Origin, Migration, and Proliferation of Human Primordial Germ Cells

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Abstract

The first histological observations about the origin of the precursors of gametes termed primordial germ cells (PGCs) in extragonadal regions and their subsequent migration into the developing gonads in human embryos date back to the early twentieth century. Fuss (Anat Am 39:407–409, 1911, Anat EntwMech 81:1–23, 1912) and Felix (Die Entwicklung der Harn- und Geschlechtsorgane. In: Keibel-Mall Handbuch der 1qEntwicklungageschichte des Menshen, vol 2. Leipzig, Hirzel, pp 732–955, 1911) were apparently the first ones to describe the extragonadal location of PGCs in human embryos. In the youngest, 2.5 mm long, embryo examined (23–26 days postfertilization), These authors described PGCs in the endoderm of the yolk sac wall as cells identifiable by their large size and spherical shape. Subsequently, Politzer (Z Anat Entw Gesch 87: 766–80, 1928, Z Anat Entw Gesch 93:386–428, 1930, Z Anat EntwGesch 100:331–336, 1933) and Witschi (Contr Embryol Carnegie Inst 209:67–80, 1948) studied the distribution of PGCs in a considerable number of embryos from presomite stages (0.3–0.8 mm, about 3 weeks) to 8.5 mm (5 weeks). Both authors described the migration of PGCs from the yolk sac to the developing gonads. Following a hot debate, it is now generally accepted that after their arrival into the gonadal anlage, PGCs give rise to the oogonia/oocytes and gonocytes (or prespermatogonia) in the embryonic ovary and testis, respectively. These germ cells enter a complex series of events that in the

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G. Coticchio et al. (eds.), *Oogenesis*,
DOI 10.1007/978-0-85729-826-3_2, © Springer-Verlag London 2013

adult end with the formation of fertilizable oocytes and sperm. Because of the inaccessibility of the human embryo to experimental investigations at these early stages, we still know little about cellular and molecular mechanisms controlling the formation, differentiation, and development of human PGCs. This chapter describes the life history of human PGCs combining old and new information and, where appropriate, making use of the most recent results obtained in the mouse.

Keywords
Primordial germ cells • Embryonic gametogenesis • Gonad development BMPs

Introduction

The differentiation and development of PGCs is an early event of the mammalian embryogenesis, crucial for assuring normal fertility of the individual and the correct transmission of the genome to the next generation. The basic principles governing these processes have been clarified and can be summarized as follows. Probably dictated by the necessity to protect the cells of the germ line from signals inducing somatic cell lineage differentiation, the precursors of PGCs are early committed and specified in the epiblast (one of the first two differentiated tissue of the embryo proper, the other being the hypoblast) before gastrulation and rapidly moved into an extraembryonic region where PGCs are determined. PGCs reenter into the embryo proper during early gastrulation to reach the developing gonads (gonadal ridges, GRs). During this journey, while undergoing proliferation, PGCs begin extensive nuclear reprogramming (activation of genes for pluripotency and epigenetic changes of the genome involving DNA demethylation and histone code) to regain differentiation totipotency and reset the genomic imprinting. These processes are completed after their arrival into the GRs. After some rounds of proliferation, PGCs finally differentiate into oogonia or gonocytes within ovaries and testes, respectively.

Despite the first morphological observations on the extragonadal formation and movements of PGCs in human embryos date back exactly 100 years ago [1–3] (Fig. 2.1 and Table 2.1), the inaccessibility of the human embryo to experimental investigations at these early stages made difficult, if not impossible, to obtain information on the complex temporal and spatial series of molecular events underlying these processes. Thanks to recent studies carried out in the mouse and the development of stem cell technologies, however, we are now obtaining precious information that can contribute to clarify crucial aspects of these processes. The present review is an attempt to summarize these results and the future perspectives.

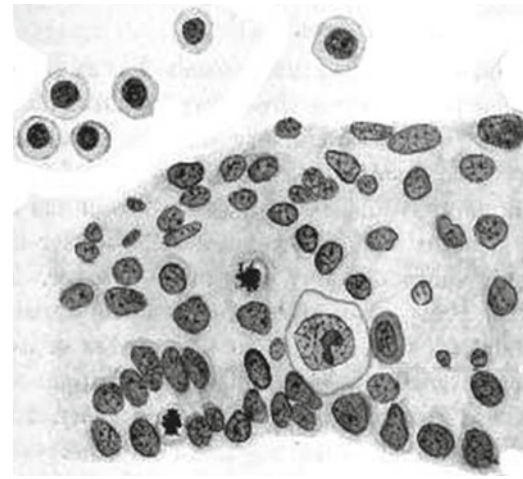
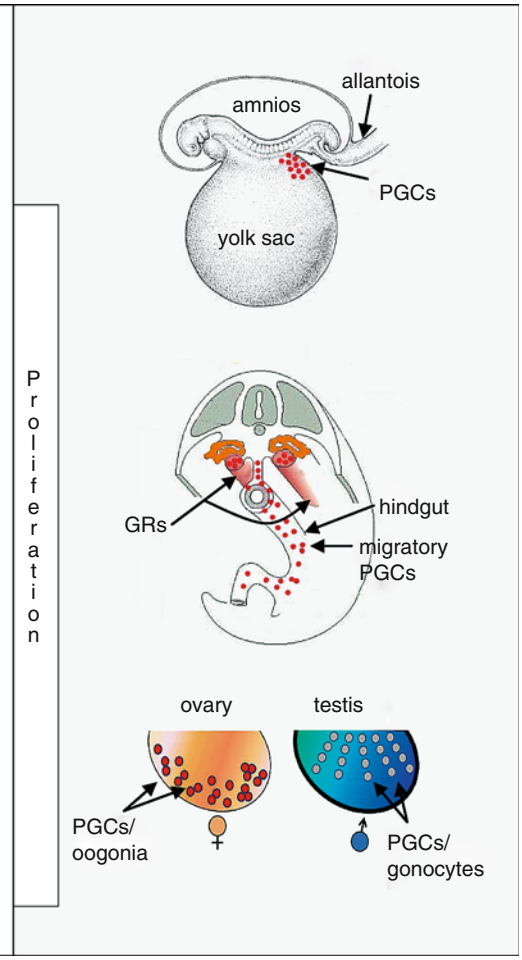


