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Epiblast-derived stem cells in embryonic and adult tissues

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ABSTRACT Pluripotent cells can be isolated from the mammalian inner cell mass (ICM) of the embryo at the blastocyst stage, and maintained in culture as undifferentiated, embryonic stem cells (ES). These cells are an important model of mammalian development *in vitro* and are the focus of a great deal of research for their use in Cell Therapy. *In vivo*, shortly after the blastocyst stage, the ICM segregates into two layers: the hypoblast which will give rise to the yolk sac, and the epiblast. Epiblast stem cells, like ES cells, are pluripotent. The epiblast will differentiate very early into germ cell progenitors, the primordial germ cells (PGC). PGCs can give rise to embryonal carcinoma cells, the pluripotent stem cells of testicular tumors. During normal embryo development, PGCs migrate into the aorta-gonad-mesonephros region (AGM). Interestingly, this region also harbors the first wave of embryonic hematopoiesis. Subsequent waves of hematopoiesis involve AGM-hematopoietic stem cell (HSC) colonization of the fetal liver, thymus, spleen and ultimately, for adult hematopoiesis, the bone marrow (BM). The BM is also source of mesenchymal stem cells (MSCs). It is accepted that the AGM region cells give rise to the mesothelial cells which are the embryonic precursors of the HSC and MSC of the BM. Recent identification of a subpopulation of cells with markers typical of PGCs in the adult BM, which are capable of differentiating into HSCs, suggests that HSCs originate from a common precursor of PGCs and HSCs derived from the epiblast. Several groups have described the presence of stem cells with the same markers in epidermis, bronchial epithelium, pancreas, retina, hair follicle, heart and dental pulp among, other organs. This presence supports the hypothesis that during early development, epiblast/germ line-derived cells are deposited in various organs which persist into adulthood. The question remains whether these pluripotent stem cells are only developmental remnants or if they continuously contribute to the renewal of tissues, and thus can be reactivated for tissue regeneration without the need for stem cell transplantation for human cell therapies.

KEY WORDS: *cell therapy, primordial germ cell, hematopoietic stem cell, mesenchymal stem cell*

Embryonic origin of the epiblast

After fertilization, the single-cell zygote divides four times originating the 16-cell morula. The morula consists of a small group of internal cells surrounded by a larger group of external cells. Most of the descendants of the external cells become the trophoblast (trophectoderm) cells, which produce no embryonic structures, but instead the embryonic portion of the placenta, the chorion. The embryo is derived from the descendants of the inner cells of the 16 cell-stage morula. These cells generate the inner cell mass (ICM), which will give rise to the embryo and its associated yolk sac, allantois and amnion. Once the commitment to become trophoblast or inner cell mass is made, different genes are expressed by the cells of these two regions.

The first segregation of cells within the inner cell mass forms two layers: the lower layer, the hypoblast which will give rise to the yolk sac, and the remaining inner cell mass tissue above it, the epiblast (Fig. 1). Epiblast stem cells (EpiSC) are pluripotent cells that will give rise to cells of the three germ layers, ectoderm, mesoderm and endoderm. Stage-specific embryonic antigens

Abbreviations used in this paper: AGM, aorta-gonad-mesonephros; BMP, bone morphogenetic protein; EG, embryonic germ cell; EpiSC, epiblast-derived stem cell, ES, embryonic stem cell; HSC, hematopoietic stem cell; ICM, inner cell mass; LIF, leukemia inhibitory factor; MAPC, multipotent adult progenitor cell; MSC, mesenchymal stem cell; PGC, primordial germ cell; PSC, pluripotent stem cell; SSEA, stage-specific embryonic antigen; VSEL, very small embryonic-like.

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(SSEA) have historically been used as markers of pluripotency. Epiblast cells express SSEA-1 (mice) and SSEA-3/4 (human). Distribution of SSEA-1 has been studied in postimplantation murine embryos, fetuses, and adult mice by immunohistochemical techniques. SSEA 1 is first detected in late eight-cell mouse embryos. It is expressed in the embryonic ectoderm, the visceral endoderm and trophoblast in early postimplantation embryos (Muramatsu and Muramatsu., 2004). In the inner cell mass, SSEA-1 expression is weak at an early stage and becomes greater at a later stage (Muramatsu and Muramatsu, 2004). During subsequent development, SSEA-1 becomes localized to portions of the brain and primordial germ cells. In addition, some sites of the urogenital anlagen are SSEA-1 positive. Interestingly, human embryonic cells express SSEA-3 and SSEA-4 instead of SSEA-1 (Henderson *et al.,* 2002). In addition, epiblast cells express *Oct-4* and *Nanog*. *Oct-4* is an embryonic transcription factor that plays a determining role in the specification of mouse pluripotent stem cells (PSC) in the inner cell mass of the blastocyst, and mouse embryos deficient in *Oct-4* are unable to form mature blastocysts (Hay *et al.,* 2004) and die around the time of implantation (Boiani & Scholer, 2005). *Oct-4* becomes downregulated during development as cells are derived to one specific cell line.

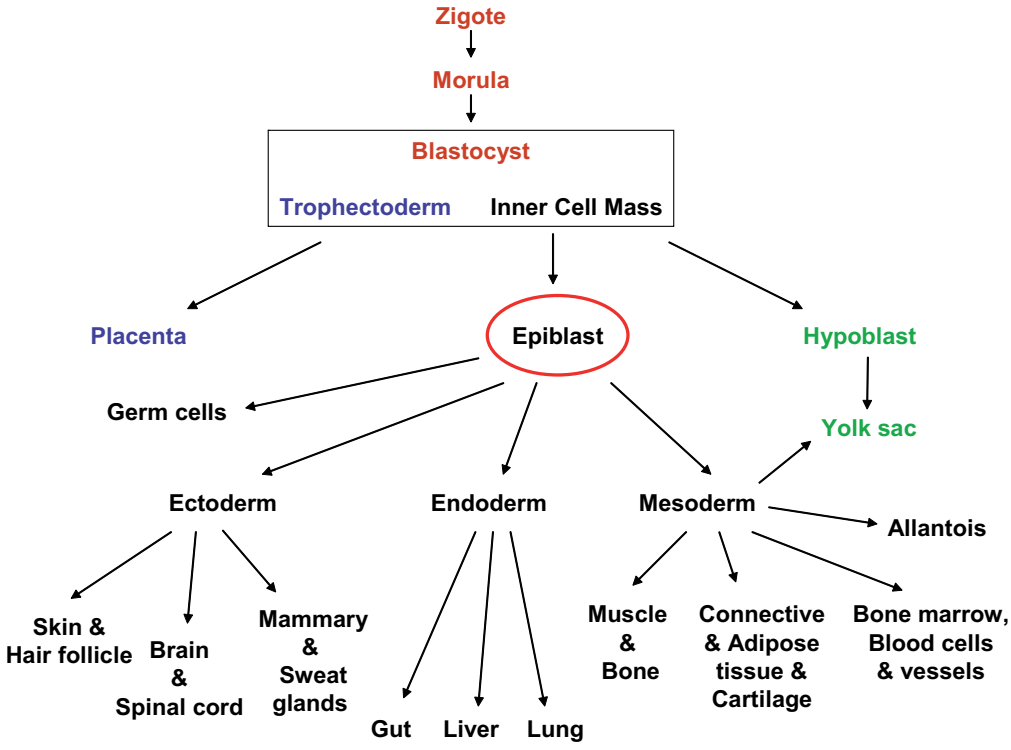

Fig. 1. Diagram showing the differentiation of adult tissues from the various embryonic anlagens.

