pathways that underlie root development, where it seems to mediate hormone activities as well [104].

Another root MADS-box gene, *ANR1*, has been identified as a gene implicated in nitrate signaling pathway and to be responsible for lateral root growth upon nitrogen deficiency [107]. Indeed, nutritional deficiency also triggers flowering [238], and might be sensed at the root [239]. Several important regulators of the transition to flowering are MADS-box genes [127]. Interestingly most of them are expressed in roots but their role in this tissue and how this relates to their role in root development and the flowering pathways is unknown. Therefore, one of the most fascinating challenges in

MADS studies will be to continue elucidating their complex integrative roles during coordinated plastic developmental responses occurring at distant parts of the plant.

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10. Chromatin remodeling and epigenetic regulation during development

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Most of the cells that integrate a multicellular organism have the same "DNA genomic set". Differences between cells arise very early in development due to a variety of processes that control which genes will be expressed in determined cells contributing to cell fate determination and maintenance. These processes gather in the term "epigenetics" and represent the first control of how does a genotype becomes a specific phenotype through the epigenome. Eukaryotic genomes reside inside the cell nucleus in a complex protein-DNA conformation called chromatin. All genomic metabolism takes place immerse in a chromatin environment and the molecular machinery that participates has evolved in tight relation with it. In recent years, this structure has shown to represent a primordial level of genome function regulation. On the other hand, the incorporation of epigenetics to the understanding of human diseases, as cancer, has opened a whole new analysis scheme. It has been recently postulated that the epigenotype represents a second code that is much more sophisticated and complex than the genetic code. The deciphering of such second code will certainly contribute to better design early diagnostic protocols and improve the prognostic, monitoring, treatment, and even prevention of cancer.

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Epigenetics have now turned into an essential framework to look at a myriad of processes during organism development as cellular plasticity, imprinting, differentiation, self-renewal, ageing and its abnormal counterparts.

Introduction

Organism development involves a sophisticated network of genetic information in combination with various and interdependent epigenetic events. Genetics and epigenetics are constantly interplaying in order to achieve proper expression programs in the cell and errors in any of these components could compromise cellular function (Fig 1).

In addition to its requirement for the assembly and packaging of the genome inside the nucleus, chromatin structure is a central component for several nuclear processes as DNA replication, DNA repair and recombination among others. The interplay between different epigenetic regulators such as the covalent modification of histones and ATP-dependent chromatin remodelers, are important in controlling gene expression and many other biological processes. The amino terminal domain of histones are exposed on the nucleosomal surface and are rich in post-translational modifications such as serine and threonine phosphorylation, lysine and arginine acetylation and deacetylation, methylation and demethylation of the same residues, lysine ubiquitination and sumoylation and ADP-ribosylation [1, 2, 3]. Additional complexity is added to the system when we consider that methylation of histones can be found in mono-, di- and tri-methylated forms, each of which is generated by a specific histone methyltransferase that can be removed in a regulated way by specific demethylases [3]. Furthermore, and with the exception of histone H4, all core histones have variants which in some cases differ in surprisingly few amino acids like the H3.3 histone variant which is associated to transcription sites and more recently, to nucleosomal dissociation [4, 5].

Several interdependent models have been proposed by Allis and collaborators, starting with the “histone code” referred to the combinatorial covalent modifications of histone tails, followed by the “cross-talk” model to explain the spreading of epigenetic modifications among poly-nucleosomes and the “barcode hypothesis” that incorporates histone variants to define different chromosomal domains and try to explain the concept of epigenetic memory [6, 7, 8]. Now the relevant question has turned into the mechanisms and targets of all these processes during early development.

Historically, transformation of a normal cell to a cancer cell involves multiple events where genetic defects emerge [9]. Those genetic

abnormalities can be grouped as gene mutations, loss of heterozygosity, translocations, deletions, recombination, and others. Knudson hypothesis based on the "two-hits", explaining the origin and progression of retinoblastoma, attracted our attention on the genetic predisposition to certain types of cancers [10, 11]. Nowadays, the origins and progression of cancer cannot be understood exclusively from the genetic perspective. Epigenetics are coming of age supported by a large and novel amount of knowledge directly associated with chromatin structure.

The classical view that both alleles must be genetically inactivated to allow tumor progression is now evolving based on epigenetic silencing mechanisms, in particular of tumour suppressor gene expression [12, 13]. Bird defined epigenetic regulation as the mitotically and/or meiotically heritable changes in gene expression that cannot be explained by changes in DNA sequence [14]. This type of regulation incorporated an additional concept that lead to the notion that regulatory signals were transmitted from one cell generation to another and that represents a sort of "memory" that should be maintained post-mitotically. The "epigenetic memory" concept represents one of the most attractive and challenging research topics in the field and its understanding will certainly contribute to learn more about cell transformation and tumour progression with a much better position to design and develop novel therapeutic strategies.

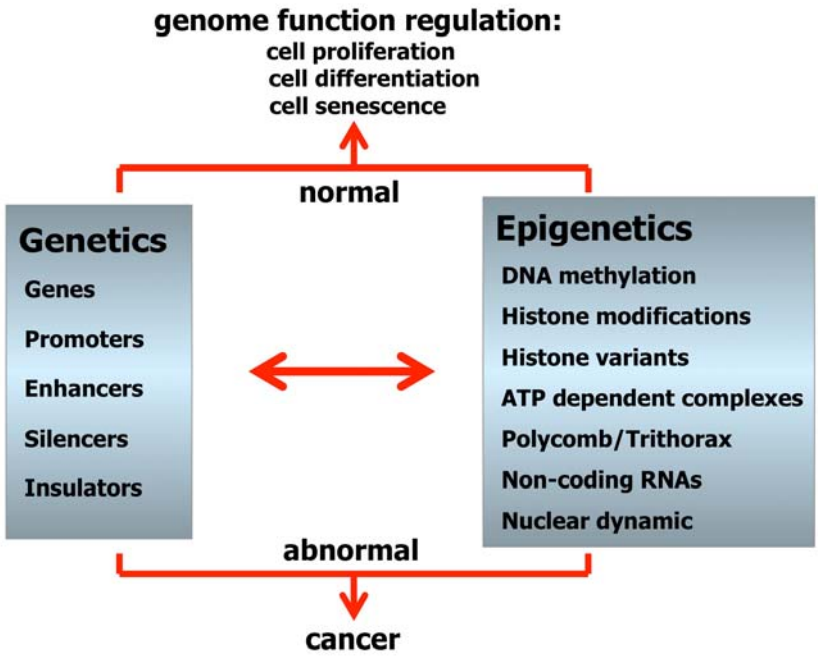

Figure 1. Genetic and epigenetic elements regulate genome function and deregulation of both processes could lead to cancer.

Chromatin structure is intimately involved in epigenetic regulation and its understanding in terms of positive and negative modulation of gene expression will provide a good stand point to address the origins and progression of cancer. Epigenetic regulatory elements can be grouped in different categories that include, DNA modifications like DNA methylation, chromatin structure modulation, non-coding RNA or intergenic transcripts and nuclear dynamics. All of them contribute to propagate genetic information in tight relationship with epigenetic regulatory mechanisms.