Fig. 2.1 Reproduction of the original drawing by A. Fuss [2]. The German scientist was likely the first to identify PGCs in the endoderm of the wall of the yolk sac in one human embryo 2.5 mm long (23–26 days postfertilization); in the center of the drawing, a PGC is clearly recognizable for its large size and spherical shape among several somatic cells

Table 2.1 Chronology of human primordial germ cell development

Age from conception (week)	
III	PGCs in the dorsal wall of the yolk sac near the developing allantois
IV	
V	PGCs into the midgut and hindgut endoderm
VI	
VII	PGCs into the midgut and hindgut endoderm; PGC migration through the dorsal mesentery into the gonadal ridges
VIII	
IX	Gonadal ridge colonization and sex differentiation (ovaries and testes)
X	
	PGC differentiation into oogonia (ovary) or gonocytes (testis)

Proliferation



Comparing Mouse and Human PGC Formation

In animals, the formation of PGCs basically occurs by one of two distinct mechanisms: inheritance of germ plasma or inductive signaling (for reviews, see [4, 5]). In most organisms, including invertebrate species such as *Drosophila* and *Caenorhabditis* and nonmammalian vertebrates such as frogs and fishes, germ cell arises through the former mechanism. Germ plasma is a maternally derived collection of cytoplasmic RNAs, RNA-binding proteins, and various organelles assembled within the mature oocyte and segregated during the first divisions of the embryo to the cells fated to become PGCs. In contrast, probably in all mammals, PGCs arise shortly before or during gastrulation through a process of inductive signaling. Specific signals secreted by neighboring cells induce the commitment and specification of PGC precursors among the epiblast cells before gastrulation. Shortly afterward, such precursors are determined as PGCs in an extraembryonic region. But when and where do these signals exactly take place, and what molecules are involved?

Recent elegant research carried out in the mouse (for a review, see [6]) has shown that in the pregastrulation period, interactions between

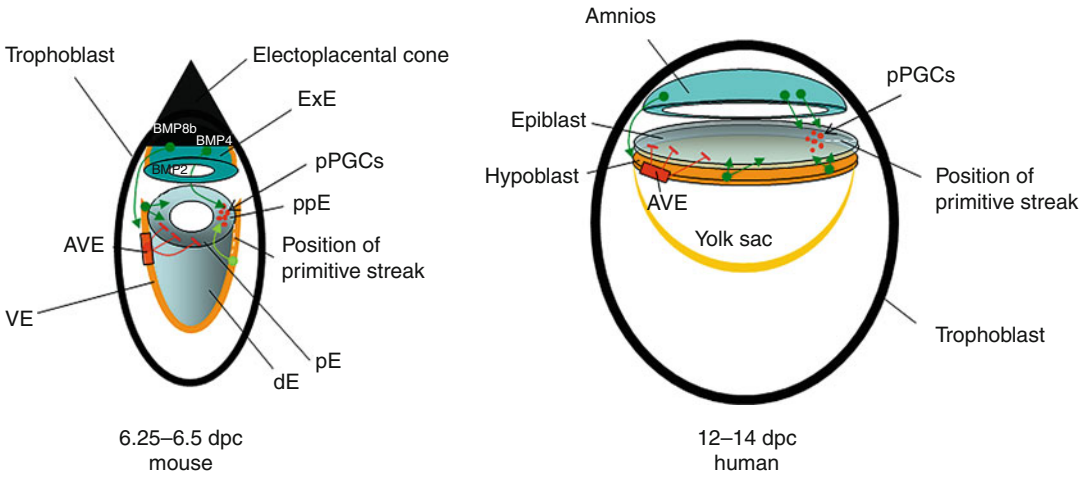


Fig. 2.2 Schematic drawings of the main tissues and BMPs involved in PGC specification in the mouse embryo. For comparison, a hypothetic scenario of PGC specification in the human embryo is drawn. For details, see text. *ExE* extraembryonic ectoderm, *ppE* posterior proximal ectoderm, *pE* proximal ectoderm, *dE* distal ectoderm, *VE* visceral endoderm, *AVE* anterior visceral endoderm, *pPGCs* primordial germ cell precursors

two extraembryonic tissues, the extraembryonic ectoderm (ExE), and visceral endoderm (VE) are crucial for the germ-line commitment and specification. In particular, in the mouse embryo around 6.25 *days post coitum* (dpc), six PGC precursors are set aside in the posterior proximal epiblast cells near the region where the primitive streak will form (Fig. 2.2). Members of the transforming growth factor β1(TGFβ1) super family, namely, bone morphogenetic protein 8a and 4 (BMP8a and BMP4) secreted by ExE and BMP2 produced by the VE, induce these early processes. The expression of the transcriptional repressor B-lymphocyte-induced maturation protein 1 (BLIMP1) (also known as PR domain-containing 1, PRDM1), closely followed by that of the companion PRDM14 and upregulation of fragilis (also known as interferon-induced transmembrane protein 3 or IFITM3), marks the emergence of PGC precursors. These BLIMP1-positive cells increase in number and begin to move out of the embryo through the forming primitive streak. During this period, PGCs are specified, and the expression of the putative RNA/DNA binding protein stella (also known as developmental pluripotency-associated 3, DPPA3, or primordial germ cell 7, PGC7) marks the event. Around 7.25 dpc, the PGC precursors are determined as PGCs in the extraembryonic mesoderm at the basis of allantois. PGCs form a cluster of about 40 cells held together by E-cadherin and expressing high levels of tissue nonspecific alkaline phosphatase (TNAP) and stella (for reviews about the formation of mouse PGCs, see [7–9]).