ICM and ES cells

Pluripotent cells can be isolated from the mouse and human inner cell mass (ICM) of the blastocyst and maintained in culture as self-renewing, undifferentiated, embryonic stem cells (ES cells, Evans & Kaufamn, 1981). These cells are an important model of mammalian development *in vitro* and are the focus of a great deal of research aimed at generating differentiated cell types for Cell Therapy. The markers that have been used to characterize human ES are carbohydrate epitopes on proteoglycans or sphingolipids, such as stage-specific embryonic antigen (SSEA)-3 and -4. The expression of SSEA-3 and -4 is tightly regulated during preimplantation development and on ES cells. Using inhibitors of sphingolipid and glycosphingolipid (GSL) biosynthesis to block the generation of SSEA-3 and -4 in hES showed that depletion of these antigens and their precursors had no effect on their differentiation *in vitro* or in teratoma formation. These experiments demonstrated that the GSLs recognized as SSEA-3 and -4 do not play critical functional roles in maintaining the pluripotency of hESs, but instead suggested roles for this class of molecules during cellular differentiation (Brimble *et al.,* 2007).

Recently, in an elegant review, Thomson (Zwaka and Thomson, 2005), pointed out that it is remarkable that permanent pluripotent embryonic stem cell lines can be derived from preimplantation embryos at all, because, *in vivo*, pluripotent cells of the early mammalian embryo proliferate only briefly before becoming cells with a more restricted development potential. It had been previously suggested that they be called “embryo-derived stem cells”, a more precise term that would distinguish between these new pluripotent cell lines and cells within the embryo (Rossant and Papaioannou, 1984).

Although ES cell lines are generally derived from the culture of the ICM, some experiments suggest that ES cells more closely resemble cells from the epiblast (primitive ectoderm). For example, isolated mouse epiblast gives rise to ES cell lines at a higher frequency than does isolated ICM. Moreover, the culture of epiblast allows the isolation of ES cell lines from mouse strains that have been previously refractory to ES cells isolation (Zwaka and Thomson, 2005; Tesar *et al.,* 2007). However, although these experiments suggest that ES cells are more closely related to epiblast than to ICM, they do not reveal whether ES cells more closely resemble epiblast or a cell derived from it *in vitro* (Zwaka and Thomson, 2005). The expression of *Oct-4* is maintained in only a small proportion of derived ES cells (Buehr and Smith, 2003), which also suggests that only a minority of epiblast cells can transit to a new stable, proliferative pluripotent state, and, subsequently, be expanded as ES cells. These results could be due to an inherent heterogeneity of the primitive ectodermal cell population. In fact, recent data indicate that even the earliest ICM is heterogeneous and consists of a mixture of cells that express either *Oct-4* or *Gata6* (Rossant *et al.,* 2003). Recently, two papers have come to strengthen the hypothesis that human ES more closely resemble epiblast cells (EpiSC) than ICM-derived mouse

ES cells (Tesar *et al.,* 2007; Brons *et al.,* 2007). First, the pattern of gene expression is more similar between mouse EpiSC and human ES than between mouse and human ES. Second, human ES cells and mouse EpiSC depend on the activin/Nodal signaling pathway to maintain their pluripotent status, whereas mouse ES cells rely on leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), as the signaling pathway maintaining pluripotency. Third, mouse EpiSC can not give rise to germ-line transmitting chimeras, as mouse ES do (the germline chimerism of human ES cells is of course untestable). And fourth, epigenetic regulation and stability of human ES cells and mouse EpiSC is more similar between them than to mouse ES (Tesar *et al.,* 2007; Brons *et al.,* 2007). In a recent paper (Chou *et al.,* 2008), new cell lines named FAB-SCs were derived from mouse ICM, by culturing ICM in a cocktail of GF media previously used for EpiSC derivation. FAB-SCs are different both molecular and functionally from ES and EpiSCs. They maintain pluripotency markers such as *Oc-t4*, *Nanog* and *Sox2*, but fail to form embryoid bodies *in vitro*, teratoma *in vivo,* or contribute to chimeras when injected into blastocyst. Interestingly, these pluripotency restrictions can be reverted by *in vitro* exposition to LIF and BMP4.

The epiblast will differentiate very early into germ cell progenitors (Fig. 1). Shortly before the epiblast is about to give rise to all three germ layers (ectoderm, mesoderm, and endoderm), the first morphologically identifiable precursors of PGC in mice become specified at 6.0 to 6.5 days post coitum (dpc) in the proximal part of the epiblast (McLaren, 2003). Thus, precursors of PGC are the first population of stem cells that is specified in the embryo at the beginning of gastrulation. PGC in mice subsequently move for a short period of time first to the basis of the allantois, which is located in the extraembryonic mesoderm and then migrate into the embryo proper toward the genital ridges (see below), where they will undergo developmental differentiation to oocytes or spermatocytes respectively (Molyneaux and Wylie, 2004).

There are several molecular markers that distinguish early germ cells from other pluripotent cells of the early embryo. One marker, tissue-non-specific alkaline phosphatase (TNAP), is strongly expressed by early germ cells and by ES cells, but is weakly expressed by the epiblast and other surrounding embryonic cells (Ginsburg *et al.,* 1990). Two new markers for early germ cells, *Fragilis* (Ifitm3) and *Dppa3* (also know as *Stella* or *PGC7*), have recently been identified, which allow the better separation of early germ cell precursors from their differentiated neighboring cells (Saitou *et al.,* 2002). Several recent reports describing the differentiation of mouse ES cells into cells that express markers of mature male and female germ cells (Hubner *et al.,* 2003; Geijsen *et al.,* 2004) are important for our understanding of the origin of ES cells. In each of these reports, germ cell markers were expressed by ES cells themselves, including those, such as *Dppa3*, which help distinguish germ cells from primitive ectoderm. Only the expression of more mature germ cell markers (such as *mouse vasa homolog*, *Mvh*) enabled *in vitro*-derived germ cells to be distinguished from ES cells themselves. In one study that examined the differentiation of human ES cells into germ cells (Clark *et al.,* 2004), the expression of each of eight genes characteristic of early germ cells was detected in human ES cells, but the expression of each of six genes that are characteristic of later germ cells was not detected. Using immunocytochemistry, it was also shown that most individual human ES cells in a population express the early germ cell markers *Stella*-related (*StellaR*) and deleted in azoospermia-like (*Dazl*), indicating that a minor subset of randomly differentiating cells in a mixed population is not responsible for the expression of germ markers in ES cell cultures. Importantly, it was also shown that at least one germ cell-specific gene, *Dazl*, was expressed by human ES cells but not by human ICM (Clark *et al.,* 2004). The existing gene expression data are consistent with the idea that the closest *in vivo* equivalent to ES cells is not the ICM or even the epiblast, but an early germ cell (Zwaka and Thomson, 2005).

Some of the properties of ES cells, however, suggest that they are not merely the equivalent of early germ cells. For example, the earliest PGC do not self-renew for prolonged periods of time, but instead follow a series of differentiation steps, beginning with germ cell migration and ending in the highly morphological specialization of sperm or egg (Wylie, 1999). Also, although ES cells can differentiate into more mature germ cells *in vitro*, they do so relatively inefficiently. In addition, isolated PGCs have never been demonstrated to contribute to chimeras when injected into blastocysts, so an exact equivalence to ES cells is unlikely (Zwaka and Thomson, 2005).

