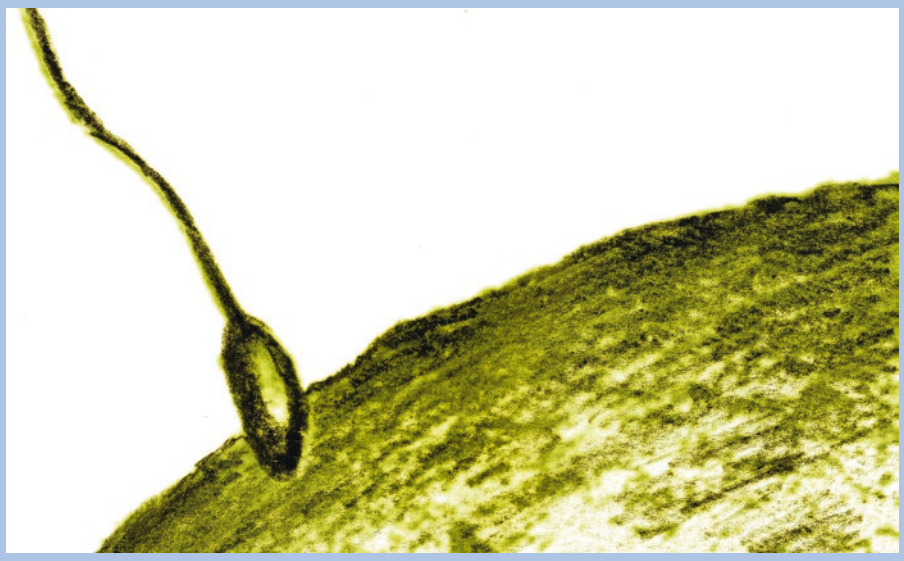
Origins of Pluripotency: From Stem Cells to Germ Cells

Maria Gomes Fernandes and Susana M. Chuva de Sousa Lopes



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What Will You Learn in This Chapter

In this chapter, the basic concept of pluripotency is explored. You will learn about the origins of pluripotent stem cells, both in mice and in human. The embryonic origins of stem cells will be discussed. The differences and similarities between naïve and primed embryonic stem cells will be described, and you will learn about the gene regulatory networks and signalling pathways that regulate each of them. This will relate to different culture conditions and the use of small molecules to keep pluripotency in vitro. You will also learn about the origin and development of primordial germ cells, the signalling pathways involved and how studies regarding pluripotency in vitro and in vivo helped to establish protocols for the generation of primordial germ cell-like cells in vitro.

Learning Objectives

After completing this chapter, students should be able to:

1. Distinguish different types of pluripotent stem cells and the signalling needed to maintain them in vitro
2. Pinpoint the differences between mouse embryonic stem cells and human embryonic stem cells
3. Understand the origins and development of primordial germ cells and the main differences and similarities between animal models
4. Understand the main signalling pathways and markers associated with specific stages of germ cell development
5. Understand the need for developmental biology studies together with stem cell biology studies for the development of in vitro protocols for the derivation of primordial germ cells and vice versa

Important Concepts Discussed in This Chapter

- Naïve and primed pluripotency
- The ground state of pluripotent stem cells
- Gene regulatory networks
- Epigenetic state of naïve and primed pluripotency
- Signalling pathways and relationship with pluripotency
- Primordial germ cells

3.1 Early Mouse Embryonic Development

Fusion of a mature oocyte with a sperm cell results in the formation of the zygote at embryonic day (E)0, and the life cycle of an organism (and of its germ cells) starts once again. The zygote has the ability to generate all the cells of the embryo, including the extraembryonic cells, essential to support proper embryonic development. The zygote is therefore referred to as totipotent. After a series of cleavage divisions, a morula (4–16 cells) is generated (E2.0–E2.5, in mice). In mice, the cells of the uncompacted morula can still give rise to all extraembryonic and embryonic tissues, and are still considered totipotent.

At the 32-cell stage, the morula undergoes a process called compaction followed, at E3.0, by cavitation – the formation of a central cell-free cavity. In mice, the E3.5 blastocyst has a distinctive inner cell mass (ICM) and an outer layer of trophectoderm cells (TE) [1, 2] (Fig. 3.1a). Expression of the transcription factor *Cdx2* directs TE

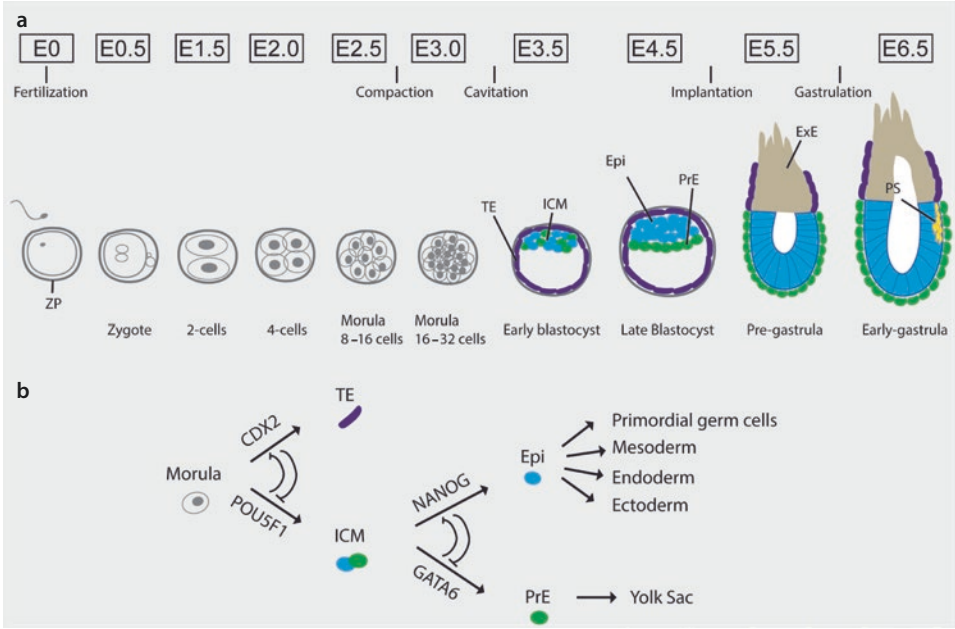

Fig. 3.1 Mouse early embryonic development and lineage choices. **a** Overview of mouse early embryonic development from the moment of fertilization, at embryonic day (E)0, until the onset of gastrulation with the appearance of the primitive streak (PS) at E6.5. **b** Main lineage choices and transcription factors that regulate those choices in the pre-implantation mouse embryo. *CDX2* drives trophectoderm (TE) differentiation while *POU5F1* keeps pluripotency in the inner cell mass (ICM). Later, *GATA6* drives primitive endoderm (PrE) fate while *NANOG*-positive cells give rise to the epiblast (Epi). The epiblast will form the primordial germ cells and the germ layers of the embryo (mesoderm, endoderm, ectoderm). Abbreviations: TE trophectoderm, ICM inner cell mass, Epi epiblast, PrE primitive endoderm, ExE extraembryonic ectoderm, PS primitive streak, E embryonic day

differentiation, while ICM fate is directed by *Pou5f1* (or *Oct4*) expression [3, 4]. At this stage, the ICM can only give rise to the embryonic germ layers (ectoderm, endoderm and mesoderm), extraembryonic mesoderm and germline, and consists of pluripotent cells [5]. In mice, E3.75 ICM is composed of two distinct populations of cells: *Gata6+Nanog-* cells and *Gata6-Nanog+* cells. *Gata6+* cells are the primitive endoderm (PrE) progenitors that, at around E4.0, become lineage restricted and are located between the blastocoel cavity and the *Gata6-Nanog+* epiblast (Epi) [6, 7] (Fig. 3.1b). Thereafter, in mice at E5.0, implantation with the formation of the egg cylinder begins, and at E5.5, the anterior–posterior axis of the embryo is defined. Gastrulation, during which the three embryonic germ layers form, begins around E6.0 (Fig. 3.1a). The Epi loses its functional pluripotency around E8.0 [8–10], becoming the multipotent ectoderm layer that can only give rise to ectoderm derivatives [11].