The place where PGCs were first identified in human embryos around the end of the third week is the same as in the mouse: the wall of the yolk sac at the angle with the allantois (Table 2.1). Exactly 100 years ago, Fuss [1, 2] and Felix [3] were apparently the first to describe the extragonadal location of PGCs (or *Urkeimzellen*) in human embryos. These cells were distinguished by their large size, spherical shape, and the presence of abundant glycogen granules in the cytoplasm (Figs. 2.1 and 2.3). In the youngest, 2.5 mm long, embryo examined (23–26 days postfertilization, 13–20 somites), Fuss described PGCs in the endoderm of the wall of the yolk sac. The extragonadal origin of PGCs was confirmed by the subsequent studies by Kohno [11] and Hamlett [12] and above all, by Politzer [13–15] and Witschi [16] who studied the distribution of PGCs in a considerable number of embryos from presomite stages (0.3–0.8 mm, about 3 weeks, 17 embryos) to 8.5 mm (5 weeks, 23 embryos). Both authors described the migration of PGCs from

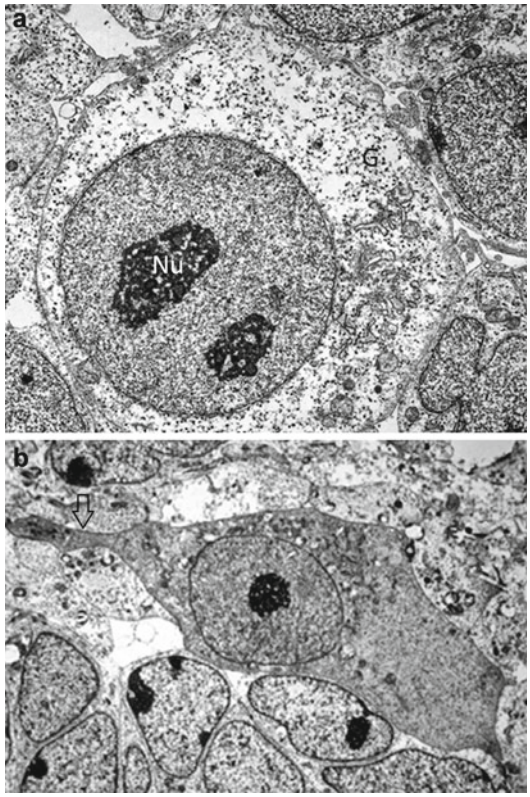


Fig. 2.3 Electron microphotographs of human PGCs between 5 and 6 weeks of gestation. (**a**) A stationary PGC in the hindgut around 5 weeks; note the presence of glycogen (*G*) aggregates and a few mitochondria (*M*) in the cytoplasm; *Nu* nucleolus. (**b**) An actively migrating PGC among mesenchymal somatic cells around 6 weeks; note its elongated shape and a pseudopodium (*arrow*) (Courtesy of Prof. Stefania Nottola, Department of Human Anatomy, University of Rome La Sapienza [10])

the yolk sac to the gonadal ridges. In an embryo 0.6 mm long (about 3 weeks), Politzer [13–15] counted 40 PGCs which increased to 600 in the 4 mm long embryo (4 weeks). Similarly, Witschi [16] counted 30–50 PGCs in the endoderm of the yolk sac around the end of the third week and 109 PGCs in an embryo a couple of days older. Histochemical methods for the identification of PGCs were first applied successfully to human PGCs for periodic acid- Schiff (PAS)-positive materials and mainly TNAP activity by Mc Kay and colleagues [17]. The presence in the cytoplasm of distinct morphological element called "nuage" was another characteristic later described in human PGCs [18]. This material is a conserved feature of germ cells in species across the animal kingdom. The "nuage" is distinct from germ plasma and under the electron microscope, appears as electron-dense granules localized to the cytoplasmic face of the nuclear envelope. There is currently a lack of information about the function of this material. In flies and mouse, an interesting possibility is that it might be involved in microRNA-pathways necessary for maintaining the germ cell lineage and for transposon repression [19, 20].

In humans, there is a lack of studies tracing back the germ-line origin to the earliest stage of development before gastrulation. In particular, no information is available about the inductive processes controlling PGC specification and determination. We can postulate that the basic principles governing human PGC origin are similar to those in mouse and other mammals as well. In humans, however, the timing and mode of formation of the extraembryonic tissues is significantly different from the mouse. This might have some implications for the formation of PGCs (Fig. 2.2).

Immediately before gastrulation (6.0 and 6.5 dpc), the mouse embryo can be visualized as a thick-walled cup of tissue (the epiblast or embryonic ectoderm), which will give rise to the entire fetus and some of the placental membranes. A second thick-walled cup of tissue (the extraembryonic ectoderm) placed overturned on the epiblast will give rise to the main part of the placenta. Both cups are enclosed in a thin bag of primitive endoderm. Taking into account only tissues involved in the formation of the germ line, in the mouse between 4.5 and 5.5 dpc, the primitive endoderm gives rise to the visceral endoderm (VE). In this epithelium, a specialized region termed anterior visceral endoderm (AVE), crucial for determining anterior-posterior embryo polarity, forms. During the same period, the extraembryonic ectoderm (ExE) arises from the polar trophectoderm and makes contact with the underlying epiblast. At 6.5 dpc, gastrulation starts with the formation at the posterior region of the embryo of the primitive streak. Epiblast cells migrating first through this structure include the PGC precursors expressing BLIMP1/stella and form

the extraembryonic mesoderm. In humans, at the beginning of the second week, the embryo consists of a bilaminar disc, the epiblast, and the primitive endoderm (or hypoblast). Polar trophectoderm above the epiblast differentiates into the syncytiotrophoblast that invades the uterine tissue and the cytotrophoblast contacting the epiblast. Within the latter, the amniotic cavity forms lined by the amnioblasts derived from the epiblast cells. The primitive endoderm forms the roof and the wall (Heuser's membrane) of the primary yolk sac. On day 10–11, extraembryonic mesoderm of uncertain origin appears between the cytotrophoblast and the yolk sac. Around the end of the second week, the definitive yolk sac is formed by a new wave of cells migrating from the primitive endoderm and displacing the Heuser's membrane. At the beginning of the third week, the primitive streak appears and the gastrulation begins. In humans, the primitive endoderm can be considered equivalent to the mouse VE, while no structure equivalent to the mouse ExE apparently exists (Fig. 2.2). Moreover, the formation of the extraembryonic mesoderm appears to precede gastrulation. However, reexamining the pregastrulation human embryos in the Carnegie collection, Lucket [21] observed that the caudal margin of the primitive streak develops precociously between 12 and 14 days and that this appears to be the source of all the extraembryonic mesoderm.

The significance of these differences for the formation of human PGCs will remain unclear until molecular markers for the human PGC precursors will be identified and a fate map of the human epiblast will become available.