In the present chapter we will first describe the epigenetic mechanisms that control gene expression mainly based on chromatin remodeling process. The second part will analyze the role of epigenetics in early development and germ line specification. Finally, we discuss how epigenetic processes are involved in ageing and human diseases.

General mechanisms of epigenetic regulation

The central aspect of epigenetic regulation is undoubtedly related to eukaryote genomic organization into chromatin. Chromatin structure has evolved to allow large genomes to be incorporated in the cellular nuclei causing a natural repressive environment for transcription activity and the subsequent transmission of genetic traits. To allow regulated gene expression, such highly compacted chromatin structure needs to be coordinately remodeled [15]. Chromatin is defined as a set of DNA-protein and protein-protein interaction in which the fundamental unit is the nucleosome where the DNA is wrapped around encompassing 147 base pairs (bp) of genomic DNA [16]. Nucleosome is organized into a histone octamer composed of two copies of histone H3, H4, H2A and H2B corresponding to the minimal level of genome compaction [17, 18]. Nucleosomes contribute to the higher-order chromatin organization of the genome through a highly sophisticated and not completely understood mechanism. Importantly, the 30 nm chromatin fiber also known as solenoid constitutes the physiological substrate for the great majority of the epigenetic regulatory events [17, 19]. Thus, based on all the progress in understanding how the genome is organized into chromatin and how such natural occurring structure influences gene expression, normal and aberrant epigenetic regulation needs to be taken seriously into account.

To comprehend the relationship between epigenetics and human diseases it is first desirable to learn how chromatin structure is modulated. Chromatin structure is remodeled at different interdependent levels that include: 1) one of the most studied epigenetic modifications that is DNA methylation, 2) histone covalent modifications, 3) the expanded field of histone variants, 4) members of the Polycomb and Trithorax family of chromatin regulators, 5)

the novel research field incorporating non-coding RNA's and RNA interference (RNAi) pathway and 6) the contribution of the nuclear architecture and dynamics. Due to the relevance of these remodeling activities we will describe their function during development and their relationship with human diseases.

DNA methylation as a central epigenetic process

One of the central components of epigenetic regulation, based on repression processes, is DNA methylation [14, 20, 21]. In mammals, DNA methylation occurs at the carbon-5 position of the cytosine in the context of symmetrical CpG dinucleotides and is present in 70-80% of all CpGs in a human somatic cell [22]. Evolutionary, DNA methylation has been proposed to contribute to genome stability repressing the chromatin structure and avoiding the mobilization of retrotransposons, endogenous viruses or repetitive sequences [23]. Interestingly and to a certain point contradictory, unmethylated CpG sequences are found normally into the genome corresponding to the so-called CpG-islands [24]. It has been estimated that around 60% of human gene promoters correspond to unmethylated CpG-islands and those promoters correspond either to housekeeping or tissue-specific genes [24]. In particular, the great majority of tumour suppressor gene promoters and surrounding genomic sequences correspond to CpG-islands [24, 25].

By definition a CpG-island corresponds to a DNA sequence between 300 and 650 bp with an average content of G+C of 60 to 70%. More recently, CpG-rich and CpG-poor promoters have been described to be both sensitive to DNA methylation [24]. Observations from our laboratory have shown that the CpG-abundance does not always correlate with their quantitative and qualitative silencing effects (De la Rosa et al., 2007; Soto-Reyes and Recillas-Targa 2010). At this point it is relevant to point out that one of the most frequent abnormal epigenetic events in human diseases is the DNA hypermethylation of tumour suppressor gene promoters [25, 26, 27].

DNA methylation has also been critical in early developmental stages and genomic imprinting [20, 28, 29]. DNA methylation participates on reprogramming specific epigenetic patterns of gene expression during early mouse development, of both maternal and paternal genomes. Even though several aspects remain unsolved, after fertilization and until blastocyst stage there is an active genome demethylation event (Fig 2). After implantation there is an active *de novo* DNA methylation [30, 31, 32, 33]. Interestingly, imprinted genes are not subject to such epigenetic variations in terms of DNA methylation. The active demethylation process remains a mystery in terms of

the enzymes responsible and the mechanisms of demethylation. Until today no-demethylation enzymatic activity has been discovered. At the present time it is well established that early DNA methylation patterns require both *de novo* and maintenance DNA methyltransferase enzymatic activities (see below).

Several models have been proposed to explain DNA methylation variations during early development. Based on the "epigenetic memory" theory it has been suggested that demethylation allows a rapid erase of specific parental methylation patterns, of course with the exception of imprinted genes. Furthermore, such demethylation will favor open chromatin environments facilitating the expression of a large set of genes that could be grouped in genomic clusters [34]. Such scenario should facilitate early patterns of gene expression and cell lineage differentiation. A complementary function for DNA methylation has been associated with an intense wave of DNA methylation beginning at birth. It has been discussed that such strategy facilitates a fast and stable silencing, in a large genomic scale, of embryonic genes that are no longer required for the subsequent developmental stages of an organism (Fig 2) [35, 36].

Histone code and cross-talk

As previously mentioned, histones can be post-translationally modified at both the core and the amino terminal tail. The combinatory of covalent modifications give rise to different biological outputs that reside at the centre of chromatin-regulated processes and are still under intense investigation. Histone covalent modifications can alter chromatin structure in different ways. One occurs when certain modification (i.e. acetylation) alters the histone electrostatic charge hence changing the local charge of nucleosomes. Such modifications can result in the alteration of histone-DNA and histone-histone interactions changing the properties of the chromatin fiber. A different phenomenon takes place when different post-translational modification patterns act as platforms to recruit effector proteins that modify chromatin structure. This final property has lead to the "histone code" hypothesis, in which histone modifications profiles provide recognition flags to specific nuclear factors [6]. Later on such concept has been extended into a global landscape in which different chromatin domains in a chromosome are determined by the differential amounts of histone modification profiles so that the code becomes meaningful when looking at broader chromatin regions. The previous idea supports that there is a cross-talk between nucleosomes that contributes to the definition and regulation of specific genomic regions [37].

Over the last decade, different chromatin-modifying enzymes have been identified. Some of these conserved protein complexes are able to establish

histone modifications like lysine acetylation and methylations, arginine methylation and serine or threonine phosphorylation. Other protein complexes can remove such modifications contributing to chromatin status reprogramming. Therefore, regulation of chromatin-modifying enzymes is required in order to maintain a balanced state that allows fine-tuning of gene expression.

ATP-dependent chromatin remodeling complexes

Another mechanism to modify the intrinsically repressive chromatin status without covalent modifications is performed through the recruitment of "remodeling" complexes that use the energy from ATP hydrolysis to reorganize the chromatin fiber. Chromatin-remodeling enzymes can expose sequences facilitating the interaction of other proteins with nucleosomal DNA or stabilize repressive chromatin conformations [38]. Remodeling activities are necessary in different contexts such as transcription, replication, recombination, histone variants replacement and repair, and distinct complexes have been characterized linked to these processes.