From ICM to AGM

Clonal analysis of the lineage potency of epiblast cells revealed that some cells in the proximal epiblast of the pre-to-early streak stage embryo can give rise to SSEA and TNAP-expressing cells that colonize the allantoic mesoderm and the hindgut endoderm. The location of PGC precursors in the proximal epiblast suggests that only epiblast cells in this region of the early gastrula may possess germ-line potency. However, cells that may contribute descendants to the PGC population are not localized specifically to any area in the proximal epiblast. *Oct-4* expression is widely localized in the epiblast of the early gastrula and shows no regionalization to the proximal epiblast (Pesce *et al.,* 1998).

The lack of a pre-determined population of PGC progenitors implies that germ-cell formation is unlikely to be restricted to subsets of epiblast cells. This idea has been elegantly verified by a series of experiments testing the ability of epiblast cells that are localized outside the proximal epiblast to form PGCs. Distal epiblast cells that normally display a neuroectodermal fate transplanted to the proximal region of the epiblast were able to differentiate into cells showing the typical PGC pattern of TNAP activity (Quinlan *et al.,* 1995). In the reciprocal transplantation, proximal epiblast colonized the host neural tissue but did not form PGC-like cells after grafting to the distal epiblast (Tam & Zhou, 1996; Tsang *et al.,* 2001). These results suggest that cells in the distal and the proximal epiblast are equally competent to form PGCs provided they are placed in an environment that is appropriate for germ cell specification. This raises the intriguing possibility that PGC formation is subject to local environmental influences unique to the proximal epiblast (Tsang *et al.,* 2001). However, since these are necessarily short-term experiments, evidence is lacking that such cells are actually able to give rise to functional mature gametes. More importantly, since there is a very high degree of cell mixing in the epiblast from early postimplantation, descendants of all its founder cells are likely to be present throughout the tissue, including its proximal and distal extremities. Hence, the possibility that only a subset of epiblast

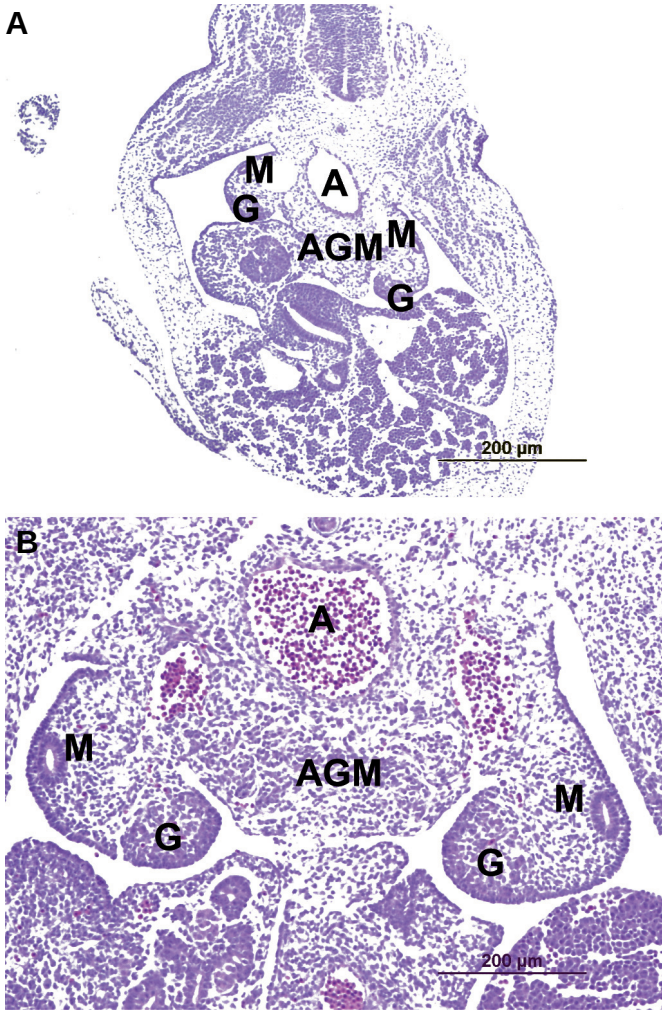

Fig. 2. AGM region in mouse embryos. *Mouse embryonic transversal sections at 10.5 dpc* **(A)** *and 11.5 dpc* **(B)** *at the level of the AGM region. A, aorta; G, gonad; M, mesonephros.*

founder clones contain cells that are competent to be induced to form germ cells cannot be discounted at present. Interestingly, descendants of these proximal epiblast cells do not only contribute to the presumptive PGCs but also to other somatic tissues such as the extraembryonic mesoderm.

PGC are the founder cell population of the gametes. During gastrulation, the PGCs that originate from the posterior epiblast ingress though the posterior primitive streak together with the allantoic, intermediate and lateral plate mesoderm (Lawson *et al.,* 1991; Tam and Zhou, 1996; Kinder *et al.,* 1999). In the mouse (McLaren, 2003), they arise early in embryogenesis, first becoming visible in the extraembryonic mesoderm, posterior to the primitive streak, at 6-6.5dpc in mice (Molyneaux & Wylie, 2004), and can be identified by expression of tissue non-specific alkaline phosphatase (TNAP) activity. Recently, Blimp1 (B –lymphocyte-induced maturation protein 1, also known as Prdm1) a potent transcriptional regulator, has been identified as a key factor for the specification of the germ cell lineage. Blimp1 positive cells at the early stage of 6.25 dpc constitute the lineage restricted PGC precursors. Blimp1 is essential for repressing nearly all the genes normally down-regulated in PGCs. They also express SSEA-1 and *Mvh* on the surface (Toyooka *et al.,* 2000) and intracellular *Oct-4*, *Stella*, *Fragilis*, *Smad1*, *Nobox* and *Hdac68* (Yabuta *et al.,* 2006).

Afterwards they are incorporated into the epithelium of the hindgut, from which they start to move at day 9.5 dpc first into the dorsal mesentery (10.5 dpc). The mesoderm of the embryo gives rise to an anterior lateral region termed the para-aorta-splanchnopleura, which later contributes to the future aorta, gonads and mesonephros (hence, it is termed the AGM region). PGCs migrate from the hindgut, reaching the AGM region at 10.5 dpc (Fig. 2) then into the genital ridges that lie on the dorsal body wall (11.5 dpc). During their migration, primordial germ cells express the SSEA-1 antigen and TNAP on their cell surfaces. These cells can be isolated and cultured for up to a week, maintaining their phenotype (De Miguel & Donovan, 2000).

From AGM to PGCs, EC and EG cells

As stated above, at the early-somite stage, PGCs congregate in the endoderm of the prospective hindgut of the embryo. Subsequently, PGCs are relocated from the ventral to the dorsal aspect of the gut, and over the next 3-4 days, migrate from the hindgut endoderm through the mesentery migrating through the AGM region so they can later colonize the urogenital ridges. At that time, around 12.5 dpc in the mouse, the gonads start to show morphological differences between sexes (De Miguel *et al.,* 2000; Donovan & De Miguel, 2005). During this migration PGCs erase imprints by demethylation (see below) and then re-establish them depending on their XX or XY chromosome content.

PGCs that are found outside the genital ridge after 13.5 dpc do not seem able to re-activate the X-chromosome and display only one active X-chromosome (Tsang *et al.,* 2001). Some of these ectopic germ cells, located in the mesonephros and adrenal glands (Francavilla and Zamboni, 1985), will proceed to meiosis at about 16.5-17.5 dpc despite the absence of the re-activation of the X-chromosome (Tam and Zhou unpublished, in Tsang *et al.,* 2001). These ectopic germ cells, however, will not form functional germ cells and are thought to degenerate postnatally (Upadhyay and Zamboni, 1982; McLaren, 2003).