Bona Fide Human PGCs from Stem Cells In Vitro

In order to compensate for the lack of information about the molecular mechanisms of PGC formation in the human embryo, in vitro culture systems able to reproduce some of these processes are now becoming available. These systems are based on the possibility to induce embryonic stem cells (ESCs) derived from blastocysts or induced pluripotent stem (iPS) cells produced from differentiated somatic cells of various origin and to develop into specific cell lineages including germ line. Identification of markers and genes expressed in cells of the germ line at the very early stages of their formation is indispensable to trace back their origin and the mechanisms underlying their formation. Beside TNAP and PAS positivity, molecular markers for human migratory PGCs now include the key pluripotency transcription factors octamer-binding transcription factor 4 (OCT4, [22–24]), Nanog [23, 24], and the tyrosine kinase receptor c-KIT [25] (Table 2.2). Moreover, the surface oligosaccharide, the stage-specific embryonic antigen 1 (SSEA1), and the RNA-binding protein dead box polypeptide 4 (DDX4, also called Vasa) have been reported to be expressed in migratory (5–6 weeks) PGCs by some authors [23, 55], but not by others [32, 56]. The chemokine receptor type 4 receptor (CXCR4) might be also expressed by human PGCs at this stage [60]. After penetrating into the GRs, PGCs continue to express these markers at least until differentiation into oogonia and prespermatogonia. In addition, they express SSEA4 and possibly the RNA-binding proteins, nanos homolog 2 and 3 (NANOS2 and NANOS3 [48]), deleted in the azoospermia (DAZ), and DAZ-like (DAZL) [23, 56]. While SOX2, another key pluripotency transcription factor expressed in early mouse PGCs and ESCs [63], is quite surprisingly not expressed in human PGCs [24], other transcription factors crucial for the formation and specification of mouse PGCs, such as BLIMP1, PRDM14, NANOS1, and stella (see above), have not yet been described in human PGCs in vivo (Table 2.2). They might represent suitable markers to trace back the PGC precursors in the human embryo. At the moment, they are being used to monitor the possible formation of PGCs from stem cell lines *in vitro*. Intriguingly, human ES and iPS cells express a panel of markers common to human and/or mouse PGCs such as TNAP, SSEA4, OCT4, Nanog, stellar (stella-related), BLIMP1, DAZ, DAZL, NANOS1, NANOS3 (in some but not all ESC lines), and c-KIT, but not SSEA1, CXCR4, and DDX4 or synaptonemal complex protein 1 and 3 (SCP1

Table 2.2 Main markers of human PGCs, EG, ES, and EC cells

Marker	PGCs (3–5 weeks)	PGCs (6–9 weeks)	EG cells	ES cells	EC cells
TNAP	+++ [17]	++ [17]	+++[26]	+++ [27]	+++ [28]
PAS histochemistry	+++ [17]	– [17]	++ [29]	++ [30]	ND
SSEA1	Variable [23, 31]	+++ [23]	+++ [26, 32–34]	– [27, 35]	– [35, 36]
SSEA3	ND	Variable [31]	+++ [26, 32–34]	+++[27, 35]	+++ [35, 36]
SSEA4	+++[23]	++[23]	+++ [26, 32–34]	+++[27, 35]	+++ [35, 36]
E-cadherin	ND	ND	ND	+++ [37]	+++ [38]
Fragilis	ND	ND	ND	++ [39]	ND
TRA1-60	– [23]	– [23]	+++ [26, 32–34]	+++ [27, 35]	+++ [40, 41]
TRA1-81	– [23]	– [23]	+++[26, 32–34]	+++ [27, 35]	+++ [40, 41]
OCT4	+++ [22–24]	++ [22–24]	+++ [26, 32–34]	+++[27, 35]	+++ [42, 43]
NANOG	+++ [23, 24]	++[23, 24]	++ [33]	++ [43–45]	++ [44, 46]
NANOS1	ND	ND	ND	+ [47]	ND
NANOS3	ND	ND	ND	++ [48]	ND
SOX2	– [24]	– [24]	ND	+++ [49, 50]	+++ [51]
Stella/stellar (DPPA3, PGC7)	ND	ND	ND	+/–[a] [39, 44, 45, 47, 52]	+ [52, 53]
BLIMP1 (PRDM1)	ND	++ [54]	±/+	±/+[a] [39, 44, 45, 47, 52]	– [54]
DDX4 (Vasa)	++/– [55, 56]	+++ [55, 56]	ND	– [39, 44, 45, 47, 52]	++ [57]
DAZ/DAZL	ND	++ [23, 56]	ND	+/–[a] [39, 44, 45, 47, 52]	– [54, 58]
c-KIT	++ [25]	++ [25]	ND	+ [59]	– [59]
CXCR4	++ [60]	ND	ND	–/+ [61]	++ [62]

[a]Depending on the ES cell lines

and SCP3) markers of pre- and meiotic germ cells [44, 48, 60, 64]. On the other hand, ES and iPS cells express some markers that human PGCs do not, such as SSEA3, tumor rejection antigen 1–60, 1–81 (TRA1-60, TRA1-81), and SOX2 [23, 24, 65] (Table 2.2). Using these in vitro systems, PGC differentiation has been diagnosed primarily by the analysis of germ cell gene and protein expression and more recently, by the use of reporter constructs with the expression of green fluorescent protein (GFP) under control of the DDX4 or OCT4 promoters. Progressively increasing numbers of studies show that human ES and iPS cells can spontaneously differentiate into PGC-like cells, albeit at a low frequency (around 5 %) [47, 66–68]. Most interesting, the efficiency of spontaneous differentiation to PGCs can be increased with the addition of BMP4, 7, and 8b [69, 70], the same growth factors governing the formation of mouse PGCs (see above). Small changes in stem cell culture conditions [60] or coculture with human fetal gonad stromal cells [71], or mouse embryonic fibroblasts (MEF) in the presence of basic fibroblast growth factor (bFGF) [72], have been also reported to favor the formation of putative human PGCs *in vitro*. In addition, silencing the *DAZ* family [70] and *NANOS3* [48] genes in human ESCs resulted in a marked reduction in the capability to give rise to PGC-like cells. These PGCs show ongoing removal of parental imprinting, erasure of global DNA methylation, and histone modifications typical of mouse PGCs [67, 70] supporting the PGC identity.