Chromatin-remodeling enzymes can be categorized into different families depending on their ATPase subunit. The best characterized are the ISWI family that mobilizes nucleosomes along the DNA and the SWI/SNF family that alters nucleosome structure exposing DNA-histone contacts. Other complexes as Mi-2 and INO-80 have been linked with compact chromatin stabilization [39]. ATP-dependent remodeling complexes have several subunits besides the ATPase that allows them to carry out their function in a regulated and specific manner. One example is the mammalian NuRD complex in which a histone deacetylase (HDAC) forms part of the complex [40]. Another example comes from the *Drosophila* NURF complex that contains NURF301 subunit, which interacts with transcription factors [40].

Defects in chromatin-remodeling complexes have been implicated in cancer. For example, a loss of SNF5, a member of the human SWI/SNF complex, has been observed in pediatric cancer. In addition, the ATPase subunit BRM and BRG1 of the same complex, have been found mutated in a variety of cancer cell lines and primary tumours that are associated with a poorer prognosis in patients with non-small-cell lung cancer [41].

Polycomb and Trithorax family of regulatory proteins

Polycomb (PcG) and Trithorax (TrxG) group of proteins represent one of the main effectors that modify chromatin in response to cellular signals transforming developmental cues into epigenetic memory. These proteins were first identified in *Drosophila* were they participate in *Hox* gene cluster

regulation as their mutants cause homeotic transformations. Since then, PcG and TrxG complexes haven been shown to be crucial regulators in essential cell processes as proliferation, cell identity (providing cellular memory) and lineage commitment. Additionally, PcG and TrxG proteins are involved in promoting chromatin transitions in response to a variety of signals as morphogens and growth factors.

PcG and TrxG proteins play antagonistic roles. PcG complexes are involved in maintaining a silenced chromatin state and TrxG proteins act preferentially propagating an open chromatin conformation that is transcriptionally permissive. This is achieved by a variety of proteins that are highly conserved between eukaryotes and perform diverse functions including most of the epigenetic mechanisms previously described. PcG genes encode products that include DNA-binding proteins (e.g. YY1), histone modifying enzymes (e.g. EZH 2) and other chromatin associated factors with chromodomains that recognize repressive chromatin marks as H3K27 me3 (e.g. PC). TrxG genes include products as ATP-dependent remodelers (e.g. Brahma), transcription factors (e.g. GAGA) and histone lysine methyl transferases (e.g. Ash1) [39]. These proteins are components of different complexes that stabilize either an open or closed chromatin conformation.

How do PcG/TrxG proteins are recruited to their chromatin target sites is still a prevailing question. In *Drosophila*, PcG proteins are localized to DNA elements named PREs (Polycomb Response Elements) located kilobases away from PcG target genes promoters. PREs are necessary in order to conserve the heritable chromatin repression exerted by PcG complexes. However, how do PcG proteins get to PREs and how do they repress their final target genes is currently unknown. Furthermore PRE sequences have not been found in mammalian organisms.

Current models propose PcG recruitment through the interaction with DNA-binding factors and further silencing stabilization through chromodomain containing proteins that associate with H3K27me3 modified nucleosomes [42]. However, evidences have emerged showing that PREs are nucleosome poor sequences and that PcG proteins can interact with unmodified histones *in vitro* [43, 44]. Thus, further research is needed to understand how these complexes are localized to their target genes and maintain their chromatin- mediated repression in different organisms.

Even less understood is the mechanism by which TrxG proteins are recruited to their final targets in the chromatin template. Some recent evidences have implicated a RNA-based pathway in order to initiate Ash1 (MLL in mammals) recruitment to their target promoters and intergenic transcription to preserve the permissive chromatin status [45].

Balance between heterochromatin and euchromatin is a key factor to conserve genome stability and cell functional integrity. Alterations in PcG and TrxG proteins have been reported in several developmental abnormalities and even cancer. For example, the increase in EZH2 and/or MLL is associated with increased risk of breast cancer, multiple myeloma or leukemia and prostate cancer [46, 47].

In summary, PcG and TrxG proteins are critical epigenetic modulators that participate in vital cell functions from keeping stem cell pluripotency, to specifying cell fates and balancing the chromatin status of the eukaryotic genome.

Non-coding transcripts and RNA interference

Eukaryotic and prokaryotic genomes are composed of DNA sequences some of which encode proteins and some of which does not. Eukaryotes have large abundance of non-coding sequences that are transcribed and perform diverse functions as RNA molecules (43% in the human genome) [48]. Besides transference RNA (tRNA) and ribosomal RNA (rRNA) there is a vast amount of non-coding transcripts that could be implicated in gene expression regulation. In humans, non-coding transcription generates the 98% of all cellular transcripts including intronic RNAs, intronic and exonic RNA from non-coding genes (as rRNA and tRNA) and intergenic RNAs [49].

Intergenic transcription was considered the result of an excess of RNA polymerases activity, however there is increasing amount of evidence that these transcripts have a fundamental role in gene expression regulation at both the chromatin state and the spatial organization of the genome inside the nucleus [50]. One of the most conserved examples of non-coding RNAs, are small RNAs. Two main types have been broadly studied, interference RNAs (RNAi) and microRNAs. Both of them are processed and interact with different machinery ultimately leading to gene silencing and chromatin compaction.

RNAi was first described as a conserved host defense mechanism that breaks down dsRNA species into small RNA molecules known as short interference RNA (siRNA). dsRNA is processed by the enzyme DICER and then, the enzymatic complex (RISC) (RNA Induced Silencing Complex) interacts with the siRNAs and degrades the corresponding mRNA. A different mechanism occurs when the siRNAs inhibit translation through post-transcriptional silencing (PTGS).

More recently, siRNA has been implicated in gene silencing at transcriptional level through heterochromatin formation from yeast to mammals and to be a central player in maintaining genome stability [51, 52]. The clearest picture of how intergenic RNAs can contribute to heterochromatin formation comes from studies in *Schizosaccharomyces pombe*. First evidences that linked

RNAi machinery and heterochromatin was that mutants in Ago1 (member of RITS complex) presented segregation abnormalities. Subsequently it was found that mutants in all Dcr1, Rdp1 and Ago1 lead to defects in heterochromatin formation at centromeres and loss of H3K9me at the mating-type locus [53]. The proposed model is that intergenic transcription promotes dsRNA processing by DICER generating siRNAs that interact with RITS complex. siRNA-RITS complex recruits RDRC (a complex composed by an RNA dependent RNA polymerase and other proteins) to the nascent transcript. Silencing propagation seems to be mediated by Clr4 (Suv-3-9h) interacting with RNA Polymerase II (Pol II). This interaction promotes H3K9 methylation by Clr4 as transcription progresses. H3K9 methylation provokes Swi6 (HP1 in mammals) binding and hence heterochromatin formation [53]. siRNAs interact with other remodeling machineries promoting DNA methylation of repetitive sequences in telomeres and centromeres. In the case of *Tetrahymena* siRNAs are implicated in DNA elimination. After conjugation of two organisms a new macronucleus is formed and the old one is eliminated through a complex recruited by RNA molecules [54].

