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Primordial Germ Cell Reprogramming

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Abstract

Primordial germ cells (PGCs) are the embryonic precursors of the gametes. Thus, they are unipotent cells. However, PGCs share some common features with pluripotent stem cells. Among them, PGCs show alkaline phosphatase activity and express stage-specific embryonic antigens and pluripotency factors Lin28, Oct4, Sox2, and Nanog. Under specific conditions, they undergo spontaneous reprogramming *in vivo*. Moreover, they can be easily reprogrammed *in vitro* into pluripotent embryonic germ cells (EGCs) by culturing them in the presence of basic fibroblast growth factor or the epigenetic modulator trichostatin A. Previous work in our laboratory has also proven that hypoxia alone can reprogram PGCs into hypoxia-induced embryonic germ-like cells, which have a pluripotent phenotype but which do not show self-renewal capacity. Therefore, PGCs are an interesting model to further comprehensively understand the process of cell reprogramming. This chapter reviews various methods to achieve PGC reprogramming, as well as the molecular pathways involved. We focus on soluble factors and genetic strategies to obtain pluripotent cells from PGCs. Special emphasis will be given to factors implied in energetic metabolism, epigenetics, and cell signaling transduction, both *in vitro* and *in vivo*.

Keywords: cellular reprogramming, ROS, glycolysis, autophagy, primordial germ cells, pluripotency, metabolism, hypoxia, epigenetics

1. Introduction

During normal embryogenesis and throughout the life of an organism, cells maintain or restrain their developmental potential. This potential refers to the ability to give rise to various types of cells. After fertilization, the resulting zygote and the blastomeres are totipotent until the four-cell stage, meaning they can develop a complete organism alone, including

extraembryonic tissues such as the placenta [1]. Pluripotent stem cells, like embryonic stem cells (ESCs), are defined by their potential to differentiate into cells of the three germ layers (endoderm, mesoderm, and ectoderm), but they do not give rise to extraembryonic structures. In a more differentiated state, we find multipotent cells capable of differentiating into several types of cells. This capability is common in progenitor cells in adults, which give rise to diverse tissue cells.

In some processes, differentiated cells revert to a less differentiated and higher potential state. These phenomena are called reprogramming and are shown both *in vivo* and *in vitro*. *In vivo* reprogramming can be demonstrated by the appearance of cancer stem cells, and *in vitro* reprogramming has been achieved by several methods, such as somatic cell nuclear transfer, cell fusion between somatic and pluripotent cells, and treatment with pluripotent cell extracts, among others [2].

The most important and well-studied method of *in vitro* reprogramming in the last decade has been the derivation of induced pluripotent stem cells (iPSCs) from somatic cells by transduction of specific transcription factors, Oct4, Sox2, Klf4, and c-Myc [3]. These cells represented a revolution in stem cell research because they eliminated the ethical concerns about the use of ESCs and allowed access to an endless and personalized source of pluripotent cells. The clinical potential of this discovery is enormous, with the possibility of generating patient-derived iPSCs with applications not only in autologous transplants, but also in disease modeling and regenerative therapy. The use of iPSCs is extensive, and new ways to improve their derivation are being studied to increase efficiency and to overcome the risks for their clinical use. Even with the progress that has been made, there is still much to understand about the mechanisms of reprogramming.

In the study of pluripotency induction, an important model could be the use of primordial germ cells (PGCs), which can give rise to pluripotent cells called embryonic germ cells (EGCs). This reprogramming is achieved relatively easily, without the need for gene transduction, thus avoiding the risks related to gene manipulation [4–6]. PGCs are the embryonic precursors of gametes, giving rise during normal development to either spermatozooids or oocytes. These cells have limited self-renewal ability and are unipotent, are incapable of forming pluripotent embryoid bodies or contributing to teratomas or chimeras [7]. However, PGCs are considered developmentally pluripotent, given they generate the whole totipotent zygote after fertilization. This dual identity of both differentiated and pluripotent stem cells make PGCs a unique model to study cell fate and flexibility.

PGCs suffer reprogramming both *in vivo* and *in vitro*. *In vivo*, PGCs can give rise to embryonal carcinoma cells (ECCs), which are the pluripotent stem cells of testicular tumors [7]. *In vitro*, PGCs are easily reprogrammed into pluripotent EGCs with a specific cocktail of growth factors [4].

The reprogramming ability of PGCs can be explained by their similarity to pluripotent cells and their latent totipotency. PGCs innately express several transcription factors related to pluripotency, such as Oct4, Sox2, Nanog, and Lin28 [7]. Some of these factors are retained during PGC development from the zygote, whereas others such as Sox2, Nanog, and Klf2 are reexpressed or upregulated [8].