These data have provided the first experimental evidence that BMPs, and probably bFGF, are involved in the formation of human PGCs and that DAZ and NANOS3 proteins function at some stages of their development. Concerning DAZ and NANOS3, recent studies in the mouse have shown that disruption of *DAZL* gene resulted in postmigratory, premeiotic reduction in PGC number

accompanied by aberrant expression of pluripotency genes and failure to erase and reestablish genomic imprinting in germ cells [73]. Moreover, Gill and colleagues [74] found that in the absence of this gene, PGCs form and migrate to the GRs but do not develop either male or female features. Instead, they remain in a sexually undifferentiated status similar to that of migrating PGCs. Other studies have implicated NANOS3 in the maintenance of mouse PGCs during migration *via* suppression of apoptosis [75].

A Model for the Formation of Human PGCs In Vivo

Taking into account the knowledge reported in the previous sections, a hypothetical model for the human PGC formation can be drawn. It seems plausible that in the human embryo, the precursors of PGCs are set aside within the epiblast between day 10 and 11 following the action of BMP signals coming from the primitive endoderm and cytotrophoblast and/or amnioblasts lining the epiblast. On days 12–14, these precursors move together to the forming extraembryonic mesoderm out the embryo proper and reach the region of the wall of the definitive yolk sac where the allantois originates around day 16. Here they are specified as TNAP-expressing PGCs. Alternatively, these last processes might be delayed for a couple of days. In such a case, PGC precursors would leave the epiblast at the beginning of gastrulation (on day 14 and 15) together with the first wave of cells that replace the primitive endoderm and form the definitive endoderm.

Migration of Human PGCs

During the fourth week, when the embryonic disc undergoes a process of folding, PGCs are passively incorporated into the embryo together with the yolk sac wall. They become transiently segregated as single cells among the endodermal cells of the primitive hind- and midgut epithelium, near the aorta (Fig. 2.2a). The GRs are visible as a distinct structure at the beginning of the fifth week. At this time, PGCs are seen to penetrate the mesenchyme surrounding the gut epithelium through breaks in the basal lamina. In the 5-week-old embryo, PGCs reach the dorsal mesentery and continue to move laterally around both sides of the coelomic angle, pass beyond the primitive mesonephros bodies, and eventually enter the GRs [16, 17, 76–78] (Fig. 2.2b). PGCs colonize the GRs during the latter part of the fifth week or at the beginning of the sixth.

Electron microscopic studies describe human PGCs in vivo as having an irregular appearance and possessing pseudopodia during their journey toward the GRs. These features are interpreted as a manifestation of active migration [16, 77, 79] (Fig. 2.2b). An interpretation confirmed by several in vitro observations reporting that human PGCs, as those of mouse, show several features of motile cells and are able to move actively both on cellular and extracellular matrix substrates [80–83] (Fig. 2.4). The in vivo time-lapse experiments with confocal microscopy by Molyneaux and colleagues [84] in slices of mouse embryos appeared to definitively confirm that in mammals, PGCs reach the GRs by active migration. However, Freman [85], reinterpreting these and other observations reported above, concludes that morphogenetic movements and local cell divisions rather than active migration are mainly responsible for PGC displacement in the different regions of the embryo. Even Freman, however, admits that human PGCs might migrate actively to cover a distance of approximately 50 μm separating the preaortic region from the GRs.

The mechanisms by which not only human PGCs but also those of other mammals are finally delivered to and colonize specifically the GRs remain largely unknown. Contact guidance with somatic and/or extracellular molecules (ECM) and attractive (chemotaxis) and repulsive signals are two unmutually exclusive mechanisms suggested by evidence in the mouse and other vertebrate species (for a recent review, see [86]). In the human embryo, migratory PGCs appear to be surrounded by extracellular matrix components in which mesenchymal cells are immersed. PGCs seem to interact with the mesenchymal cells through different type of junctions, such as

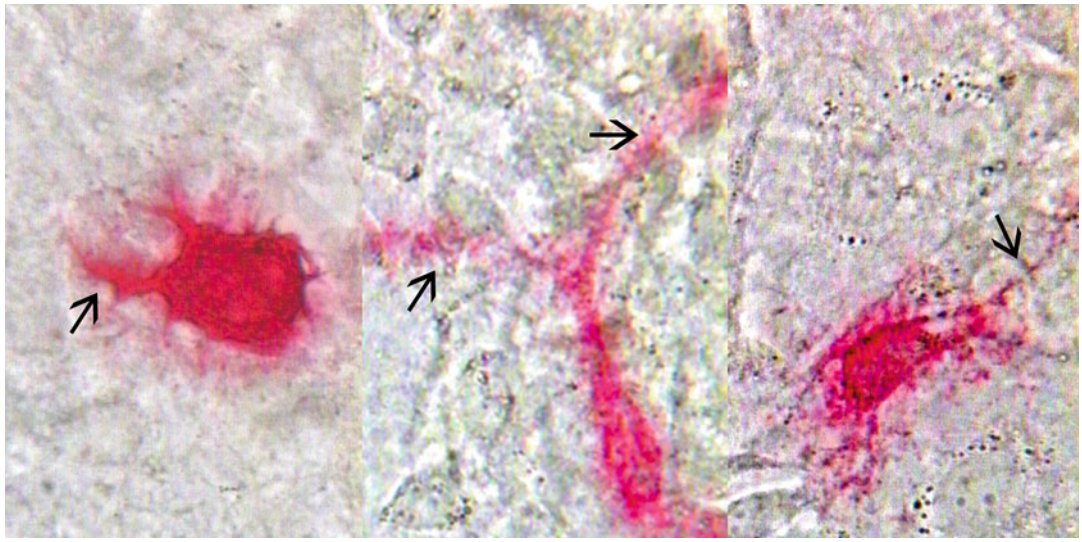


Fig. 2.4 Human PGCs isolated from 6–7 week embryo and cultured in vitro for 2 days onto STO fibroblasts identified by TNAP staining. PGCs usually appear elongate with pseudopodia and fine filopodial extensions (*arrows*)

desmosomes, gap junctions, and focal contacts [77, 87]. Glucosaminoglycans have been histochemically detected in the extracellular matrix surrounding migratory human PGCs ([87] and references here in). Moreover, several results demonstrated that mouse PGCs may use various types of integrins for dynamic adhesive interactions with extracellular matrix molecules such as fibronectin, laminin, and collagen IV (for a review, see [88]). In this regard, it is important to report that mouse PGCs lacking β1 integrins fail to migrate normally to the GRs [89].