3.2 Mouse Embryonic Stem Cells

Mouse embryonic stem cells (mESCs) were first established from the ICM of E3.5 blastocysts in 1981 [12, 13] (Fig. 3.2). These cells have an unlimited capacity for self-renewal during in vitro culture, can be expanded clonally and retain pluripo-

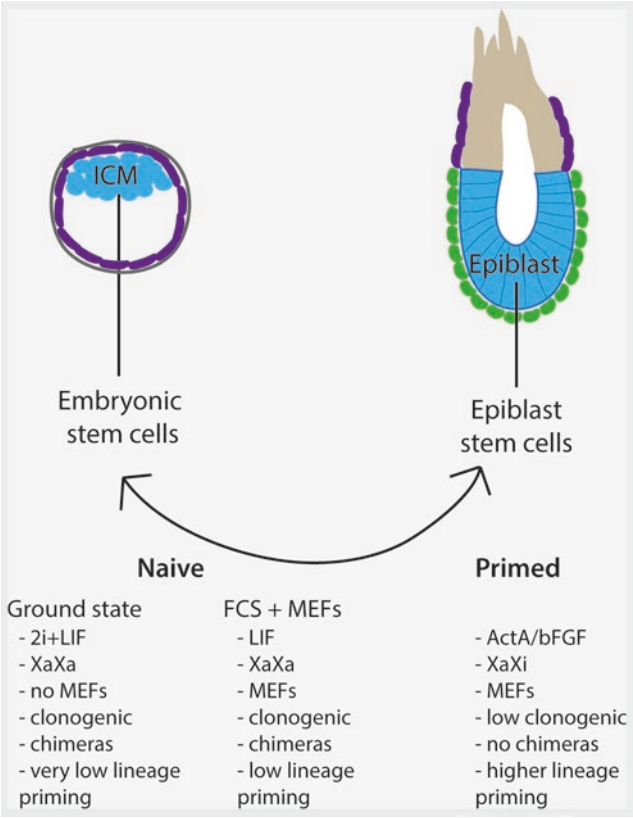

Fig. 3.2 Different types of mouse embryonic stem cells. Pluripotent cell lines can be derived from the inner cell mass (ICM) of the mouse blastocyst or from the epiblast (Epi) of the early implanting embryo. These lines reflect their embryonic origin and have different signalling requirements in culture. Ground state and naïve mouse embryonic stem cells (mESCs) are LIF-dependent. Ground state mESCs are MEFs and FCS-free. Epiblast embryonic stem cells (EpiSCs) are FGF-dependent and have one inactivated X chromosome (Xi) contrary to the two active X (Xa) in ground and naïve mESCs (reflecting the X status of the ICM and epiblast). Although EpiSCs can differentiate into the three germ layers in vitro, they do not generate chimeras and have lower clonogenicity than naïve mESCs. Abbreviations: Epi epiblast, ICM inner cell mass, mESCs mouse embryonic stem cells, MEFs mouse embryonic fibroblasts, FCS foetal calf serum, EpiSCs epiblast embryonic stem cells, LIF leukaemia inhibitory factor, FGF fibroblast growth factor

tency, as they are capable of generating all the cells of the adult body in chimeras, but not TE-derived cells [14]. The characteristics of mESCs in culture also reflect their biological origin: they express ICM-associated genes such as *Pou5f1*, *Nanog* and *Sox2*, and can be differentiated in vitro into derivatives of mesoderm, endoderm and ectoderm, but they do not form TE-derived cells [14–16]. Pluripotency of mESCs is usually demonstrated through two classical in vivo experiments: (1) when injected into immunodeficient mice, mESCs are capable of generating tumours containing derivatives of the three embryonic germ layers called teratomas ("teratoma assay"); and (2) when injected into a blastocyst or after morula aggregation, mESCs can contribute to the three embryonic germ layers and the germline of the resulting chimeric embryo.

3.2.1 States of Pluripotency In Vitro

The first in vitro culture methods for mESCs involved the conditioning of medium by teratocarcinoma stem cell lines as the main source of growth factors [12, 13]. Later, it was demonstrated that the cytokine leukaemia inhibitory factor (LIF) was one of the growth factors required for mESC self-renewal and to inhibit differentiation [17, 18]. LIF signals through the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway, particularly the JAK/STAT3 axis [19, 20]. Nevertheless, mouse embryonic fibroblasts (MEFs) and foetal calf serum (FCS) were usually still required as the source of other important factors. mESCs derived from the E3.5 ICM are referred to as naïve pluripotent stem cells. They differ from another type of pluripotent mouse stem cells derived from the E5.5 post-implantation epiblast, known as mouse epiblast stem cells (mEpiSCs) [21, 22] (◘ Fig. 3.2). Despite being pluripotent in vitro, mEpiSCs do not give rise to chimeras very efficiently; thus, they are considered to be in a primed state [23, 24]. mEpiSCs require ActivinA (ActA) and fibroblast growth factor 2 (FGF2) to maintain pluripotency rather than LIF and FCS, and mEpiSCs are highly prone to undergo apoptosis when passaged in single cells [22, 24]. Naïve mESCs cultured in the presence of MEFs and FCS show a high degree of heterogeneity and transit between the naïve-like and primed-like pluripotency states [25, 26].

In the absence of FCS, LIF alone is not sufficient to block mESC commitment to differentiation. However, this is bypassed by the addition of two small-molecule inhibitors (called 2i): CHIR99021 and PD0325901. CHIR99021 acts by inhibiting glycogen synthase kinase 3 beta (Gsk3β) and consequently activating the WNT/β-catenin signalling pathway. PD0325901 is a specific inhibitor of the extracellular signal-regulated kinase (ERK1/2)/mitogen-activated protein kinase (MAPK) signal transduction pathway [27]. mESCs cultured in LIF+2i medium can be maintained without FCS and MEFs, and are in the ground state of pluripotency. The identification of this ground state allowed not only the maintenance of mESCs under chemically defined culture conditions but also the derivation of mESCs from "non-permissive" mouse strains such as C57Bl6 [28]. Ground state mESCs are phenotypically more homogeneous than mESCs grown in FCS and MEFs, show lower levels of DNA methylation and lower expression of lineage specific-associated genes, and hence are more similar to E3.5 ICM cells than mESCs grown in FCS and MEFs [29, 30] (◘ Figs. 3.2 and 3.3).

3.2.1.1 Gene Regulatory Networks in Mouse Naïve Pluripotent mESCs

The core gene regulatory network associated with pluripotency in naïve mESCs consists of *Nanog*, *Sox2* and *Pou5f1* [31]. It is the delicate transcriptional balance between these core regulatory genes that maintains naïve pluripotency and prevents differentiation during in vitro culture [32]. In addition to these core genes, other transcription factors such as *Klf2, Klf4, Zpf42, Myc, Prdm14, Sall4, Tfcp2l1, Esrrb, Tcf3, Gbx2, Dppa3* and *Tbx3* are also involved in the maintenance of pluripotency in naïve mESCs [33–40]. The balance between self-renewal and differentiation in mESCs is also regulated by factors that include *Id1* and *Dusp9*, both downstream targets of the bone morphogenic protein (BMP) signalling pathway [41, 42].