Several evidences have accumulated supporting the idea that intergenic transcripts are also involved in chromosome dynamics, chromatin domain organization, epigenetic memory and imprinting [53]. Long non-coding transcripts direct X inactivation in mammals and X activation in *Drosophila*. In both cases the non-coding RNA interacts with remodeling factors and histone modifying enzymes in order to generate heterochromatin and euchromatin, respectively. In addition non-coding transcription has been implicated in contributing to form active chromatin domains *in vivo*. Such is the case for the human β-globin gene locus in which intergenic transcripts all along the domain have been reported to play a critical role in preserving the open chromatin conformation of the domain when globin gene expression is needed [55].

In conclusion, the RNA molecule is widely involved in different epigenetic mechanisms that range from maintaining repetitive sequences silenced and compact to avoid incorrect recombination events, degrading unwanted mRNAs, and acting at the domain or chromosome level in order to promote an open or closed chromatin conformation.

Cell nucleus and epigenetics

For many years cell nucleus was thought to be a passive genome container. In the last decade, evidences from different groups have dramatically changed this notion and has positioned the cell nucleus architecture as a key epigenetic factor involved in almost all genomic

regulatory processes. Chromatin inside the nucleus appears to be organized in chromosomal territories (CT) [56]. CTs are observed in interphase nucleus where each chromosome occupies a defined three-dimensional space. Differences in the compaction levels of autosomal CTs have been reported among several chicken haematopoietic cell lines, reflecting distinct differentiation stages [57].

The initial proposal of this model suggested that chromosome territories surfaces were rather smooth and separated by interchromosome domains (ICD). Genes localized to the periphery of the CT in order to approach the machinery in ICDs such as nuclear speckels, nuclear bodies and transcription factories (see below). These view has know turn into a more complex landscape in which CTs resemble a “chromatin sponge” with DNA free channels interspersed in between like lacunae. This space has been named interchromatin compartment (IC), which eliminates the “interchromosome” localization idea of ICDs [58]. The observation of different CTs has shown that chromosome distribution inside the nucleus depends on genic density having gene-reach chromosomes localized to the nuclear center and gene-poor chromosomes to the nuclear periphery. Additionally the CT-IC model suggests that nuclear architecture is highly dynamic where genes within a chromosome territory are localized differently depending on their expression levels. Coding sequences and their regulatory elements must relocate to the CT periphery and loop out to the IC in order to interact with transcriptional machinery (or others depending on the process) and be correctly expressed [58].

Simultaneously to the understanding of chromosome territories inside the nucleus and their link with the regulation of distinct epigenetic processes, nuclear specific compartments containing different molecular machineries were described. An outstanding example is the notion of “transcription factories”. Microscopic analysis of transcriptionally active sites in HeLa cells nucleus revealed ~10^4 sites of ongoing transcription, 8000 of them containing RNA Pol II and the rest RNA Pol III. These clusters measure ~80 nm and where named transcription factories [reviewed in 59]. More recently, a series of three-dimensional techniques have provided major insights into transcription factories function. Using a combination of these methods, Dr. Peter Fraser’s group has shown that active genes can localize to the sites of active transcription (and not vice-versa) and furthermore, that widely separated active genes can co-localized in one factory [60]. Moreover it has been shown that factories remain in the absence of transcription arguing in favor of a more “stable” structure than previously recognized [61].

How does DNA template gets into transcription factories is still under investigation. Several proposals have been made. One possibility is that RNA

Pol II gives the driving force in order to pull in its DNA template. Alternatively molecular motors as nuclear actin and myosin can be involved in recruiting sequences into factories. Finally, intergenic transcription at regulatory elements has been proposed as a critical step to set the factories gears into motion [reviewed in 59]. More recently, transcription initiation and elongation seem to be responsible for the relocation of DNA templates to transcriptional active foci [61]. For all the previous, it has become indispensable to analyze all epigenetic processes into the light of nuclear architecture and its impact on gene regulation.

Based in all this background it is not surprising that epigenetic processes are involved in several aspects of development, going from the early to the late stages of an organism life. In the next section we will address some of the most important epigenetic features.

II- Epigenetic reprogramming

Maternal and paternal genomes undergo a dramatic epigenetic reprogramming after fertilization. Gametes are terminally differentiated cells that are capable of initiating all the genetic and epigenetic processes necessary for a new organism development [62, 63]. Immediately after fertilization both maternal and paternal genomes suffer a progressive and generalized DNA demethylation by until now an unclear mechanism (Fig 2) [32]. However, imprinted genes are an exception to this global demethylation process keeping intact their parental epigenetic imprints. This allows specific mono-allelic expression of a selected group of genes required for the earliest stages of development (Fig 2) [29]. At the blastocyst stage, in particular in the inner cell mass of the blastocyst, differential patterns of *de novo* DNA methylation occurs in tight association with cell differentiation programs that give rise to the entire organism [33]. During early embryo development, both the *de novo* (Dnmt3a and Dnmt3b) and maintenance (Dnmt1) DNA methyltransferases are needed to establish proper post-blastocyst patterns of DNA methylation [64]. Why is so important to erase, with the exception of the imprinted genes, all DNA methylation patterns? One possibility comes from the need to erase some of the epigenetic marks coming from the inherited gametes. Such scenario may certainly allow, at the genome scale, the desilencing of the great majority of genes, facilitating the coordinated establishment of genetic and epigenetic programs early during cell differentiation. This model is consistent with the need of complementary epigenetic processes like histone modifications, the action of PcG proteins, and ATP-dependent remodeling complexes during early embryogenesis [65]. For example, H3K27me3, a characteristic repressive PcG-dependent

modification, has been found co-localizing with the H3K4me3 chromatin open mark in ES cells [66, 67]. This co-localization suggested the term “bivalent chromatin” distribution on embryonic stem cells referring to a chromatin state capable of rapid activation or repression of specific group of genes in response to precise developmental cues. For all the previous observations, it seems that DNA methylation and other epigenetic patterns should be transmitted from cell to cell ensuring the integrity of each genomic program in post-implantation embryos in order to achieve a proper embryonic development.