However, recent publications have demonstrated the presence of cells able to give rise to pluripotent ES-like cells in postnatal murine testes (Kanatsu-Shinohara *et al.,* 2004; Guan *et al.,* 2006), and to oocytes both in murine bone marrow (Johnson *et al.,* 2005) and porcine fetal skin (Dyce *et al.,* 2006) as well as the presence of cells able to give rise to spermatogonia in bone marrow (BM) (Nayernia *et al.,* 2006).

Historically, it has been described that PGCs can give rise to two types of pluripotent stem cells. *In vivo*, PGCs can give rise to embryonal carcinoma (EC) cells, the pluripotent stem cells of testicular tumors (Donovan & De Miguel, 2003). Cultured PGCs exposed to a specific cocktail of growth factors give rise to embryonic germ (EG) cells, pluripotent stem cells that can contribute to all lineages of chimeric embryos including the germline (Matsui *et al.,* 1992; Resnick *et al.,* 1992). The conversion of PGCs into pluripotent stem cells is a remarkably similar process to nuclear reprogramming in which a somatic nucleus is reprogrammed in the egg cytoplasm (Donovan & De Miguel, 2003). Both EC and EG cells, like ES cells, share the specific markers

SSEAs, *Oct-4* and TNAP (De Miguel & Donovan, 2000).

In fact, the derivation of ES cells was based on studies of teratocarcinoma cells. The transplantation of genital ridges or of egg-cylinder-stage embryos into ectopic sites, such as under the kidney capsule of adult mice, gave rise to teratocarcinomas at a high frequency in strains that did not spontaneously produce these tumors (Stevens, 1970). The stem cell of these tumors is the embryonal carcinoma cell, the EC cell, which can be serially transplanted between adult mice (Solter *et al.,* 1981). If the EC compartment disappears, the resulting tumor develops as a benign teratoma. Indeed, EC cells injected into mouse blastocysts can contribute to either the normal tissues of the resulting chimera (Brinster, 1974) or in some cases, to tumors (Rossant and McBurney, 1982).

EG have been derived from pre- and postmigratory as well as from migratory PGC in both mice and humans and are pluripotent (Matsui *et al.,* 1992; Shamblott *et al.,* 1998). That is, EG in contrast to PGC fully contribute to blastocyst complementation giving rise in the developing embryo to all somatic lineages and germ cells. To explain this phenomenon at the molecular level, it is known that the pluripotency of PGC nuclei depends on the methylation status of genomic imprinted genes (e.g., *H19, Igf-2, Igf-2R, Snrpn*; Mann, 2001; Yamazaki *et al.,* 2003). Until 9.5 dpc, PGC display a somatic imprint (paternal and maternal pattern of methylation) of *H19, Igf-2, Igf-2R* and *Snrpnt,* which is crucial to maintaining their pluripotency. This imprint, however, is erased by demethylation, during the migration of PGCs towards the genital ridges at 10.5 dpc (Lee *et al.,* 2002). The erasing of the methylation (imprint) in early PGC could be envisioned as one of the mechanisms that shuts down PGC developmental pluripotency and makes these cells resistant to potential parthenogenesis or formation of teratomas (Oosterhuis and Looijenga, 2005). A proper somatic imprint is subsequently reestablished later in sperm and oocytes, so that a fertilized egg expresses a developmentally proper somatic imprint of these crucial genes. That PGC-derived EG cells are pluripotent demonstrates that the re-establishment of a proper somatic imprint is possible.

Mouse EG cell lines are remarkably similar to mouse ES cell lines (Donovan and De Miguel, 2003). Since during germ cell migration and maturation the somatic status of imprinted genes is progressively erased (Yamazaki *et al.,* 2003), EG cells isolated at various stages of migration retain some of these differences, such as the reduced methylation of many imprinted genes, including *H19* and *Snrpn*. The analysis of mouse PGCs at 10.5dpc suggests that methylation erasure has already begun by this time (Yamazaki *et al.,* 2003). This study showed that imprinted genes exhibit imprinted (somatic) expression patterns in 9.5 dpc PGCs, but by 10.5 dpc, they have switched to a bi-allelic mode of expression (Yamazaki *et al.,* 2003). Since the genes expressed in ES cells exhibit somatic imprinting status, these data suggest that if ES cells are derived from germ cells, this derivation must occur before 9.5 dpc in the mouse.

Similar to PGC, other epiblast derived stem cells deposited in the developing tissues undergo erasure of their somatic imprint.

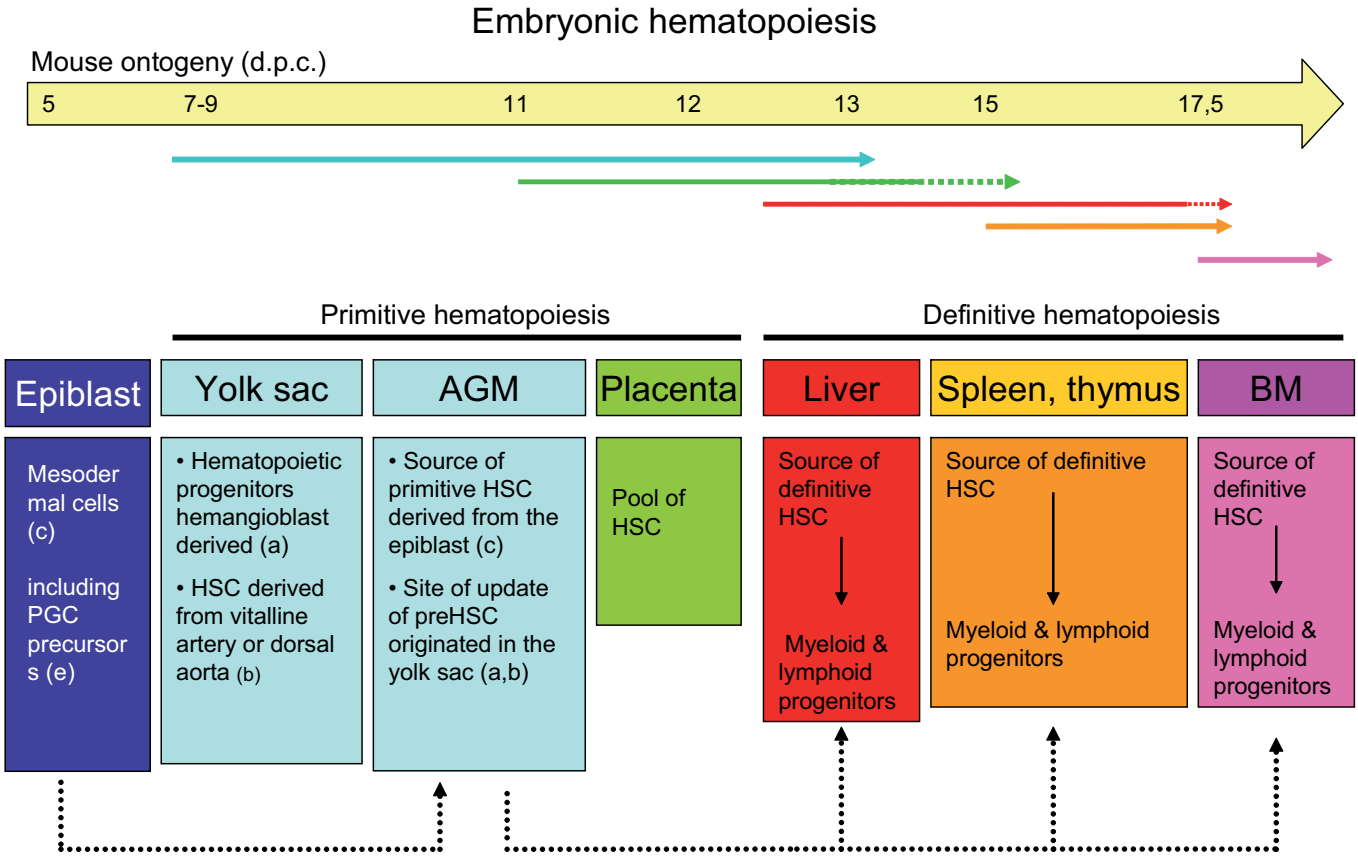

Fig. 3. Diagram of the ontogeny of hematopoietic stem cells. *Different letters show distinct hypotheses. Dotted arrows represent alternative migration pathways.*