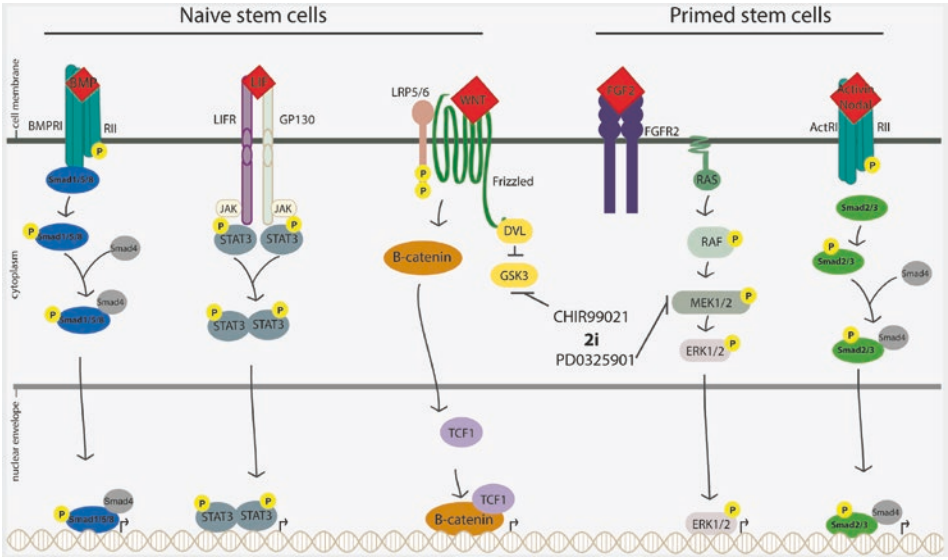

Fig. 3.3 Signalling pathways in naïve and primed pluripotency. The major signalling pathways in pluripotency include the BMP, LIF, WNT, FGF and ActivinA/Nodal signalling pathways. Typically, the ligands (red) bind and bring together their transmembrane receptors and co-receptors. Those complexes usually include a kinase that is able to phosphorylate (P) the cytoplasmic effectors, which are then able to translocate to the nucleus and regulate transcription of target genes. Abbreviations: BMP bone morphogenic protein, BMPRI BMP receptor type 1, RII receptor type 2, P phosphorylation, LIF leukaemia inhibitory factor, LIFR LIF receptor, GP130 glycoprotein 130, JAK Janus kinase, STAT signal transducer and activator of transcription, WNT wingless/integrated, LRP low-density lipoprotein receptor-related protein, DVL dishevelled, GSK glycogen synthase kinase, TCF T-cell factor, FGF fibroblast growth factor, FGFR2 FGF receptor 2, RAS rat sarcoma oncogene, RAF rapidly accelerated fibrosarcoma, MEK mitogen-activated protein kinase kinase, ERK extracellular signal-regulated kinase, ActRI activin receptor type 1

LIF Signalling in mESCs

The LIF signalling cascade is initiated with the binding of LIF to its receptor LIFR in association with its co-receptor subunit glycoprotein 130 (gp130). LIFR and gp130 heterodimers activate associated tyrosine kinases such as the family of Janus kinases (JAKs). JAKs then phosphorylate gp130 promoting the recruitment of STAT3. STAT3 is also phosphorylated by JAKs, dimerizes and translocates to the nucleus where it regulates the transcription of target genes [43, 44] (Fig. 3.3).

In mESCs, LIF triggers several different signalling pathways: the JAK/STAT3 pathway; the PI3K (phosphoinositide 3-kinase)/PKB (protein kinase B) pathway; and the SHP2 (SH2 domain-containing tyrosine phosphatase 2)/MAPK pathway [45, 46]. Nevertheless, only the STAT3 pathway is essential for LIF-mediated mESCs self-renewal [47, 48]. Downstream targets of LIF are, for example, *Myc*, *Klf4, Pim1/3, Prr13, Gbx2, Pramel7, Pem*/*Rhox5, Jmjd1a* and *Tfcp2l1* [36, 40, 49–54].

BMP Signalling in mESCs

BMP is part of the larger transforming growth factor (TGF)-β family. These proteins are involved in the regulation of cell proliferation, differentiation and apoptosis, thus playing key roles during embryonic development and pattern formation [55]. The

BMP-SMAD canonical signalling pathway depends on the activation and subsequent heteromerization of its receptors by BMP ligands. There are two types of BMP receptors: receptor type I ALK2 (or AcvR1A), ALK3 (or BMPRIA) and ALK6 (or BMPRIB), and receptor type II (BMPR2, AcvR2A and AcvR2B), both necessary to mediate BMP signalling. Once receptor type I is activated by phosphorylation by receptor type II, it can bind and phosphorylate the downstream intracellular receptor (R)-SMADs 1, 5 and 8. The activated R-SMADs complex with the common-mediator SMAD4 and can be then translocated to the nucleus where the complex binds to specific target sequences to regulate transcription of target genes [56] (◘ Fig. 3.3).

BMP4 cooperates with LIF to promote self-renewal of mESCs in 2i-culture conditions by blocking neuronal differentiation. In the presence of LIF alone (no BMP4), mESCs cannot be maintained undifferentiated, but undergo neuronal differentiation [41, 57]. On the other hand, in the presence of BMP4 alone (no LIF), mESCs undergo differentiation to mesoderm, endoderm and *Cdx2*+ derivatives, presumably from the trophoblast lineage, instead [58].

BMP-SMAD signalling is, nevertheless, dispensable for self-renewal, since mESCs knockout for *Smad1* and *Smad5* self-renew at the same rate as the wild-type lines [42]. This double KO mESC line remained pluripotent, but showed high levels of DNA methylation and high propensity to differentiate, highlighting a role of BMP-SMAD in the regulation of lineage priming, rather than self-renewal.

WNT Signalling in mESCs

In the presence of WNT ligands, the transmembrane receptor frizzled (FZ) and LRP6 or LRP5 (low-density lipoprotein receptor-related protein 5 or 6) form a complex. This WNT-FZ-LRP5/6 complex recruits dishevelled (Dvl), and this event promotes the phosphorylation of LRP5/6 and activation and recruitment of the Axin complex. This inhibits Axin-mediated β-catenin phosphorylation, promoting the stabilization of β-catenin and its accumulation in the cytoplasm. β-Catenin will then translocate to the nucleus, forming complexes with TCF/LEF, regulating the expression of WNT targeted genes [59] (◘ Fig. 3.3).

WNT/β-catenin signalling insures the maintenance of naïve pluripotency in mESCs by multiple mechanisms and tight synergy with other signalling pathways like BMP-SMAD, FGF-ERK and TGFβ-ActA [60–62]. In addition to effects on DNA methylation by regulating the expression of TET proteins [63], the activation of WNT signalling results in the upregulation of *Stat3*, *Klf2* and *Tfcp2l1* [64, 65], and the suppression of neuroectodermal differentiation by downregulation of *Tcf3* [66]. Fluctuations of β-catenin have been correlated with *Nanog* and *Pou5f1* expression in naïve mESCs [67, 68].