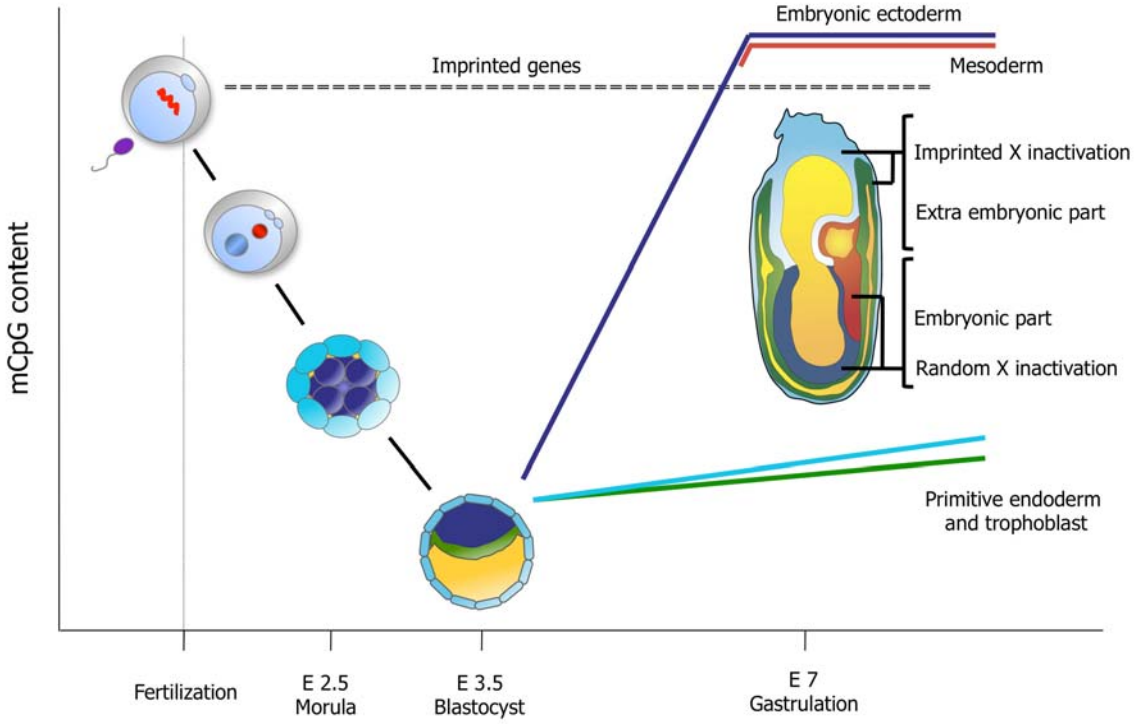

Figure 2. CpG methylation status during early mouse development. After fertilization the bulk genome undergoes demethylation. The lowest level of demethylation is reached at the preimplantation blastocyst stage at E 3.5. After implantation the whole genome becomes *de novo* hypermethylated in embryonic ectoderm and mesoderm (blue and red respectively) whereas the genome of extra-embryonic cells so as the primitive endoderm (green) and throphoectoderm (Light blue), remain hypomethylated. The parental imprinted genes escape demethylation. X inactivation is imprinted in extraembryonic tisuues and random in embryonic ones [modified from 20].

III- Embryonic development: Epigenetics of the zygote

One of the most important features of post-fertilization and pre-implantation stages, are the zygote totipotency and the embryonic stem cells pluripotency, respectively. The zygote is a totipotent cell meaning that it can give rise to the whole embryonic and extra-embryonic tissues of the

organism. This cell divides forming blastomeres (34-64 cell stage), which retain totipotency but lack self-renewal capacity. In contrast in a later stage of development the cells originated within the inner cell mass of the blastocyst are capable to self-renew when cultured and are pluripotent, meaning that they can differentiate into every cell type of the embryo but not to extra-embryonic tissues. This takes place through the interdependency of genetic and epigenetic information that specify distinct differentiation profiles.

In addition, in the post-implantation embryo the pluripotent epiblast cells differentiate into somatic and pluripotent germ cells [68]. This latter case is very interesting since activating or repressive signals are needed for pluripotent epiblast cells to undertake a differentiation decision to establish a somatic cell program or to maintain the pluripotency identity as a germ cell (Fig 3) [65]. Evidences coming from different studies have brought to conclude that development and cell fate acquisition requires coordinated action between genetic and epigenetic instructions to activate or repress specific set of genes.

The zygote and its totipotency

Maternal and paternal epigenomes are surprisingly different. For instance, the paternal genome is structured in a highly compacted arrangement, through the incorporation of protamines, which are rapidly replaced by histones. Then, one of the earliest epigenetic events is the incorporation of the histone variant H3.3, also associated to active transcription sites into the genome [69]. As mentioned before, the zygote genome becomes rapidly demethylated in association with diverse patterns of histone post-translational modifications (Fig 2). From the maternal point of view, the zygote receives a well defined heritage composed by chromatin remodeling components as the PcG proteins EZH2 and EED among others, Brg1 that is a sub-unit of an ATP-dependent chromatin remodeler and key transcription factors associated to the pluripotency like Oct3/4 and Sox2 [70]. In fact, Brg1 loss can cause zygote arrest at the two-cell stage [70]. In addition, the epigenetic identity of the zygote is gradually reached with the progressive acquisition of H3K9me2 chromatin mark [71, 72]. All these epigenetic changes are critical for the determination of zygote's totipotency needed for the establishment after a finite number of cell divisions, of the pluripotent embryonic stem cells and primordial germ cells (see below).

Later the epigenetic environment that is generated within the zygote activates the synthesis and function of critical transcription factors for blastocyst development. Oct4 and Cdx2 are two of the most important factors needed for inner cell mass (Oct4) and outer cells (Cdx2) development [73]. Another key factor is Nanog which is a homeodomain protein whose function is restricted to the inner cell mass [74, 75]. The epigenetic requirements at this early

developmental stage are exemplified by *nanog* expression regulation by Carm1 that is a histone-specific arginine methyltransferase [76]. Based on stage-specific synthesis of regulatory factors and epigenetic modifications, cell fate decisions are progressively made until the identity of the pluripotent embryonic stem cells is reached in the blastocyst at E3.5. Then, dramatic epigenetic differences are seen between the inner cell mass and trophoectoderm cells in the blastocyst at both the histone post-translational modifications and the incorporation of different DNA methylation patterns (Fig 3). DNA methylation occurs only in the ES cells, instead in the trophoectoderm, the genome stays basically hypomethylated [65].