This mechanism of erasure of methylation of somatic imprinted genes protects the developing organism from the possibility of teratoma formation. However, it will affect some aspects of the pluripotentiality of these cells (e.g., potential of these cells to contribute to blastocyst development or teratoma formation after transplantation into immunodeficient mice). Interestingly, as demonstrated experimentally, EpiSC are also pluripotent but do not retain germ lineage potential (Tesar *et al.,* 2007).

From AGM to embryonic and adult hematopoiesis

Hematopoietic stem cells (HSCs) play an indispensable role in the maintenance of blood cell homeostasis by their life-long self-renewal activity and multipotent differentiation potential into all classes of hematopoietic cells (Kondo *et al.,* 2003). In adult mice, and conserved through all vertebrates, hematopoiesis occurs mainly in the bone marrow. However, during ontogeny in mouse fetuses the site of hematopoiesis is modified several times (Cumano *et al.,* 2001). A first wave of hematopoiesis is observed in the yolk sac and the AGM region at 7–9 dpc, lasting until 13 dpc (Moore and Metcalf, 1970; Kikuchi & Kondo, 2006). HSC activity is also detected in the placenta after 11 dpc (Gekas *et al.,* 2005). A second wave of hematopoiesis starts in the fetal liver at 12 dpc and then moves towards the spleen, and later to the BM (for a review see McGrath & Palis, 2008).

Respect to HSC migration during ontogenesis, there are several hypotheses. In the embryo, the first blood cells originate from the epiblast after gastrulation, although they do not appear as such until 7-9 dpc in the mouse embryo (Fig. 3). The mesodermal cells of the embryo will contribute in part to the extraembryonary structures, including the corion, the amnios and the yolk sac, (Fig. 1). In particular, the yolk sac derives from the mesoderm and the primitive endoderm. In the embryonic mesoderm, some structures named "blood islands" develop, consisting in erythroid cells and surrounding endothelial cells, which are formed in the vascular plexus of the yolk sac. The emergence of erythroid and endothelial precursors at the same location and at the same time suggests that they share the same ontogenetic origin, the hemangioblast (Fig. 3, hypothesis a, for a review see McGrath & Palis, 2008). However, the relationship between these progenitors and embryonic HSCs has not been sufficiently demonstrated, and there is still some controversy with regard to the yolk sac being a source of HSCs. The main data arguing against the yolk sac as the source of HSCs come from explant culture studies, which show that early yolk sac explants do not have the potential to generate adult reconstituting HSCs or lymphoid progeny (Cumano *et al.,* 2001). An alternative theory is that, yolk sac HSCs could be derived from the vitelline artery or upper dorsal aorta, which are upstream of the yolk sac in the circulation (Fig. 3, hypothesis b, De Bruijn *et al.,* 2002).

Multiple studies have documented that the AGM region is a source of definitive HSCs (Medvinsky & Dzierzak, 1996; De Bruijn

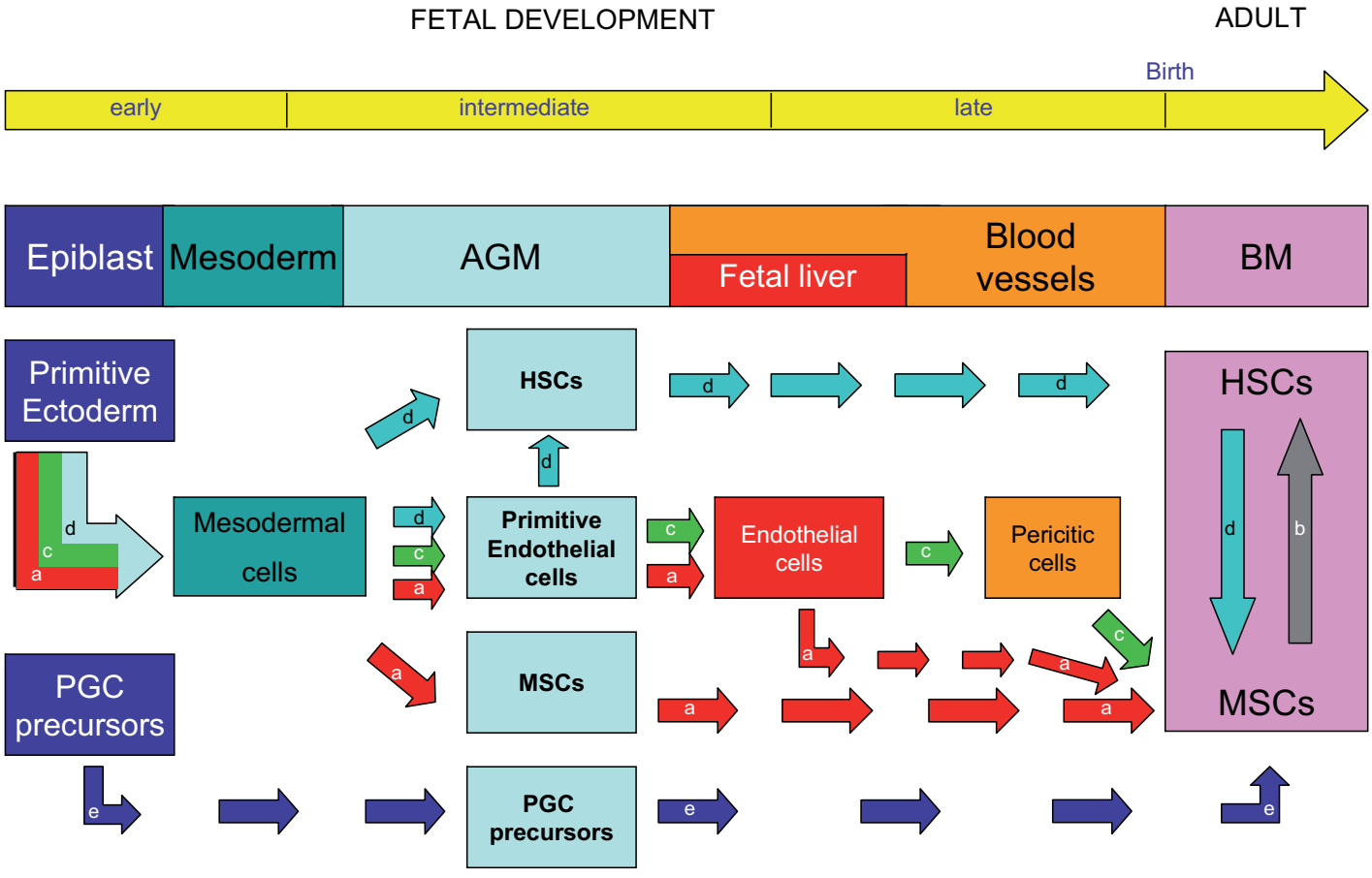

Fig. 4. Diagram of the ontogeny of mesenchymal stem cells. *Different arrows colors and letters show distinct hypotheses.*