3.2.1.2 Gene Regulatory Networks in Mouse Primed Pluripotent mEpiSCs

The core transcriptional regulatory genes, *Pou5f1*, *Nanog* and *Sox2*, are still expressed in mEpiSCs, although there is a downregulation of *Nanog* [69]. Nevertheless, the similarities end here. Whereas genes like *Klf2*, *Klf4, Klf5, Zpf42, Esrrb, Dppa3, Tfcp2l1, Fgf4, Tbx3* and *Cdh1* are highly expressed in naïve mESCs, mEpiSCs express genes associated with early lineage specification like *Dnmt3b, Fgf5, Pou3f1, Meis1, Otx2, Sox11, Sox17, T* and *Gdf3* [70]. *Essrb* expression is reduced in mEpiSCs, due

to the translocation of *Tfe3* from the nucleus to the cytoplasm during conversion to the primed state [71]. Primed pluripotency requires both the TGF-β signalling pathway, via the ligands ActA/Nodal and FGF2 (or bFGF) for self-renewal [21, 22].

FGF Signalling in mEpiSCs

The FGF family contains 22 genes divided into 6 subfamilies. Each FGF ligand binds to specific splice variants of the FGF receptor (FGFR) and uses either heparin-like glycosaminoglycans or transmembrane Klotho enzymes as co-factors. Upon ligand–receptor interaction, autophosphorylation of the intracellular region of the FGFR occurs, and this can activate four distinct pathways: JAK/STAT, PI3K, PLCγ (phosphoinositide phospholipase C) and Erk pathways [72].

FGF2 uses heparin-like glycosaminoglycans as co-factors [73]. FGF2 appears to stabilize the primed pluripotency state by dual inhibition of differentiation to neuroectoderm and blocking the reversion to a naïve state [62] (◘ Fig. 3.3). FGF2 also has an indirect effect on the maintenance of primed pluripotency by stimulating MEFs to produce ActA [74]. Interestingly, it has been shown that FGF4, which also uses heparin-like molecules as co-factors, promotes self-renewal of mEpiSCs without exogenous stimulation of ActA/Nodal [70]. mEpiSCs cultured with FGF4 were more homogeneous regarding *Pou5f1* expression [70].

ActA/Nodal Signalling in mEpiSCs

ActA and Nodal are members of the TGF-β superfamily [75]. ActA and Nodal ligands signal via the same receptors and effectors, and in the majority of the cases, the resulting signalling is the same. They bind to receptor type II AcvR2A and AcvR2B (both also BMP receptors), leading to the recruitment of the specific receptor type I ALK4 (or AcvR1B) and ALK7 (or AcvR1C) [76]. AcvR2A/2B and ALK4/7 then trigger the phosphorylation of the R-SMADs 2 and 3, which complex with the common-mediator SMAD4 and translocate to the nucleus regulating gene expression of specific targets [75] (◘ Fig. 3.3). Activation of the ActA/Nodal pathway promotes self-renewal of EpiSCs via direct activation of *Nanog*, whereas inhibition of this pathway induces neuroectodermal differentiation [62].

3.2.1.3 Epigenetics in Naïve Versus Primed Pluripotency in Mice

There are important epigenetic differences between mESCs and mEpiSCs [30, 77]. Female mESCs have two active X chromosomes, while in female mEpiSCs, X chromosome inactivation has occurred, so they present one inactive or silent X chromosome [24]. This reflects the different embryonic origins of naïve and primed pluripotency. In general, the genome of mEpiSCs is hypermethylated when compared to mESCs [29, 78]. Similarly, there is a reduced prevalence of the repressive histone mark H3K27me3 (histone 3 lysine 27 trimethylation) at promoters and fewer bivalent domains in naïve mESCs [30]. Most importantly, there are also differences in the enhancer usage of genes between naïve and primed cells. This occurs not only in differentially expressed genes but also in commonly expressed genes, such as *Pou5f1*. The distal enhancer (DE) of *Pou5f1* is used in naïve mESCs, whereas its proximal enhancer (PE) is methylated. By contrast, in primed mEpiSCs, the *Pou5f1* PE is used and the *Pou5f1* DE is methylated [79]. Also, development-associated enhancers, called seed enhancers, convert from a dormant to an active state in mEpiSCs, and this is thought to regulate lineage priming [80].

3.3 Human Embryonic Stem Cells

The first pluripotent human embryonic stem cells (hESCs) were derived in 1998 and initially maintained in culture on MEFs and in medium containing FCS [81]. Although derived from the same embryonic-stage blastocyst embryos, hESCs display primed pluripotency and thus show important differences to naïve mESCs: flattened colonies that are highly sensitive to single-cell passage; different associated pluripotent markers like SSEA-3, SSEA-4 and TRA-1-81 instead of SSEA-1; different signalling requirements in culture (dependency on FGF/TGFβ instead of LIF/STAT3) and an inactive X chromosome in female hESCs [24, 82] (Fig. 3.4).

The similarities between mEpiSCs and hESCs suggest that the isolated human ICM displays a more advanced embryonic state, acquiring an EpiSC-like signature in culture. Alternatively, the primed signature reflects the fact that human ICM does not have the ability to undergo diapause (pause development), unlike the mouse, and epithelializes in culture. In agreement, an epithelialized post-ICM intermediate

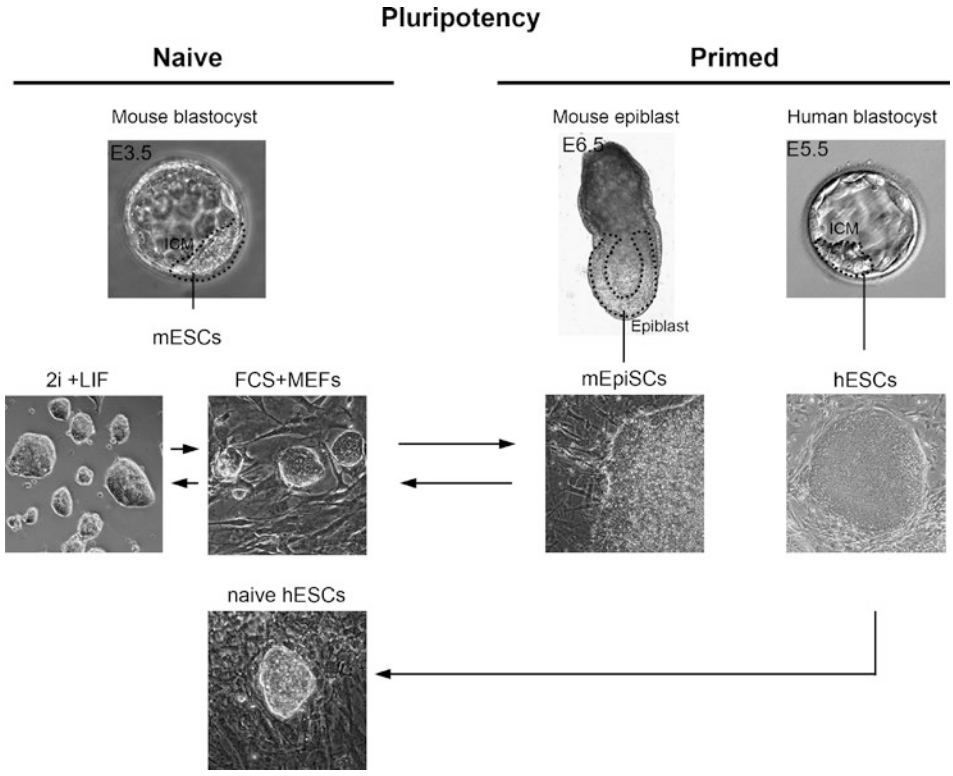

Fig. 3.4 Origins of mouse and human embryonic pluripotent stem cells. Naïve mESCs (mouse embryonic stem cells) are derived from the inner cell mass (ICM) of E3.5 blastocysts. mESCs represent naïve pluripotency in 2i+LIF (ground state) and FCS and MEFs culture conditions. Primed mEpiSCs (mouse epiblast stem cells) are derived from the epiblast of E6.5 embryos. mEpiSCs can be converted to mESCs and vice versa. Primed hESCs (human embryonic stem cells) are derived from the ICM of E5–E7 human blastocyst. hESCs can be reverted to a naïve-like state. Abbreviations: ICM inner cell mass, mESCs mouse embryonic stem cells, hESCs human embryonic stem cells, MEF mouse embryonic fibroblasts, FCS foetal calf serum, mEpiSCs mouse epiblast embryonic stem cells, LIF leukaemia inhibitory factor, E embryonic day