In the opinion of Witschi [16], the coelomic epithelium in the region of the GRs releases specific molecules to attract PGCs. The use of migratory cell assays similar to those used for mouse PGCs [90, 91] could help to identify attractants for human PGCs. The tumor growth factor-β (TGFβ) [92], stromal-derived factor1 (SDF1) [93], and stem cell factor (SCF, or c-Kit ligand, KL) [91] have been proposed as chemoattractants for mouse PGCs. KL and SDF1 could be also implicated in directing the migration of human PGCs. The KL receptor c-KIT is expressed by human PGCs [25], while putative PGCs obtained from human ESCs express the SDF1 receptor CXR4 [60].

The difficulty in identifying specific chemoattractants in PGCs suggests that the underlying mechanisms of migration are complex and are likely to involve morphogenetic movements, interactions with ECM molecules and the surrounding cells, and attractive and repulsive signals as well. PGCs might recognize more than one attractive and repulsive signal at particular locations of their pathways and/or recognize different signals at different locations. The expression of transcripts for member of the olfactory receptor gene family in human PGCs from 10-week-old embryos makes this class of receptor additional candidates for PGC attractants [94]. Finally, in a recent study, Møllgard and colleagues [78] observed that human PGCs preferentially ascended from the mesentery of the hindgut to the gonadal anlage by migration along autonomic nerve fibers close to the Schwann cells and proposed that these nerve fibers and/or Schwann cells may release chemoattractants supporting PGC migration.

During migration, human PGCs contain a large PAS-positive cytoplasmic store of glycogen and several lipid droplets [16, 76, 77]. In other species, these cytoplasmic inclusions were not observed. Following their arrival in the GRs, the glycogen content is diminished. Round mitochondria with a pale matrix and small tubular vesicular cristae were observed near the nucleus.

They significantly increase in number during PGC migration and settlement in the GRs. Migratory PGCs have less than 10 mitochondria, while 100 mitochondria are present in ovarian PGCs and 200 in oogonia [95]. These observations suggest that PGCs might prevalently employ an anaerobic metabolism during migration and undergo a transition in their energy metabolism after reaching the GRs.

An interesting issue concerning PGC migration is the fate of the misallocated cells which fail to reach the gonads. While those remaining nearer the gonads were observed to enter meiosis irrespective of the sex [96–98], most of the other misallocated cells are believed to undergo apoptosis [98, 99] or to give rise to germ cell tumors (GCTs) after birth. Human GCTs are a heterogeneous group of tumors that may occur both in the gonads and at extragonadal midline sites such as the coccyx, the pineal gland, and the mediastinum [100, 101]. Runyan and colleagues [102] have recently identified in the caudal region of the mouse embryo a population of undifferentiated ectopic PGCs that might be the population of origin for sacrococcygeal tumors. The possible causes of the PGC transformation into tumorigenic cells will be discussed in the next section. Another possibility is that ectopic PGCs enter near the aorta and are distributed to various tissues throughout the embryo. In birds and reptiles, the vascular system is a normal way to deliver PGCs to the GRs [103]. In mouse [104] and bovine [105] embryos, electron microscope observations showed cells morphologically identifiable as PGCs entering or circulating in the bloodstream. Because of their intrinsic pluripotency, under certain circumstances, these cells might participate to normal tissue differentiation or enter a quiescent status to later give rise to tumors. In my view, such a possibility represents an intriguing working hypothesis for future investigations.

Proliferation of Human PGCs

Human PGCs proliferate during migration, mostly after reaching the GRs. Once relocated in the GRs, PGCs are rapidly surrounded by cords of somatic cells. The differentiation of PGCs into oogonia occurs apparent during the ninth week. Oogonia show a higher mitotic activity and possess a regular and smooth cellular profile. In the cytoplasm, lipid inclusion and glycogen granules are markedly reduced while the number of mitochondria is increased (see above) [106, 107]. In addition, oogonia tend to form clusters of dividing cells joined by rims of cytoplasm, termed intercellular bridges, originated by incomplete division of the cell body during cytodieresis [108, 109]. The mitotic proliferation of oogonia lasts several weeks and overlaps the period of their entry into meiosis (10–11 weeks). In fact, until the fifth month of fetal life, mitotic oogonia and primary oocytes in different stages of meiosis coexist [110, 111].

In the male, after reaching the developing testis, PGCs are usually termed gonocytes. While being enclosed within seminiferous sex cords, gonocytes assume some distinct morphological features, such as a large nucleus and a prominent nucleolus [112]. Male sex determination is marked by the expression of sex determining region Y (SRY) and SRY-box 9 (SOX9) genes at 5–6 weeks [113]. Thereafter, sex cords, formed from Sertoli cells and gonocytes, become increasingly evident within the testis from the seventh week during late embryonic life. During the first trimester, gonocytes are mitotically active (they correspond to the M-prospermatogonia of Wartenberg's classification [114]). They appear to form a quite homogenous single, round cell population both morphologically and histochemically [115]. Of relevance, gonocytes continue to express markers typical of pluripotent cells and PGCs such as OCT4, SSEA1, DDX4 (Vasa), Nanog, and c-KIT [23, 114]. This means that despite some morphological differences, gonocytes are basically equivalent to PGCs. During the second trimester, most but not all gonocytes progressively lose mitotic activity together with the pluripotency and PGC markers. At this time, two new types of germ cells have been described, intermediate germ cells still able to proliferate and mitotic quiescent prespermatogonia or T (transition)-spermatogonia. The former are mostly in pairs, while the latter form groups

of cells interconnected by cytoplasmic bridges [114–116]. Interestingly, a few cells maintaining gonocyte characteristics seem to remain among the prespermatogonia [23, 115]. Similar results have been recently reported in the mouse [117]. The fate of these undifferentiated cells could be relevant in establishing the spermatogonia stem cell population in the postnatal testis and for the development of testis tumors.

Considering the observations and the results reported above, we can estimate that the period of human PGC proliferation in the female lasts from the beginning of the fourth week to about the ninth week, when oogonia become clearly recognizable within the fetal ovary. In the male, if we consider the gonocytes equivalent to PGCs, proliferation continues probably for a little longer period until about the end of the first trimester (10–12 weeks), when the most part of gonocytes differentiate into prespermatogonia. Significantly, in both sexes, the occurrence of intercellular bridges appears to mark the differentiation of PGCs into oogonia and gonocytes into intermediate germ cells and prespermatogonia. In the ovaries, oogonia continue to proliferate until the fifth month. During the same time in the testes, intermediate germ cells proliferate while prespermatogonia become progressively mitotically quiescent.