et al., 2002). Within this region, HSCs are thought to arise from hemangioblast precursors located in the ventral wall of the dorsal aorta. The study by Samokhvalov *et al.,* (2007) demonstrated the migration of hematopoietic progenitors from the yolk sac to the umbilical cord, the AGM region and subsequently the embryonic liver and thymus. This raises the possibility that some HSCs associated with major embryonic vasculature are derived from yolk sac precursors. It is conceivable that yolk sac-derived pre HSCs acquire functional HSC characteristics in the AGM/umbilical artery and umbilical vein region (Samokhvalov *et al.,* 2007). The AGM region could be the site of an "update" of HSC towards their new adult environment (Samokhvalov *et al.,* 2007). Alternatively, the AGM could be a source of HSC deriving from the epiblast (Fig. 3, hypothesis c). Using a genetic tracing strategy, a recent study (Zovein *et al.,* 2008) has demonstrated that at least mouse HSCs originate at the AGM region exclusively from endothelial precursors, excluding the possibility of an origin from the adjacent mesenchyme. These cells do migrate in the circulation towards the fetal liver and then to the bone marrow. At both sites they expand and undergo multilineage hematopoietic differentiation.

In addition, the discovery of murine placenta harboring a large pool of multipotent progenitors and HSCs during midgestation indicates that the placenta may also play an important role in the establishment of HSCs (Fig. 3, hypothesis d, Alvarez-Silva *et al.,* 2003). It remains controversial whether these cells come from the allantoic mesoderm or originate from the AGM as the main vascular route by which blood cells from the dorsal aorta circulate into the fetal liver through the umbilical vessels and the placenta (Mikkola & Orkin, 2006).

Definitive hematopoiesis is better known, and most scientific groups agree in that involves the AGM-HSCs colonization of the fetal liver, thymus, spleen, and ultimately the bone marrow. Hematopoiesis is detected in the fetal mouse liver at 12 dpc and in the spleen at 15 dpc (Kikuchi & Kondo, 2006). Until 17.5 dpc, HSC activity is not detected in BM (Christensen *et al.,* 2004), and from then on, BM hematopoiesis is sustained throughout the life of an animal, while the rest of the tissues are no longer hematopoietic (Fig. 3). In definitive hematopoiesis, HSCs give rise to myeloid progenitors and lymphoid progenitors in all tissues where it is established, that is, in the liver, spleen, thymus and bone marrow (Fig. 3).

Recently, differentiation experiments from PGCs to HSCs (Rich, 1995) and the presence of a small subpopulation of SSEA-4, *Oct4* and *Nanog* positive cells (markers typical of PGCs) in the adult BM, which are capable of differentiating into HSC (Kucia *et al.,* 2006a) suggest that these cells are the embryonic and adult HSC precursors that originate from a common precursor of PGCs and HSC derived from the epiblast. In the case of HSCs they will migrate to the AGM and then astray towards the hematopoietic organs (Fig. 3, hypothesis e).

From AGM to BM-MSC

The bone marrow is a well-known source of adult stem cells, providing not only hematopoietic stem cells but also nonhematopoietic stem cells located in the stroma, known as mesenchymal stem cells (MSCs). MSCs support hematopoiesis and are able to differentiate towards the mesodermal lineage to generate osteoblasts, condrocytes and adipocytes, all of them cellular types present in the adult bone marrow (Friedenstein *et al.,* 1970; Dennis *et al.,* 1999). MSCs are a heterogeneous population with several subgroups of cells with different proliferative and differentiation potentials.

Friedenstein *et al.* (1970) was the first group to isolate this type of cells by means of their ability to adhere to culture plastic, obtaining a heterogeneous population comprising both hematopoietic progenitor cells as well as nonhematopoietic stromal cells. Several groups have tried to find a specific marker that allows identification and isolation of the mesenchymal stem cells from the rest of the BM population. Stro-1, an antibody recognizing a surface antigen of unknown function, has been used to isolate clonogenic stromal cells (Gronthos *et al.,* 1994). Unfortunately, Stro-1 is also present, at low levels, in endothelial or pericytic cells (Bianco & Cossu, 1999). Another group has postulated that the human MSCs can be isolated based on the expression of CD49a, the α1- integrin subunit of the very late antigen-1, a receptor for collagen and laminin (Deschaseaux *et al.,* 2003). This population $CD49a^{high}$ $CD45^{med/low}$ contains all CFU-fibroblast progenitors present in the bone marrow (Deschaseaux *et al.,* 2003). However, this population is also heterogeneous and contains many hematopoietic cells, which are $CD45^{med}$, whereas the $CD45^{low}$ would identify the CFU-F or MSC (Anjos-Afonso & Bonnet, 2007). Finally, SSEA-1 in mice (Anjos-Afonso & Bonnet, 2007) and SSEA-4 in humans (Gang *et al.,* 2007) have also been proposed to identify the adult mesenchymal stem cells in the BM.

MSCs origin remains uncertain, although there are different hypotheses raised throughout the last decades. All of them agree in that the epiblast will give rise to the AGM region cells that originate the mesothelial cells that are the embryonic precursors of HSC, endothelial cells and MSC that at the end will home at the bone marrow (Fig. 4). Little is known about the path these cells follow from the AGM region into the adult bone marrow. The most accepted hypothesis states that mesenchymal cells probably follow endothelial cells from the AGM region towards the ventral embryonic aorta, where differentiated endothelial cells will begin to form the blood vessels. These vessels will invade the regions meant to develop into the long bones, carrying the cells that will be forming the primitive cartilage and also the mesenchymal cells that will form the bone marrow stroma (Pardanaud *et al.,* 1996, Fig. 4 hypothesis a). The experiments published by Anjos-Afonso & Bonnet (2007) suggest that transdifferentiation would occur from MSCs to HSCs in the BM (Fig. 4 hypothesis b), based on experiments showing that after depletion of CD45+ cells in the bone marrow stromal fraction and transplantation of these MSC cells in mice with medullar aplasia, new CD45 hematopoietic-like cells are detected in the bone marrow. Although these results are not conclusive, as the CD45+ population could have colonized the marrow coming from other parts of the body, they suggest the possibility that a common precursor of HSC and MSC is present in the adult BM.

Another theory embraced by several authors suggests the possibility that among these differentiated endothelial cells that are in the ventral zone of the aorta, there are specialized vascular cells called pericytes that at the end would give rise to the MSCs in the bone marrow. This hypothesis is supported by the fact that the expression of Stro-1 occurs in both cellular types, pericytes and MSCs (Bianco & Cossu, 1999, Fig. 4 hypothesis c). Other

authors (Lakshmipathy & Verfaille, 2005) propose the occurrence of transdifferentiation of the HSCs in the bone marrow into MSCs (Fig. 4 hypothesis d), although experiments of Wagers *et al.* (2002), Murry *et al.,* (2004) and Gang *et al.,* (2007) suggest that this hypothesis is not correct.