(PICMI) has been identified during the transition from human ICM to hESCs in culture [83] (Fig. 3.4).

hESCs and EpiSCs are not strictly identical: contrarily to EpiSCs, hESCs express pre-implantation markers such as *REX1* [84] and not post-implantation markers such as *FGF5* [85]. In addition, not all female hESCs lines have gone through X chromosome inactivation, and different X chromosome states have been described in hESCs [86–89]. Also, the pattern of DNA methylation of primed hESCs resembles more the one from naïve mESCs [90, 91].

3.3.1 Primed Versus Naïve Pluripotency in hESCs

After the identification of naïve, primed and ground states in the mouse, efforts are being made to push primed hESCs into the ground state (reviewed in [92, 93]). Most of these protocols included LIF+2i conditions, but these alone were not sufficient to induce naïve pluripotency in hESCs [84, 94–97].

The first attempts to induce a naïve state in (primed) hESCs relied on the overexpression of *KLF4*, *KLF2* and *POU5F1* in the presence of LIF and 2i [95]. These naïve-like hESCs showed high levels of phosphorylated (p)STAT3 and differentiated when exposed to a JAK inhibitor that blocks phosphorylation of STAT3, similar to naïve mESCs. Also, naïve-like hESCs did not differentiate upon addition of BMP4 or inhibition of FGF2, as primed hESCs and mEpiSCs do.

The first transgene-independent naïve-like hESCs were described by Gafni and colleagues in 2013 [96]. They developed a naïve pluripotency growth medium (naïve human stem cell medium, NHSM) to use in both MEF and MEF-free conditions. This medium contained 2i+LIF together with p38 inhibitor (p38i), Jun N-terminal kinase inhibitor (JNKi), aPKCi, RHO-associated protein kinase 1 inhibitor (ROCKi) and a low dose of FGF2 and TGFβ1 (or ActA). These converted naïve-like hESCs showed downregulation of lineage priming-associated genes including *DNMT3B, OTX2, ZIC2* and *CD24*. Other studies followed describing the conversion of primed hESCs to naïve-like pluripotency using different cocktails of molecules [84, 94, 97], but in none of them, a complete independency of FGF2 and/or TGFβ/ActA signalling was achieved. Interestingly, naïve-like hESCs under different culture conditions exhibit dependence of the mTORC2 subunit of PI3K/PKB/mTORC pathway [98]. This suggests that naïve pluripotency is different in mESCs and hESCs, although more studies are required to understand these differences.

3.3.2 Epigenetics in Naïve Versus Primed Pluripotency in Humans

Similarly with what has been shown in naïve and primed mouse pluripotent stem cells, naïve-like hESCs show lower levels of DNA methylation that primed hESCs [99]. Nevertheless, it is hard to pinpoint epigenetic differences between naïve-like and primed hESCs. The different approaches to induce naïve-like hESCs using different molecule cocktails result in hESCs that show a wide "range" of naïve properties, and are (epi)genetically unstable [100]. Moreover, the switch from the PE to the DE usage in the *POU5F1* locus, used as a hallmark of naïve and primed mouse stem cells, may not occur in hESCs. Moreover, the long-term culture conditions cause abnormal erasure of DNA methylation in imprinting regions [101].

The X chromosome inactivation state in female naïve-like hESCs does not predict the pluripotency state, as several X chromosome activation states have been described in primed hESCs [86–89, 102]. In addition, during long-term culture, there is progressive "erosion" of silencing marks throughout the silent X chromosome in primed hESCs, which can include the absence of the *XIST* cloud, deposition of H3K27me3 and DNA methylation in certain regions of the silent X chromosome [103]. When erosion occurs in the silent X chromosome of female primed hESCs, even if differentiated the cells will not reacquire the silencing marks [104].

3.4 hESCs as a Model to Study Embryonic Development

The interest in hESCs and their pluripotent state resides in the future applications for gene therapy, drug discovery and regenerative medicine. Recently, the scientific community turned its interest to the self-organization capacity of hESCs during in vitro culture, as a model to understand early human development (Fig. 3.5).

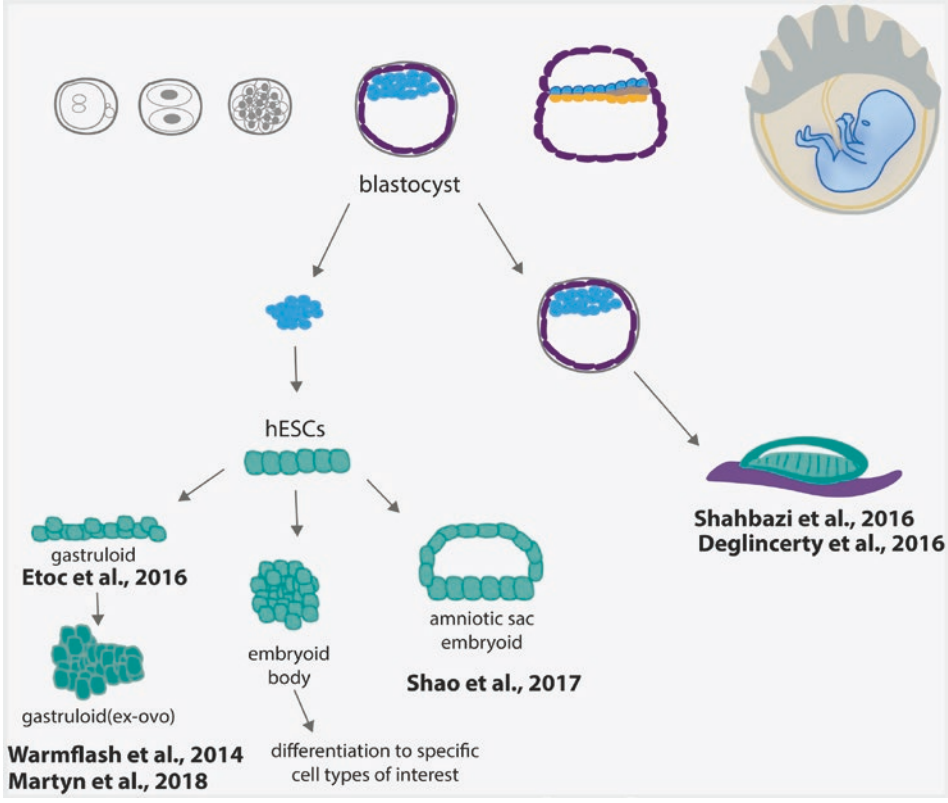

Fig. 3.5 Can human stem cells mimic some aspects of human embryology? Recent developments and protocols for the culture of hESCs (human embryonic stem cells) and human blastocysts allowed the study of human embryonic development and particularly the study of early implantation period. hESCs can self-organize in vitro into structures that mimic gastrulation (gastruloids), recapitulating some aspects of early embryonic events. hESCs can also be directed to differentiate into specific types of cells via embryoid body differentiation. Abbreviations: hESCs human embryonic stem cells

It has been shown that hESC colonies of a certain size (500 μm), in response to BMP4, pattern spontaneously into concentrically arranged zones, mimicking the arrangement of the mammalian germ layers [105, 106]. More recently, hESCs treated with WNT and ActA and grafted in a chick embryo directed the development of a secondary axis and induced a neural fate in the host, acting like the primitive streak organizer [107]. Another group has generated a synthetic human amniotic sac from hESCs, which they called post-implantation amniotic sac embryoid (PASE) [108].