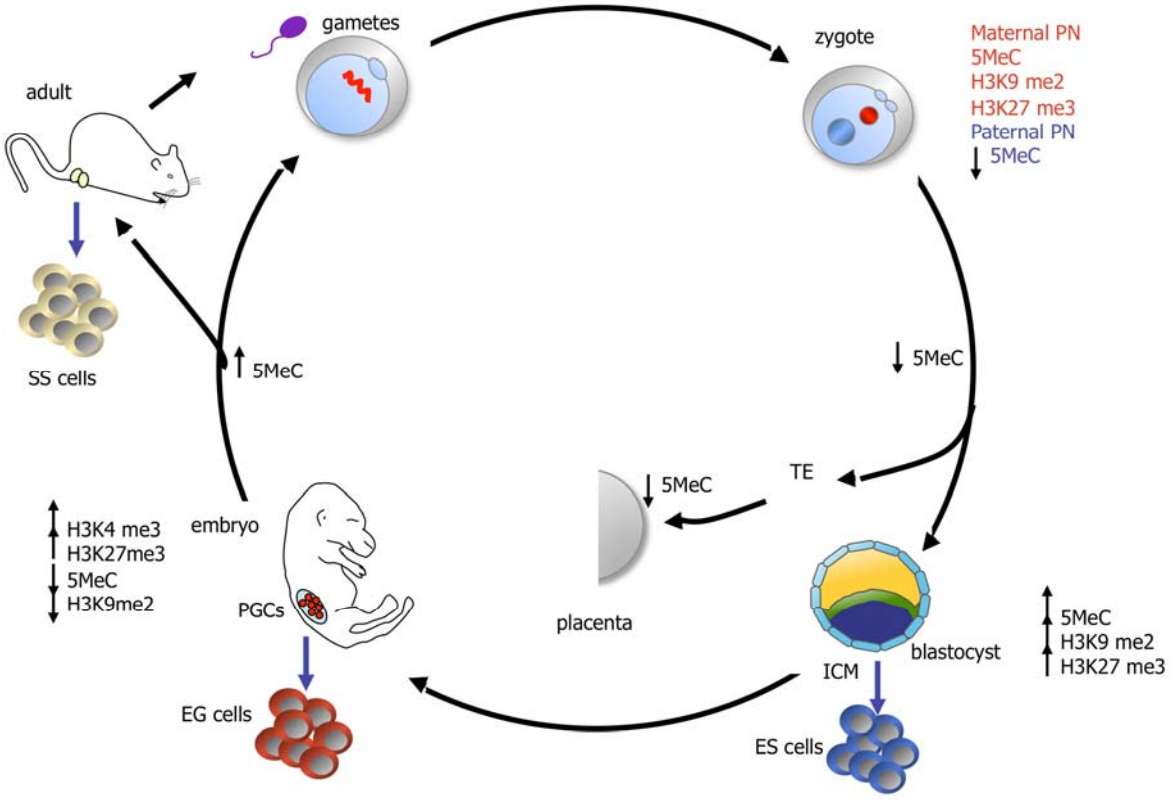

Figure 3. Epigenetic reprogramming in mammalian development and pluripotent cells derivatives. After fertilization the paternal and maternal chromatin are differently packed. The maternal chromatin contains H3K9me2 and H3K27me3 and 5MeC. The paternal chromatin lacks these histone modifications and rapidly loses DNA methylation. Passive loss of 5MeC occurs until the blastocyst stage. Then, the inner cell mass (ICM) starts to acquire high levels of all three marks. The trophoectoderm (TE) derived tissue (placenta) remains hypomethylated. PGCs undergo 5MeC and H3K9 demethylation. At later stages de novo methylation including parental imprinting occurs in germ cells. Pluripotent cell lines can be obtained from the ICM, PGCs and spermatogonia stem (SS) cells [modified from 39].

The pluripotency of the embryonic stem cells

It has been proposed that the inner cell mass represents a "niche" where signaling molecules from the surrounding cells converge and contribute to the pluripotency of the embryonic stem cells. Furthermore, those signals may contribute to a general euchromatinization of the ES cells epigenome leading to the plasticity needed for pluripotency (Fig 4). It is well established that Oct4, Nanog and Sox2 are critical transcription factors necessary for the activation and repression of a large number of target genes contributing to the pluripotency of ES cells [77]. From the chromatin structure point of view the ES cells are highly dynamic with a generalized relaxed organization that tends to compact during most differentiation pathways (Fig 4). Also and as previously mentioned, ES cells present "bivalent" histone marks [66, 78]. Interestingly, these "bivalent" domains frequently overlap genomic regions in which the binding sites for Oct3/4, Sox2 and Nanog are clustered [79]. Then this two features of ES cells chromatin, the "bivalent" domains and the association of key transcription factors, may participate in the establishment and maintenance of pluripotency on the one hand, and in the ability of ES cells to respond to differentiation signals in order to acquire specific cell identities on the other. In addition to the histone H3K27me3 repressive mark co-existing with the H3K4me3 (bivalent domain), two other members of the Polycomb PRC2 complex, Esd and Suz12, are also present on ES cells chromatin and it has been suggested that they are ready to induce repression of a subset of genes as soon as the ES cells start to undergo differentiation [77, 80, 81]. Furthermore, mutations on epigenetic regulators including EZH2, Eset, MBD3 and Dicer, perturb the pluripotency of ES cells [65, 82].

An unexplored epigenetic aspect on ES cells is the role of the recent discovered reversible status of histone methylation through the action of the LSD and Jumonji domain-containing histone demethylases [83]. The window of possibilities is now bigger since in ES cells, histone methyltransferases and demethylases can coordinate their action to keep an undifferentiated state or make decisions leading to the activation of specific differentiation programs. According to this, recent work has shown that when *Jmjd1a* is knocked-down in ES cells it causes the activation of a differentiation pathway with an increase in H3K9me2. In contrast, depletion of Jmjd2c induces differentiation with a global increase in H3K9me3 [84].

On ES cells, domain specific chromatin conformations can also be reached by the incorporation of histone variants as part of a pluripotent "bar-code" as proposed by Allis [8]. This hypothesis suggests that the relative amount of histone variants (in particular of H3), defines genomic domains with particular epigenetic features. In addition, the different characteristics of

histone variants contribute to determine nucleosome stability and thus to the flexibility of chromatin fiber [5]. This has been suggested on the basis of mutants of different histone chaperon components (HirA and CAF-1) that bring aberrant consequences in ES cells pluripotency [85, 86, 87].

Another aspect associated to ES cells epigenetics is the participation of ATP-dependent chromatin remodeling complexes, in particular, the case of NURD (nucleosome remodeling and histone deacetylation complex) and its components MBD3, that are required *in vitro* for differentiation of ES cells [88].

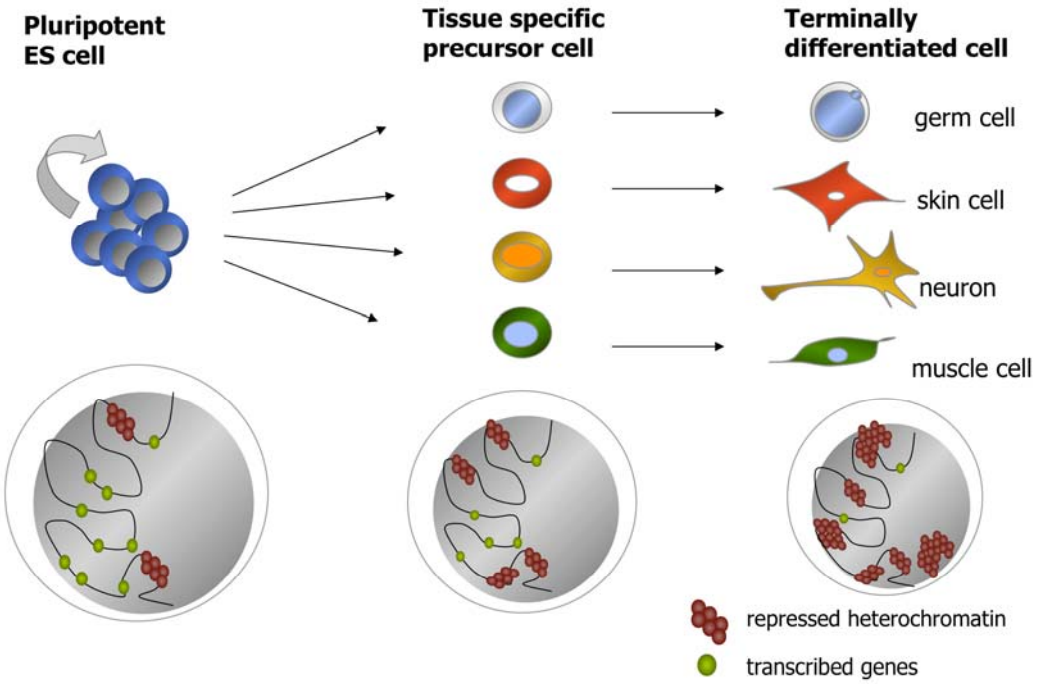

Figure 4. Chromatin status of ES cells and its derivatives. ES cells are pluripotent stem cells able to give rise all embryonic cell types. ES cells contain an "open" chromatin conformation with mostly euchromatin. As differentiation proceeds heterochromatin starts to be more abundant and finally in the majority of terminally differentiated cells, chromatin is mostly compacted and just some regions are still permissive.