Lastly, Kucia and colleagues (2006b) proposed a different pattern of migration: in the AGM region, PGC, hematopoietic and mesenchymal stem cells would have a common precursor that would travel to where hematopoiesis first appears, the fetal liver, and later to the bone marrow (Fig. 4 hypothesis e). The recent discovery of VSEL (very small embryonic-like) stem cells in the adult bone marrow expressing SSEA-4 and *Oct4* and which respond to SDF-1 gradient, supports this idea (Kucia *et al.,* 2006a; Gang *et al.,* 2007). In addition, the fact that PGC-like alkaline positive cells in the human embryo are a heterogeneous population comprising a small subpopulation of *Oct-4* positive cells (Kerr *et al.,* 2008) further supports this hypothesis.

Embryonic origin of mesenchymal cells in adult tissues

In addition to ES, EC, EG cells, epiblast and PGCs as stated above, several groups have identified SSEA positive cells in other tissues.

As described earlier, bone marrow contains a population of SSEA+ *Oct-4*+, *Nanog*+ stem cells that express markers of pluripotent stem cells (Kucia *et al.,* 2006a; Gang *et al.,* 2007). Kucia *et al.* named these cells very small embryonic-like (VSEL) stem cells. As mentioned before, it is hypothesized that they are deposited early in development in marrow tissue and are descendants of epiblast-derived stem cells (EpiSC) and perhaps some primordial germ cells (Kucia *et al.,* 2006b).

Several populations of cells that express early embryonic stem cell markers have been identified in bone marrow and cord blood (CB). These populations of cells could be different names for the same population of stem cells (Ratajczak *et al.,* 2007), or pluripotent cells in different stages:

VSEL Stem cells

These cells are *CXCR4+, Oct-4*+, SSEA-1+, Sca-1+, Lin-, CD45- (Kucia *et al.,* 2006a and Fig. 5). Generally, VSEL are believed to be a dormant quiescent population of PSC that resides in BM and that actively contribute to long-term hematopoiesis and turnover of other tissue specific (monopotent) stem cells located in peripheral niches, which after being mobilized into peripheral blood during organ injury, may contribute to tissue organ regeneration. As these cells are associated with MSC in the bone marrow, several authors hypothesize that the unexpected plasticity of MSC with fibroblastic morphology is actually due to VSEL stem cell contamination (Anjos-Afonso & Bonnet, 2007; Ratajczak *et al.,* 2007). Furthermore, a single-cell-derived population was capable of differentiating abundantly into different mesenchymal cell types *in vivo* (Anjos-Afonso & Bonnet, 2007). Since isolated VSEL express several markers of primordial germ cells (fetal-type alkaline phosphatase, *Oct-4*, SSEA-1, *CXCR4*, *Mvh*, *Stella*, *Fragilis*, *Nobox*, Hdac68, Kucia *et al.* (2006b) hypothesized that VSEL are related to a population of early PGCs. VSEL derivation would then mimic the derivation of EG cells from PGCs.

Mesenchymal stromal cells

It has also been reported that when cultured in serum-free, b-FGF-containing medium, fibroblastoid mesenchymal stromal cells obtained by plating cells from BM into culture flasks and selecting plastic-adherent cells, enhance the expression of the embryonic markers SSEA-4, *Oct-4* and *Nanog*, and give rise to multilineage differentiation (Battula *et al.,* 2007 and Fig. 6). Another article shows however that human first-trimester fetal blood, liver, and bone marrow MSC but not adult MSC express the pluripotency stem cell markers *Oct-4*, *Nanog*, *Rex-1*, SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (Guillot *et al.,* 2007). The relationship of these undifferentiated MSC to other populations of *Oct-4*+ cells in bone marrow, including VSEL, is not known.

MAPC cells

Jiang *et al.* (2002) reported that MAPC cells (multipotent adult progenitor cells) obtained from bone marrow, can differentiate, at the single cell level, not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm and endoderm characteristics *in vitro*. When injected into an early blastocyst, single

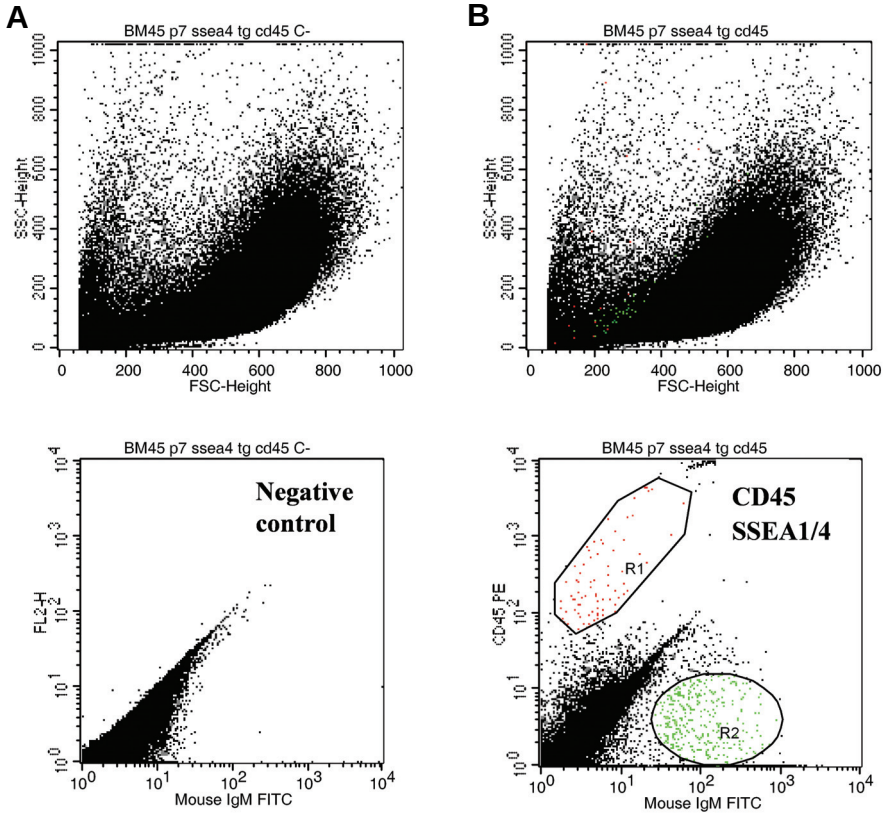

Fig. 5. FACS analysis of adult mouse bone marrow derived mesenchymal stem cells using CD45 and SSEA antibodies. (A) *Negative control.* **(B)** *SSEA positive cells isolated are shown in green (R2). Note that this population is different from the CD45+ one exposed in red (R1).*

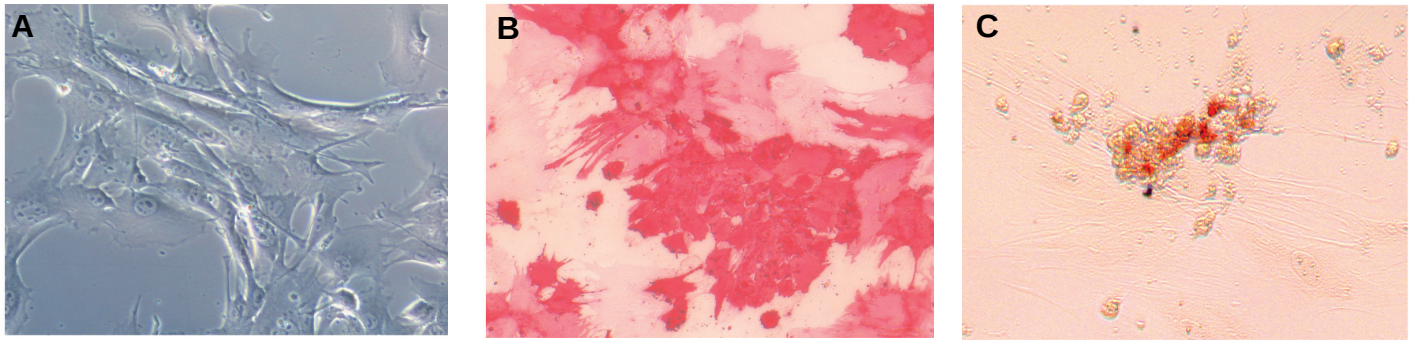

Fig. 6. Mouse bone marrow derived mesenchymal stem cells (MSC) in culture. (A) *Undifferentiated MSCs seen under phase contrast optics. 200x.* **(B)** *Osteogenic lineage cells derived in culture from bone marrow derived mesenchymal stem cells after two weeks in osteogenic-inducing medium, assessed by alkaline phosphatase staining. 100x.* **(C)** *Adipogenic lineage cells derived in culture from bone marrow derived mesenchymal stem cells after two weeks in adipogenic-inducing medium, assessed by Oil red staining. 200x.*

MAPCs contribute to most, if not all, somatic cell types (Jiang *et al.,* 2002).