Counting the number of PGCs at different stages of development provides estimation of their proliferation capability and possible sex differences. As reported in a previous section, in an embryo of 3 weeks, Politzer counted 40 PGCs that increased to 600 in an embryo of 4 weeks. Similarly, Witschi [16] counted 30–50 PGCs around the end of the third week and 109 in an embryo a couple of days older. The same authors also counted about 450 and 1,400 PGCs in two embryos of 4 weeks. In his classical study, Baker [110] estimated the number of female germ cells in a total of 12 ovaries covering 2–7 months postconception. He estimated a mean of a total 600,000 oogonia in two 9-week-old ovaries and a peak of about 6,000,000 at the fifth month. Six recent publications presenting stereological estimations of the number of germ cells in much higher numbers of ovaries and testes (overall 103) for the first two trimesters have been recently analyzed by Mamsen and colleagues [118–124]. Extrapolating the old and the new data covering the 4- to 9-week period, it results that the total number of PGCs increases from about 1,000 to about 450,000 in female and 150,000 in male (Fig. 2.5).

The regulation of human PGC proliferation in both sexes is poorly understood. The scant available information comes mainly from in vitro studies of human PGCs cultured on cell monolayers. These studies indicate that human PGCs appear to respond to the same compounds (forskolin, retinoic acid) and growth factors (SCF, bFGF, leukemia inhibitor factor, LIF) reported to stimulate the survival and/or proliferation of mouse PGCs ([26], our unpublished observations). Most importantly, like mouse PGCs, human PGCs give rise to pluripotent embryonic germ cells (EGCs) (see also next section), when cultured in vitro in the presence of a cocktail of compounds and growth factors [26], suggesting that the mechanisms controlling PGC growth in mammals are largely conserved. Hiller and colleagues [125] recently reported that the addition of recombinant BMP4 increased the number of human PGCs after 1 week of in vitro culture in a dose-responsive manner. The efficiency of EGC derivation and maintenance in culture was also enhanced.

Analysis of gene expression in human PGCs and the study of spontaneous mutations resulting in reduction or absence of fertility may help to confirm or disprove such similarities. For example, as reported above, c-KIT is expressed by both male and female PGCs. Mutations in the *c-KIT* gene affect both hematopoietic and melanocyte lineages in humans, but to date, no association of mutations in this gene with human infertility has been documented, contrary to the mouse model. However, while no mutations have been detected in codon encoding Y721 (analogous to Y719 in the murine *c-kit* gene, a residue known to be essential for a normal mouse spermatogonial proliferation) of the human *c-KIT* gene of infertile idiopathic patients [126], other results indicate that genetic variants within the genomic sequences of the *c-KIT* and *KITLG* genes are associated with idiopathic male infertility [127]. On the other

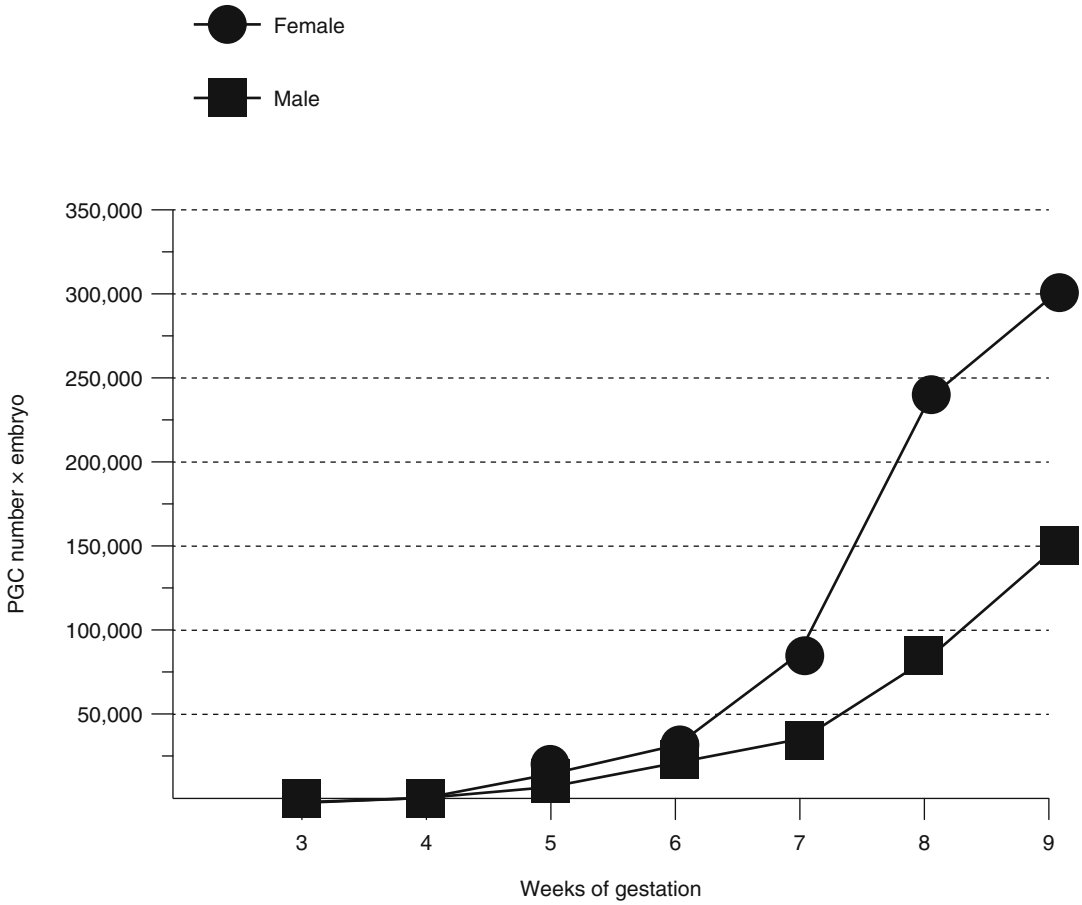


Fig. 2.5 The number of human PGCs in female and male embryos from 3 to 9 weeks of gestation extrapolated by the data of papers [14–16, 110, 118–122]. Note that from 5 weeks onward, the number of PGCs is much higher in female than in male embryos. This difference continues also when PGCs differentiate into oogonia or gonocytes/prespermatogonia. At the end of the proliferation period (around fifth month), the estimated number of oogonia is around 10,000,000 and of prespermatogonia between 3,000,000 and 4,000,000 per embryo [110, 118]