Epigenetic regulation of germ cells

At the post-implantation embryonic stages E6-E6.5 epiblast cells remain pluripotent and have the capacity to generate all somatic tissues including germ cells. In contrast to the action of key transcription factors at the early blastocyst stage, at the post-implantation time, the establishment of programmed repressive procedures is needed. Different processes that include DNA methylation, histone methylatransferase activities, repressors

and co-repressors and non-coding RNAs achieve this repression in order to guide the transition of proximal epiblast cells to primordial germ cells (PGC), which will give rise to germ cells (sperm and oocytes) in the adult organism (Fig 3).

PGCs are capable to sense and respond to extra-embryonic signals to undergo differentiation. These cells are highly specialized and distinct to ES cells since they are incapable to create chimeras when introduced into blastocysts but they retain particular pluripotent characters. PGCs originated from the proximal epiblast generate germ cells and direct specific responses to block somatic programs, in particular through the action of the transcriptional repressor Blimp1 [65, 89, 90]. Deficient *Blimp1* cells cause aberrant development of PGCs and the cease of cell proliferation.

From the epigenetic point of view, Blimp1 can interact and form complexes with several remodeling complexes including the histone methyltransferase G9a, HDAC2 and the arginine-histone methylatransferase Prmt5 [91]. Therefore the covalent modifications H2A and H4R3me2 seem to be relevant for PGC formation and germ cell specification [65]. Consistent with the role of Prmt5, mutation of its *Drosophila* homologue, Casuleen/daut5, causes dramatic deleterious effects on germ cells [92,93]. Then, one key epigenetic aspect of the germ cell lineage is the maintenance of some of the features of pluripotency therefore, the great majority of the epigenetic processes should focus on the generation of such specific type of pluripotency. Accordingly, it has been proposed that PGCs have some of the zygote and some of the ES cells epigenetic features. In the future it will be interesting to decipher which are the epigenetic programs that are exclusive to the germ line.

IV- Genomic imprinting during development

Genomic imprinting is the phenomenon associated to the differential gene expression of paternally and maternally inherited alleles. Imprinted genes are often required for tissue and developmental processes during early development [29]. Notoriously, imprinted genes are distributed in clustered domains that often include both paternally and maternally silenced genes. Mono-allelic gene expression is in part determined by parental epigenetic marks defined during gametogenesis [94]. Importantly, imprinting is not present in all organisms and it has been suggested to appear in marsupials around 201 million years ago [35, 95].

One key component of imprinted loci that is tightly associated to epigenetic regulation, is the allele differentially marked *cis* element defined

as the imprinted control region (ICR), also named as differential methylation domain (DMD) [29]. It is now well established that differential DNA methylation of paternal or maternal alleles at the DMD is one of the key processes leading to mono-allelic expression of imprinted loci. In the present chapter we will not go through all the aspects associated to genomic imprinting, instead we would describe the latest epigenetic mechanisms associated to allele-specific gene expression.

CTCF and imprinting mechanisms

The *Igf2/H19* locus has been the paradigm of imprinted domains. In this domain, the fetal growth factor insulin-like growth factor 2 gene (*Igf2*) is paternally expressed and the *H19* gene is maternally expressed. Both genes are located around 100 kb from each other and are regulated by enhancers downstream of the *H19* gene (Fig 5) [29]. These genes are mainly expressed in mesodermal, endondermal and extraembryonic tissues in the developing fetus, with a downregulation around 3 weeks of postnatal development. Between *Igf2* and *H19* genes there are 2 kbs corresponding to the imprinted control region. The DMD is DNA methylated from the paternal derived germ line, in contrast to the unmethylated state on the maternal allele. The differential DNA methylation of the DMD is essential to achieve monoallelic gene expression. Interestingly at the maternal allele, the DMD is acting as an enhancer-blocking element that is dependent on multiple CTCF binding sites [97]. CTCF is exclusively bound to the maternal allele and its binding is sensitive to DNA methylation. Therefore, when CTCF is binding to the DMD, the downstream enhancer only trans-activates the *H19* gene, while in the paternal allele, the DMD is methylated, CTCF can not bound and then the enhancer now acts over the *Igf2* gene (Fig 5).

More recently, Reik and collaborators demonstrated that in addition to the enhancer blocking mechanism, there is differential formation of chromosome loops that explain allelic-specific expression at the *Igf2/H19* locus [98]. This was performed through the application of a recent developed method known as chromosome conformation capture (3C). On the maternal allele the unmethylated *H19* DMD, which is bound by CTCF, interacts with the *Igf2* DMR1 (Fig 5). This conformation results in the formation (by looping) of two topological independent chromatin domains, with the *H19* gene in an active domain where the enhancers can activate its promoter. In contrast, the *Igf2* gene is placed on an inactive domain unable to be activated by the enhancers (Fig 5) [98]. Then, the *Igf2/H19* imprinted locus exemplifies how higher-order chromatin structure can actively participate on epigenetic imprinting regulation.

Surprisingly, interchromosomal interactions are not only involved in regulating gene expression in *cis* [59]. Recent observations demonstrate that the epigenetic role of the DMD of the *Igf2/H19* locus located on the murine chromosome 7 interacts in *trans* with the imprinted locus *Wsb1/N*f1 on chromosome 11 [99]. Interstingly, and supporting its topological role, those interchromosome interactions are dependent on CTCF-binding to the maternal allele. This is consistent with the regulated loop formation at the murine β-globin locus in which CTCF mediates the long-range contacts and three-dimensional conformation of the locus [100]. Then, the epigenetic processes occurring in early stages of development do not only require the action of highly specific transcription factors; long-range contacts, chromatin fiber topology and nuclear dynamics are also participating as epigenetic mechanisms that control different networks of gene expression.

In addition to coding transcription, a large portion of the genome composed by non-coding sequences, are constantly transcribed. Then, a vast variety of non-coding RNAs (ncRNAs) represent a common feature of mammalian gene regulation with consequences during development. The classical example of ncRNA is the *Xist* RNA, which is responsible for the initial epigenetic steps leading to X-chromosome inactivation in female cells [101]. Today, there are clear evidences showing that long ncRNAs participate as well, on the silencing of imprinted genes. A growing list of imprinted loci is emerging including the *Igf2/H19*, *Kcnq1*, *Gnas*, *Air* (antisense of *Igf2*) and others [102]. It has been proposed that in some way the imprinted control regions may have something to do with the regulation and expression of ncRNAs. In all the cases, the non-coding RNAs are acting through epigenetic silencing but the mechanisms are still not clear [102, 103]. Several models can be proposed to explain the mechanism of action of this kind of RNAs; for example, the participation of the RNA interference machinery to induce repressive chromatin conformation (a model that requires the formation of double-stranded RNA intermediates) [104]. An alternative model could come from the influence of transcription of ncRNAs affecting imprinted genes in an allelic-specific manner by either opening or closing the chromatin structure. In fact in both cases we can imagine that ncRNA transcription is contributing to the formation of an open chromatin configuration that can be of more easy access to regulatory factors, either positive or negative.