Using a different approach to understand the human early development, human pre-implantation embryos have been cultured to the implantation stage (14-day limit) in the absence of maternal tissues [109, 110]. These studies open many possibilities to understand human early embryology.

3.5 Primordial Germ Cells Have an Underlying Pluripotent State

Another type of cell that seems to retain some aspects of pluripotency is the primordial germ cell (PGC). PGCs are the first embryonic cell lineage to be lineage restricted in the embryo. Due to their pluripotent-like properties, PGCs can be used to derive another type of pluripotent stem cells known as embryonic germ cells (EGCs). EGCs are derived by culturing PGCs from E8.5–E12.5 mouse embryos in the presence of LIF, FGF2 and stem cell factor (SCF) [111–113]. Like mESCs, mEGCs express the core pluripotency genes, *Pou5f1, Sox2* and *Nanog*, and can contribute to mouse chimeras showing germline transmission [111, 114, 115]. Since PGCs only give rise to oocytes or sperm in vivo, the derivation of EGCs is considered a reprogramming event. mEGCs can also be maintained in the ground state [116]. The derivation of human EGCs from human gonadal PGCs has also been attempted [117–119], but the long-term culture of hEGCs has not been achieved successfully.

3.5.1 Origin and Specification of PGCs in Humans and Other Animals

PGCs are highly specialized cells that give rise to gametes during adult life. They are the vehicle through which genetic and epigenetic information is passed from one generation to the next [120]. The mechanisms through which PGCs are specified, migrate and differentiate, first outside and then inside the gonads, differ between species.

There are two main mechanisms thought to govern the formation of PGCs: preformation and epigenesis (or induction). In animals that use preformation, germ cell precursors are defined by the direct inheritance of maternal factors (germ plasma) physically contained in the oocyte. In animals using epigenesis (or induction), germ cell fate is induced de novo during embryonic development [121–123]. Preformation has been documented, for example, in the fruit fly and chicken [124–126]. In the chick, cells expressing the post-migratory germ cell marker CVH (chicken vasa homologue) are already present in the centre of the blastoderm [126, 127] (◘ Fig. 3.6a). Epigenesis is so far common to all mammals studied, but it has also been described in the axolotl [128].

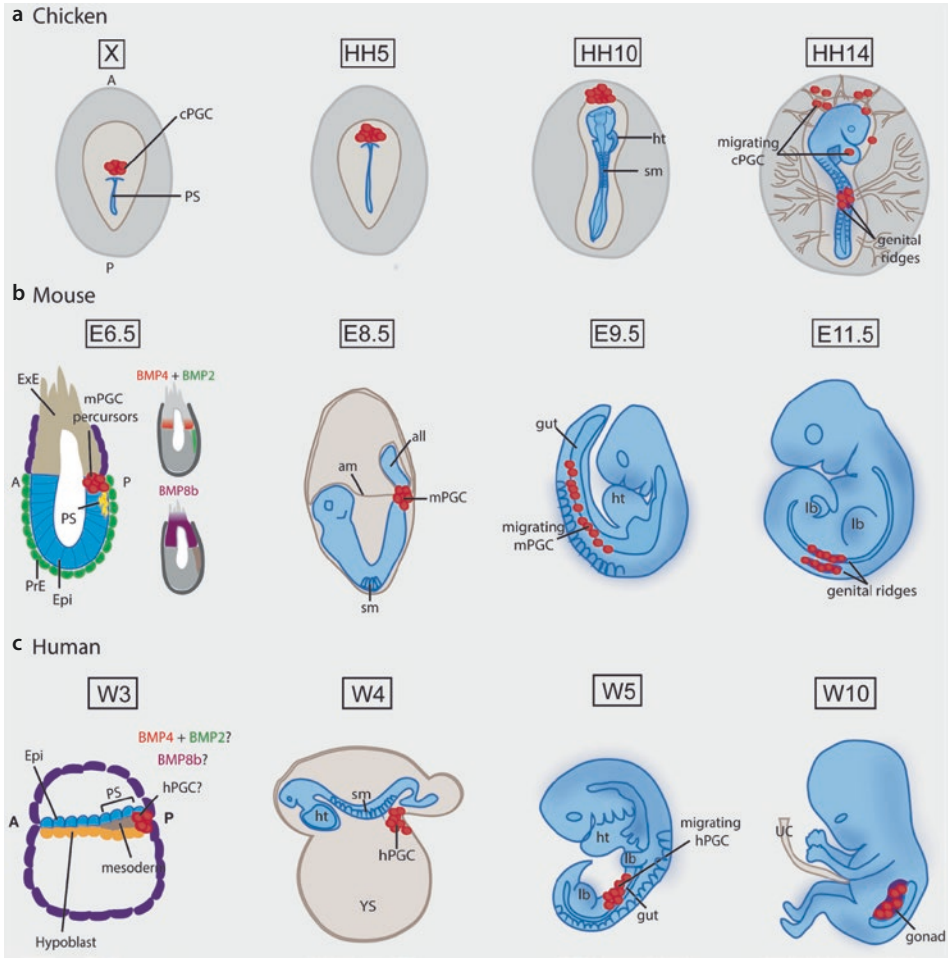

Fig. 3.6 Different origins and migratory routes of primordial germ cells. **a** In chicken, primordial germ cell (PGC) fate is passed on via germ plasma present in the egg. During gastrulation (Eyal–Giladi and Kochav stage X), the primitive streak develops, and the chicken PGCs (cPGCs) are localized ventrally and move to the anterior part of the embryo to localize to the germinal crescent, anteriorly to the head at Hamburger–Hamilton stage (HH)10. Around HH15, cPGCs migrate from the crescent to the genital ridges via the anterior vitelline veins and the aorta. **b** In mice, BMP4 and BMP8b from the extraembryonic ectoderm (ExE) and BMP2 visceral endoderm induce PGC competence at the posterior part of epiblast adjacent to the extraembryonic ectoderm at E6.5. mPGCs localized at the base of the allantois migrate via the hindgut, reaching the genital ridges at embryonic day (E)11.5. **c** In humans, the origin of the signals that induce PGC competence is unknown. hPGC competence is thought to be initiated around gastrulation (W3 of embryonic development, 12–16 days after fertilization) at the posterior part of the embryo, where the primitive streak is formed. At W5, hPGCs are located at the endoderm of the yolk sac wall near the allantois. They migrate via the gut endoderm and the dorsal mesentery to colonize the gonadal ridges around W6–7. At W10, hPGCs are encapsulated in the gonads. Abbreviations: A anterior part, P posterior part, PS primitive streak, ht heart, sm somites, ExE extraembryonic ectoderm, PrE primitive endoderm, Epi epiblast, am amnion, all allantois, lb limb bud, UC umbilical cord, PGC primordial germ cells, cPGC chicken PGCs, mPGCs mouse PGCs, hPGCs human PGCs, HH Hamburger–Hamilton stage, E embryonic day, W week, BMP bone morphogenic protein

Although the two models seem different, there is mounting evidence that in animals using preformation, an induction mechanism is also important for PGC lineage specification, and conversely, in animals with epigenesis, the oocyte may retain some maternal-inherited factors important for PGCs. Recently, a unifying model has been proposed, suggesting that all animals show a period of multipotent pre-PGCs, followed by lineage restriction by induction [123].