hand, *c-KIT* is strongly upregulated in some types of germ cell testicular tumor that are believed to originate from PGCs (for a review, see [128]), suggesting that it plays a crucial role in the control of human PGC survival/proliferation as in mice. In this regard, *OCT4* was found to be expressed in all human germ cell tumors containing undifferentiated cells [129]. In Fanconi's anemia (FA), individuals are characterized by several congenital abnormalities including decreased fertility (for a review, see [130]). Interestingly, targeted mutation of *Francc* in mice results in significantly slower proliferation of PGC [131], suggesting again shared control mechanisms of PGC proliferation in these species. Finally, in Trisomy 16 of mouse, an animal model of the human Down's syndrome leading frequently to sub- or infertility, a delay in migration and reduction of PGC number was observed [132]. Gene expression studies on single human PGC obtained from 10-week-old embryos have been reported [133, 134]. The preparation of cDNA libraries and microarrays from human PGC should be valuable resources for researchers in this field.

Human EGCs and Their Potential Uses

Derivation of human embryonic germ cell (EGCs) has been reported by several groups from PGCs obtained from 5- to 9-week-old embryos [26, 32–34]. The differentiation capability both in vitro and

in vivo of these cells into several types of tissues has been reported, and important studies toward therapeutic use of human EGCs are in progress ([135] and references therein). An important difference with mouse is that human EGCs maintain the methylated status of imprinted genes, those genes that are expressed from either the maternal or paternal allele, without undergoing the erasure of these epigenetic marks that normally occurs in PGCs [136]. Genomic imprinting is an epigenetic process that involves DNA methylation in order to achieve monoallelic gene expression without altering the genetic sequence. These epigenetic marks are established in the germ line and are maintained throughout all somatic cells of an organism. Appropriate expression of imprinted genes is important for normal development, with numerous genetic diseases associated with imprinting defects. Imprinting is erased during the PGC development and reestablished in germ cells during gametogenesis according to the sex of individuals (for a review, see [137]). In mouse, gene imprinting is progressively erased in migratory PGCs and lost almost completely by the time they complete the colonization of the GRs (for a review, see [138]). The timing of this erasure is not known in humans. In the mouse, deregulation of imprinted genes in EGCs has been reported to cause imprinting-related developmental abnormalities [139]. Similarly, mouse ESCs fail to properly control the expression of imprinted genes [140]. In humans ESCs, gene-specific differences in the stability of imprinted loci have been reported [141]. The stability of imprinting in human EGCs suggests that there may be no significant epigenetic barrier to human EGC-derived tissue transplantation. The difference between human and mouse EGCs in the imprinting status also suggests that the timing of the crucial epigenetic changes involving gene imprinting erasure might be different in human and mouse PGCs. In the latter, as reported above, gene imprinting is progressively erased and lost almost completely by the time of GR colonization by PGC (for a review, see [138]). This pattern is reflected in the variability of the imprinting status reported in mouse EGCs derived from PGCs at different developmental stages [139, 142, 143]. Deregulation of imprinted genes can be associated with tumorigenesis and altered cell differentiation capacity. Noteworthy, human EGCs have a normal karyotype and do not form tumors at least when transplanted into immunocompromised mouse hosts ([33] and references therein).

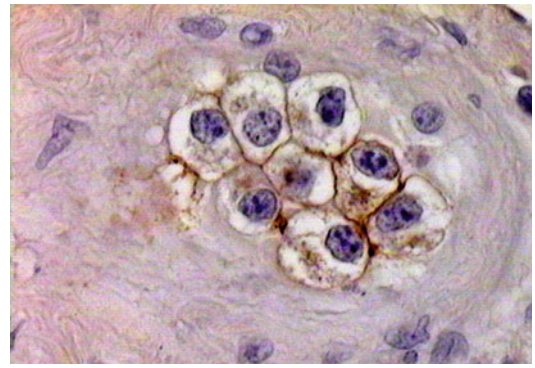


Fig. 2.6 A group of carcinoma in situ (CIS) cells inside a seminiferous tubule of a human testis. The derivation of these tumorigenic cells from PGCs or gonocytes or spermatogonia and the molecular mechanisms of their formation are still debated

Besides the use in tissue therapy, studies on human EGCs can offer clues about important aspects of cell stemness and on the mechanisms underlying the transformation of PGCs into tumorigenic cells. For a long time, as reported above, human PGCs have been believed to give rise to germ cell tumors (GCTs) both in the testis and extragonadal sites, but an experimental model was lacking. GCTs can be classified in three categories. The first group includes teratomas and teratocarcinoma and yolk sac, which occur in fetus and infants. Teratocarcinomas are malignant tumors containing undifferentiated cells known as embryonal carcinoma (EC) cells, able to propagate the tumors after host transplantation. The second group consists of adult tumors and includes both seminomas and nonseminomas. The third class characterizes spermatocytic tumors which occur in elderly men. In the mouse, early studies have demonstrated that teratomas and EC cells originate directly from PGCs [144] so that the same origin is plausible for these type of tumor cells in humans. Seminomas and nonseminomas derive from a common precursor cell, called carcinoma *in situ* (CIS) cells. It has been hypothesized that CIS cells originate from fetal germ cells [145] (Fig. 2.6). Comparative microarray studies have shown that CIS cells show a high degree of pluripotency and

are indeed very similar to PGCs and gonocytes. CIS cells are consistently aneuploidy. Moreover, CIS cells possess an abnormal chromosome described as isochromosome 12p, or i(12p), formed from two fused short arms (p arms) of chromosome 12 [146]. The arrest of PGC differentiation and their nuclear reprogramming that in mouse have been convincingly reported to occur during PGC transformation into EGCs (for a review, see [138]), might be the key first events, that may be followed by malignant transformation into EC or CIS cells associated to the acquisition of an abnormal karyotype and overt germ cell cancer later in life.

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Oogenesis
Coticchio, G.; Albertini, D.F.; De Santis, L. (Eds.)
2013, XII, 364 p. 62 illus., 42 illus. in color., Hardcover
ISBN: 978-0-85729-825-6