In summary, imprinting regulation and its influence during early stages of development is clearly a complex process. Despite the large amount of knowledge generated around imprinting there are novel and exciting results clearly illustrating the participation of epigenetic mechanisms on the regulation of imprinted loci with a determined role during development.

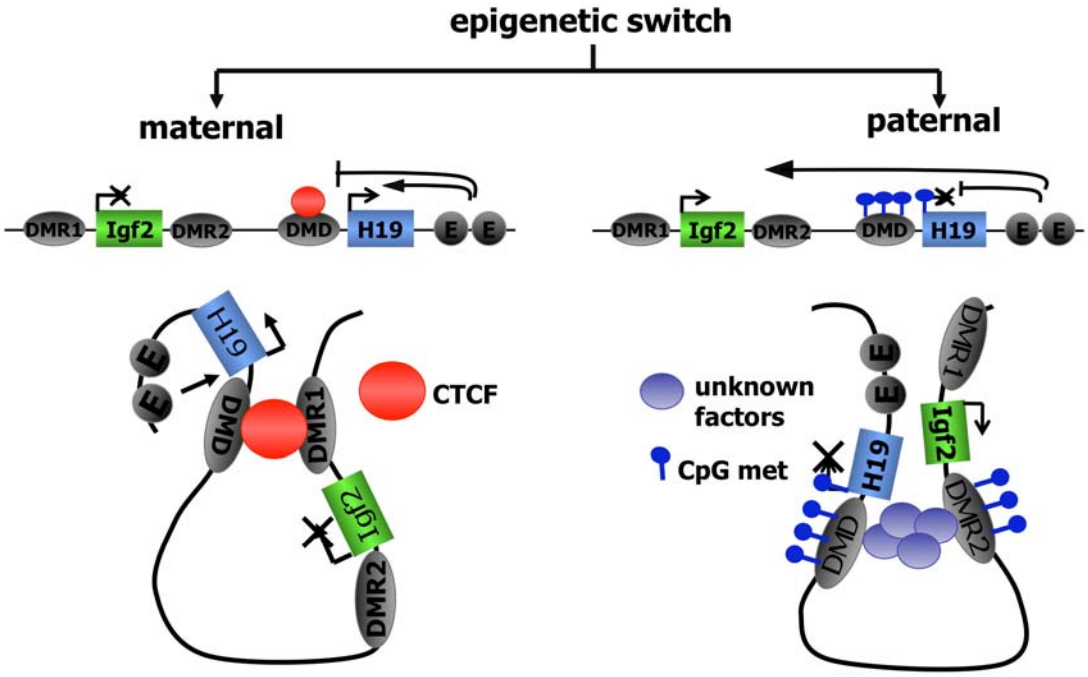

Figure 5. *Igf2/ H19* locus. In the maternal allele CTCF binds the unmethylated DMD and the DMR1 forming a loop that excludes *Igf2* gene from the enchancer's activation so that only *H19* is activated. In the paternal allele, the DMD is methylated so CTCF cannot bind. Therefore, a different loop is formed in which the enhancer can trans activate *Igf2* gene. *H19* gene is silenced by DNA methylation on its promoter [modified from 96].

V- Senescence

The development of an organism is a progressive process and after reaching adulthood another progressive process begins that leads to senescence and ageing. Ageing may be understood as the decay in the regenerative capacity of tissues and these phenomena is directly linked with a change in the progenitor cells of each tissue. Based on such view, cellular senescence in an adult organism can be a caused by the combination of the physiological loss of regenerative competence and the unbalance of tissue homeostasis that lead to cell ageing.

Cellular senescence can be described as a state of permanent and irreversible cell cycle arrest with no capacity of response to serum or growth factors. Senescent cells exhibit a large and flat morphology and are positive for the senescence-associates β-galactosidase assay. It is known that p53 and retinoblastoma (Rb) tumor suppressor genes participate in the onset and maintenance of the senescent cell state [105]. However, the precise mechanisms are still not well understood.

As mentioned, a currently unsolved question resides on which are the processes that lead to cell senescence and ageing. Different evidences have emerged arguing that senescent cells present distinctive epigenetic features thus making the epigenome analysis an appealing start point to look into senescence. As cells go through senescence, heterochromatin foci called senescence-associated heterochromatin foci (SAHF) are formed [106]. In human senescent cells there are between 30 and 50 SAHFs representative of highly compacted chromosomes [107]. These foci consist on heterochromatic spots in which histones are hypoacetylated, there is an enrichment of H3K9me3 and incorporation of the heterochromatin protein 1, HP1. In addition, SAHFs are depleted of linker histone H1 and the present deposition of the histone variant macroH2A and the high mobility group A (HMGA) proteins [for review see 108]. It has been proposed that SAHFs must represent the repressive foci in which proliferative promoting genes are silenced in senescent cells [109].

Senescence represents an attractive scenario to understand certain epigenetic mechanisms linked to tumor suppressor processes. Conceptually, a senescent cell can be viewed as incapable to respond to mitogenic stimuli thus presenting anti-proliferative properties and SAHFs are important structures in order to maintain a this state. Therefore and to certain extent, understanding the genetic and epigenetic mechanisms of senescence could give rise to alternative therapeutic strategies to obstruct malignant cell proliferation in cancer.

Discussion and prospects

Epigenetics comprehend a broad range of processes involved in every aspect of organism development, life and ageing. One of the most outstanding evidences about the importance of epigenetics during early stages of development and cell fate determination has emerged from the examination of cloned mammalian embryos [110]. A specific study revealed drastic differences on H3K9 methylation and global DNA methylation when comparing a zygote from cloned and a wild-type bovine [110]. This observation clearly demonstrates an incomplete reprogramming of epigenetic marks and that such marks are critical for preimplantation embryos, their development and adulthood. An intriguing aspect that has yet to be resolved is to understand the epigenetic mechanisms occurring at the developmental transition between pre-implantation and post-implantation. Particular attention is needed to unravel the genetic and epigenetic programs that maintain the undifferentiated state of ES cells and the initial steps they take towards the establishment of particular differentiation programs.

The interdependency between genetic and epigenetic mechanisms requires from the genetic perspective, the expression of specific network of transcription factors that blocks differentiation and promotes continuous self-renewal capacity of ES cells. From the epigenetic point of view, those transcription factors and their associated co-factors should have the responsibility to attract chromatin remodeling activities that allows the construction of the particular scenario (open and close chromatin conformation) for self-renewal and differentiation of ES cells.

More over, the understanding of epigenetic processes involved in cell senescence, ageing and disease may provide a new platform to better approach therapeutic protocols. A characteristic feature of epigenetic defects is that apparently, and the majority of the data supports this idea, they tend to be acquired in a progressive way rather than abruptly as compared to genetic ones. In the next future, these must be capitalized in terms of improved strategies for early diagnosis in diverse pathologies as cancer.

Acknowledgments

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