Miami Cells

Marrow-isolated adult multilineage inducible (MIAMI) cells, are capable of differentiating *in vitro* into mature-like cells from all three germ layers. Low pO_2 upregulated mRNAs for *Oct-4*, Rex-1, telomerase reverse transcriptase, and *hypoxia-inducible factor-1 alpha*, and increased the expression of SSEA-4 compared to normal O_2 concentration. It is speculated that primitive MIAMI cells self-renew while localized to areas of low pO_2 in the bone marrow, but tend to differentiate toward osteoblasts when they are located closer to blood vessels and exposed to higher pO_2 (D' Ippolito *et al.,* 2006).

Umbilical and neonatal cord blood SSEA positive cells

The presence of *Oct-4*+, *Nanog*+, and SSEA-3/4+ stem cells in human cord blood and umbilical cord matrix has been described (Carlin *et al.,* 2006). Recently another group purified small cells resembling a population of murine BM-derived VSEL from human CB. These CB-isolated VSEL are very small (3-5 μm) and highly enriched in a population of *CXCR4*+AC133+CD34+Lin-CD45- mononuclear cells, and they possess relatively large nuclei containing unorganized euchromatin, express nuclear embryonic transcription factors *Oct-4* and *Nanog* and surface embryonic antigen SSEA-4 (Kucia *et al.,* 2007). Further studies are needed to determine whether human CB-isolated VSEL, like their murine BM-derived counterparts, are endowed with pluripotency.

Placenta-derived multipotent cells

Placenta-derived multipotent cells (PDMCs) exhibit many markers common to mesenchymal stem cells (including CD105/endoglin/SH-2, SH-3, and SH-4) and they lack hematopoietic, endothelial-, and trophoblastic-specific cell markers. In addition, PDMCs exhibit ES surface markers SSEA-4, TRA-1-61, and TRA-1-80 (Yen *et al.,* 2005). When cultured in appropriate conditions, adipogenic, osteogenic, neurogenic and hepatic differentiations were achieved (Yen *et al.,* 2005).

SSEA cells in non-hematopoietic organs

Several groups of investigators have described the presence of *Oct-4*+, *Nanog*+, and SSEA-3/4+ stem cells in nonhematopoietic organs, such as epidermis (Dyce *et al.,* 2006), bronchial epithelium (Kim *et al.,* 2005), pancreas, (Kruse *et al.,* 2006), testis (Kanatsu-Shinohara *et al.,* 2004), retina (Koso *et al.,* 2006), amniotic fluid (De Coppi *et al.,* 2007), bulge region of hair follicles (Yu *et al.,* 2006), renal tubular epithelium (Sagrinati *et al.,* 2006), heart (Mendez-Ferrer *et al.,* 2006), human fetal liver (Dan *et al.,* 2006), and dental pulp (Kerkis *et al.,* 2006). The expression of these embryonic stem cell markers suggests that these cells could belong to the population of embryonic/epiblast derived or PGC stem cells that may be deposited early on during development in various organs.

It is possible that other stem cells not committed to becoming PGC cells, founder cells, subsequently move through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. The presence of SSEA positive cells in the MSC population of different organs supports the hypothesis that during early development epiblast/germ line-derived cells including PGC become a founder population of pluripotent stem cells (Kucia *et al.,* 2006b).These cells may be then deposited during embryogenesis in various organs and may persist in these locations into adulthood (for example in bone marrow) (Zambidis *et al.,* 2006). One of these organs may be the aorta-gonad-mesonephros. Furthermore, there is some additional evidence that some epiblast derived PGC themselves might go astray and seed to peripheral tissues during migration through the embryo proper on their way to the genital ridges (Upadhyay & Zamboni, 1982).

A similar hypothesis goes even further, and states that PGC may go astray from the main migration stream and become deposited in various tissues (Francavilla and Zamboni, 1985). On the way to the genital ridges *CXCR4+* PGC cells migrate through the embryo proper through the AGM region. Like PGC, HSC are also *CXCR4+* and their migration is regulated by *SDF-1* gradient, which becomes expressed during the second trimester of gestation in the fetal liver (Medvinsky and Dzierzak, 1996). The translocation from the liver to the BM is again mediated by the *SDF-1* gradient, which becomes expressed in developing BM tissue (Ara *et al.,* 2003). In addition to HSC, the fetal liver may also chemoattract some migrating PGC in a *SDF-1* dependent manner. Furthermore, it had even been suggested that PGC gives rise in AGM to a population of definitive HSC (Rich, 1995).

Interestingly, PGCs can give rise to cells of the hematopoietic system such as the hematopoietic stem cells cobblestone-area forming cell, and cells of the erythroid lineage (Rich, 1995). The

possibility of epiblast cells deposition in various tissues during embryonic life is also supported by the fact that oocytes were generated by putative germ cells of the BM and peripheral blood in sterilized female mice (Johnson *et al.,* 2005). Male germ cells have also been derived from bone-marrow cells (Nayernia *et al.,* 2006).

Summary and Conclusions

We have reviewed the ontogeny and specification of the epiblast derived cells towards various stem cell compartments, both *in vitro* and *in vivo*. Data is presented supporting the hypothesis that pluripotent SSEA+ stem cells, isolated from both embryonal and adult tissues are descendants of epiblast derived cells that could be deposited during embryonic life in various tissues. A great deal of controversy and further basic and preclinical research is needed to elucidate how the different types of stem cells are specified during the mammalian embryonic life.

From a Cell Therapy point of view, the question remains, whether these pluripotent stem cells are only developmental remnants from the epiblast or if they can continuously contribute in adult life to the renewal of other more committed adult stem cells.

More importantly, the presence of pluripotent stem cells in adult tissues opens the possibility of aiming at the development of techniques that will allow for their reactivation in various organs in order to regenerate them without the need for stem cell transplantation, providing a whole new path for human Cell Therapy.

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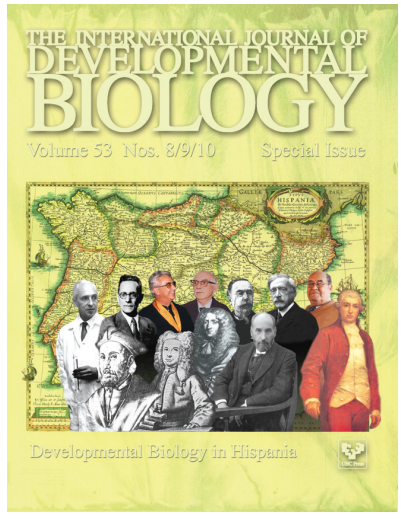

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