3.5.2 Molecular Mechanisms Regulating Specification of PGCs in Humans and Other Animals

In the mouse, competence for the generation of PGCs is set at E6.0–E6.5 in the proximal epiblast adjacent to the extraembryonic ectoderm (ExE) [129, 130] (Fig. 3.6b). This initial population can be identified by expression of *Ifitm3* (or *Fragilis*). *Ifitm3* is considered the first gene to mark the onset of competence for the PGC fate [131]. Nevertheless, not all *Ifitm3*+ cells are to become bona fide pre-PGCs. From this initial niche, about six *Ifitm3*+ cells begin to express *Prmd1* (or *Blimp1*), and these are considered the mPGC precursors or pre-PGCs [132]. These pre-PGCs become lineage restricted, when they start expressing *Prdm14*, *Tfap2c* (or *Ap2γ*) and *Dppa3* (or *Stella*) at E7.25 and acquire the characteristic alkaline phosphatase (*Alpl* or *Tnap*) activity. This cluster of about 45 founder mPGCs is embedded in the extraembryonic mesoderm and visible at the base of the allantois [133–135]. The mPGCs are positive for *Pou5f1* (via the distal enhancer of the *Pou5f1* promoter just as the ICM, in contrast to the epiblast that uses the proximal enhancer of the *Pou5f1* promoter) and start re-expressing pluripotency-associated genes such as *Sox2, Nanog* and *Sall4* [136–139].

After implantation, the mouse embryo develops as an egg cylinder while the human embryo develops as a flat disc [11, 140]. Recent studies in pig embryos, in which peri-implantation development is closer to humans than to mice, show that competence for porcine PGC specification is set initially in the posterior end of the nascent primitive streak [141]. In the cynomolgus monkey, cyPGCs were identified prior to gastrulation, at E11, in the dorsal amnion [142]. Although both pig PGCs and cyPGCs seem to depend on BMP signalling for specification and express common germ cell markers, the PGC specification takes place at different locations suggesting that the mechanism of induction of PGC fate may not be entirely conserved among mammals [141, 142]. The timing of establishment of competence for PGC differentiation and the embryonic origin of the PGC founder population in the human embryo is presently unknown, but it is expected to occur before the initiation of gastrulation at day 14 (Fig. 3.6c).

3.5.3 Migration of PGCs in Humans and Other Animals

After specification, mPGCs migrate towards the future gonads via the endoderm epithelium of the hindgut, reaching the mesentery at E9.5 and colonizing the (left and right) genital ridges at E10.5 [143–145] (Fig. 3.6b). During migration and briefly after colonization of the gonads, PGCs proliferate and undergo epigenetic

reprogramming characterized by genome-wide DNA demethylation, X chromosome reactivation (in the females) and erasure of genomic imprinting [143]. mPGCs start to express *c-Kit* and *SSEA1* during migration [146–149]. Contrary to the mouse, chick PGCs migrate through the vascular system to reach the genital ridges [150] (Fig. 3.6a), instead of using the gut.

During human development, human PGCs (hPGCs) have been identified the earliest in week (W)5 of gestation (or week 3 of development), recognized by their morphology and alkaline phosphatase activity. hPGCs were observed in the extragonadal region of the developing embryo, more specifically in the posterior-ventral part of the endoderm of the yolk sac wall near the allantois [151]. hPGCs have been shown to migrate via the midgut and hindgut endoderm and later via the dorsal mesentery to colonize the gonadal ridges around W8 [152] (Figs. 3.6c and 3.7).

Recently, the expression of germ cell markers associated with migratory and early post-migratory in hPGCs has been validated at W4.5 [153]. In this study, the migration of hPGCs in a human embryo was analysed, and specific expression of *NANOG*, *POU5F1*, *TFAP2C* and *PRDM1* in hPGCs in the AGM (aorta–mesonephros–gonadal region) was shown. On the other hand, the well-known mouse germ cell markers, like *ALPL* and *KIT*, and others, like *SOX17*, *TUBB3* and *ITGA6*, were expressed in other cell types in the AGM, highlighting that to identify hPGCs at this developmental stage, using a suitable combination of markers is essential [153].

3.5.4 Arrival and Colonization of the Gonad

After initial colonization of the gonad by E10.5, mPGCs undergo mitotic division until E12.5 [154]. By this time, *Dazl* and *Ddx4* (or *Vasa*/*Mvh*) are upregulated [155, 156], and mPGCs lose expression of early germ cell and pluripotency-associated markers like *Prdm1*, *Dppa3*, *Pou5f1*, *Sox2*, *Nanog* and *Alpl* [157–160].

In humans, long after colonization of the human gonadal ridges, around W9 of gestation, *DAZL* and *DDX4* start to become upregulated, while *POU5F1 and NANOG* expression decreases, becoming mutually exclusive [161–163] (Fig. 3.7). While in the mouse, meiosis entry is relatively synchronized and occurs in a short-lived wave of about 12 hours [157, 159], in human this process is asynchronous, taking place from W17 to birth [162, 164]. More recently, with advances in technology and access to human foetal gonads with ages varying from W7 to W20, the transcriptome and epigenome of hPGCs have been investigated by FACS-sorting hPGCs from embryonic somatic tissue using surface markers and performing single-cell transcriptomics [163–166].

3.5.5 Protecting PGC Genome Integrity

During development, mPGCs undergo a series of epigenetic reprogramming waves where epigenetic marks such as global DNA methylation and genomic imprints are erased and re-established later in a sex-specific manner [167, 168]. Throughout this reprogramming period, when global DNA methylation is low, the genome is particularly vulnerable to random integration by repetitive transposable elements (TrE) that are usually repressed by DNA methylation [169]. In addition to DNA methylation,

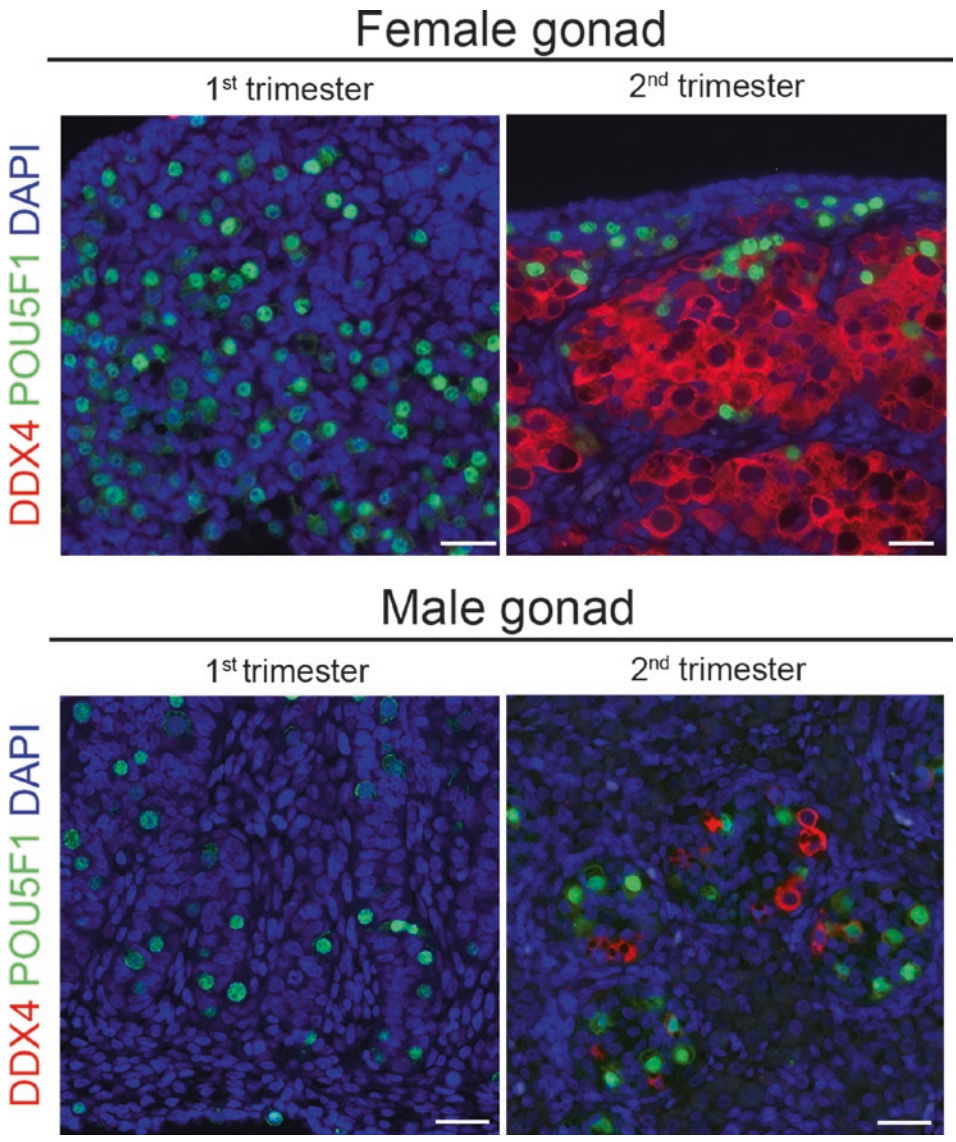

Fig. 3.7 Germ cells in first- and second-trimester human gonads. Histological sections of female and male gonads immunostained for the pre-meiotic germ cell marker DDX4 (red) and early germ cell marker POU5F1 (green) in the first- and second-trimester gonad. Nuclei are counterstained with DAPI (blue). Scale bars are 20 μm

another strategy to block the integration of TrE in the genome is targeting of TrE transcripts for degradation [169]. Targeting of TrE for degradation is achieved in complexes of Piwil (P-element induced wimpy testis-like) proteins with small non-coding RNAs that are germline-specific, the piRNAs [170–172]. These piRNA-induced silencing complexes (piRISC) recognize and cleave target TrE transcripts with complementary sequences to the loaded piRNAs. Mice have three Piwi-like paralogues (*Piwil1*/*Miwi*, *Piwil2*/*Mili* and *Piwil4*/*Miwil2*), while humans have one

extra Piwil gene, *PIWIL3* [173, 174]. Mutations in *Piwil*, in mice, have been associated with defects in meiosis, specifically in the male germline, while females remain fertile, and mutated oocytes are able to resume meiosis normally (reviewed in [175]). Retrotransposon silencing via piRISC usually occurs in a peri-nuclear cytoplasmic structure rich in mitochondria and endoplasmic reticulum surrounding a Golgi aggregate, known as intermitochondrial cement [176–178].

Specific haplotypes in *PIWIL4* and *PIWIL3* together with hypermethylation of the *PIWIL2* promoter have been associated with infertility in humans [179–181]. Due to the prominent role in human gametogenesis, the expression of the different PIWIL during male and female germline development has recently been systematically investigated [173, 174, 182]. *PIWIL1*, *PIWIL2* and *PIWIL4* have a mutually exclusive pattern of subcellular localization, particularly in female oocytes. In contrast to mice, in humans *PIWIL4*, but not *PIWIL2*, is localized to the intermitochondrial cement [182] highlighting important differences between mouse and human.

3.5.6 Protocols for In Vitro Germ Cell Development

In mouse, PGC induction is initiated by BMPs originating in the ExE and proximal visceral endoderm (VE) [183] (Fig. 3.6b). *Bmp4* and *Bmp8b* from the ExE together with *Bmp2* from the VE induce the expression of *Prdm1* [183–185]. BMP signalling is essential for the induction of PGC fate since mutations in *Bmp4*, *Bmp8b*, *Smad1* and *Smad5* result in impaired PGC development in vivo [186–188]. Similarly, protocols aiming to generate mPGCs from pluripotent stem cells in vitro require, among other factors, the addition of Bmp4 and Bmp8b [189]. In mouse, PGC-like cells (mPGCLCs) with full competence to generate functional gametes after transplantation to mice in vivo have successfully been induced from mESCs [189, 190]. The mPGCLCs were able to undergo gametogenesis leading to the formation of functional sperm or oocytes, but meiosis was only accomplished due to co-culture with gonadal tissue [189, 190]. More recently, meiosis has been completed in vitro, without the need to transplant the aggregates of gonadal tissue containing the mPGCLCs into mice, resulting in differentiation to both female functional gametes [191] and male functional gametes [192].

The studies in mice have paved the way to protocols to generate human PGC-like cells (hPGCLCs) [141, 193, 194]. These protocols rely on the comparison of gene expression between the newly generated hPGCLC and gonadal hPGCs. Primed hESCs cultured in the presence of FGF2 have low germline competence, but when grown in 4i-medium (four inhibitors: CHIR99021, PD0325901, SB203580 and SP600125) containing BMP4 or BMP2, hESCs efficiently differentiated to hPGCLCs [141, 193, 194]. W7 hPGCs and hPGCLCs have relatively similar global transcriptional profiles with the expression of *PRDM1, ALPL, DDPA3, TFAP2C, NANOS3, KIT, NANOG, POU5F1, KLF4* and, surprisingly, some other lineage-marker genes like endoderm marker *SOX17*. Surprisingly, *SOX17* and *KLF4* are specifically expressed in hPGCLCs, but not in mPGCLCs. By contrast, *SOX2* and *PRDM14* are specifically expressed in mPGCLCs and not in hPGCLCs [141, 165, 166, 193, 195, 196]. In 4i condition, hESCs develop early mesodermal characteristics and differentiate into hPGCLCs with high efficiency [193, 194]. This is similar to mPGCs that transiently upregulated mesoderm markers, such as *T*, before PGC

specification [197]. *SOX17, PRDM1* and *TFAP2C* were also upregulated during the in vitro acquisition of competence for cyPGC fate from cynomolgus monkey pluripotent stem cells [141], suggesting that there are conserved aspects between primates.

Developing protocols to mimic gametogenesis starting from human pluripotent stem cells is currently challenging, but it will surely open novel avenues to understand causes and develop treatments for human infertility and perhaps even revolutionize the way we reproduce in the future.

Take-Home Message

This chapter covers the following topics:

- We have introduced early embryonic development.
- We have explained the differences between different pluripotency stages.
- We have raised awareness of differences between animal models.
- We presented the different signalling pathways involved in pluripotency.
- We have introduced the development of germ cells in vivo and in vitro.

Review Questions for This Chapter

1. Discuss the differences between totipotency and pluripotency.
2. Discuss the differences between naïve and primed pluripotent stem cells.
3. Describe the differences between hESCs and mESCs.
4. Compare the signalling pathways important for naïve and primed pluripotency.
5. Explain the BMP signalling pathway.
6. Enumerate genes associated with naïve and primed pluripotency.
7. Describe the events that take place during pre-implantation.
8. Describe the formation of PGCs, including specification, migration, epigenetic reprogramming and genomic integrity.

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