Other markers, such as tissue nonspecific alkaline phosphatase (TNAP) and germ cell nuclear antigen, do not belong to the pluripotency network itself, but are also strongly expressed by PGCs and ESCs. Even typical germ line factors such as B lymphocyte-induced maturation protein-1 (Blimp1) and Stella are typically expressed by ESCs [9]. It has been described that derivation of ESCs from the inner cell mass (ICM) is preferably achieved from cells that express Blimp1 and other germ line markers (interferon-induced transmembrane protein 3, Lin28, Prdm14, Stella, & c-Kit) [10]. The similarities in gene expression between PGCs and ESCs lead to the idea that the closest *in vivo* equivalent to ESCs are germ cells, instead of the ICM or even the epiblast, because PGC precursors are specified within the epiblast around 6 days post coitum (dpc) in mice [11].

The proximity of the PGCs to pluripotent stem cells and the ease of their reprogramming agree with the results of experiments on iPSC derivation from differently developed cells. The more undifferentiated the cells, the more easily they give rise to iPSCs, in a more efficient manner and requiring the transduction of fewer transcription factors. Conversely, the more differentiated cells, the more difficult they are to reprogram [12]. PGCs, however, can be reprogrammed with the transduction of only one of any of the four traditional iPSC factors [13]. This result is especially interesting because PGCs already express Oct4 and Sox2, which means that a variation in the expression level of just one transcription factor can be sufficient for PGCs to become pluripotent.

2. PGC reprogramming *in vivo*

ECCs are the stem cells of testicular tumors, which can be maintained indefinitely in culture as pluripotent cells. ECCs were first established as cell lines from mouse teratocarcinomas 50 years ago. In humans, the first teratocarcinoma cell lines isolated *in vitro* were TERA1 and TERA2, but their identity as ECCs was not discovered until some years later [14].

These pluripotent stem cells share most of their characteristics with ESCs, such as self-renewal capacity, specific markers, and the ability for differentiation to any cell of the organism [15]. The main difference from other pluripotent cells is that they are usually aneuploid. The malignancy of these cells is highly dependent on the microenvironment, as has been observed in chimera formation experiments: ECCs injected into mouse blastocysts can contribute to the development of a normal chimera, or in some cases to tumors. Another difference from ESCs is the very low efficiency in colonizing the germ line, which makes them less suitable for establishing mutant rodent lines [2]. Some authors define these cells as multipotent rather than pluripotent due to limitations in the differentiation potential, which is even more limited in human lines that often show no differentiation potential at all [14]. This potential has not been consistent, changing from one ECC line to another, and varying with culture conditions, such as the F9 EC line, which was considered nullipotent until the discovery of the induction of differentiation by retinoic acid (RA) exposure [16]. Considering the definition of pluripotency as the ability to differentiate into cells of the three germ layers, most ECC lines are pluripotent, but could be considered incompletely or partially pluripotent cells.

The germ line origin of the ECCs was proven using transgenic *Steel* (*Sl*) mutant mice. These mice, carrying a homozygous mutation in the *Sl* locus are unable to express stem cell factor (Kit ligand), a growth factor required for PGC survival and proliferation [17]. When embryonic gonads of the teratogenic mouse strain 129/Sv are transplanted to the adult testis, teratoma formation occurs in wild-type mice but not in *Sl* mutants, suggesting the Kit ligand is implicated in ECCs.

Most ECC lines are derived from early mouse PGCs (8.5–10.5 dpc), and spontaneous teratomas have been described to start around 12.5 dpc. PGCs lose their ability to reprogram *in vivo* after 12.5 dpc, which is coincident with the time the PGCs are able to give rise to EGCs *in vitro*. When 129/Sv 12.5 dpc gonads were transplanted into adult testes, teratomas appear at an 80% incidence, whereas when 13.5 dpc gonads were grafted, the tumor incidence decreased to 8%. The induction of these tumors is also related to the strain used, and is far less efficient in strains other than 129/Sv [14, 15].

In vivo reprogramming of PGCs to ECCs depends on a variety of genetic factors. The best known mutation that affects the development of mice teratomas is Teratoma (Ter) [18] in the RNA-modifying gene DND microRNA-mediated repression inhibitor 1 (*Dnd1*). In homozygous 129/Sv-Ter mice, teratoma incidence increases up to 75%. This reprogramming appears to be linked with proliferation, because the PGCs of these mice continue proliferating after 13.5 dpc, whereas unmutated PGCs enter mitotic arrest. The Ter mutation appears to be caused by the surrounding somatic cells instead of the PGCs themselves. In a similar manner, doublesex-related transcription factor 1 (*Dmrt1*) mutants also develop teratomas at a high rate in 129/SV mice, but unlike the Ter mutation, this effect is achieved by the loss of *Dmrt1* in PGCs [19, 20].

Also related to PGC transformation is the phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) pathway, whose upregulation could lead to the appearance of ECCs. This outcome can be observed in the effect of the specific inactivation in PGCs of the tumor suppressor phosphatase and tensin homologue (PTEN), which leads to an activation of PI3K/Akt signaling, and, therefore, testicular teratoma formation [21]. Among the Akt targets is the tumor suppressor Trp53, whose deletion increases the incidence of testicular teratomas in mice [22].

Testicular cancers in humans are classified into three categories: (1) teratomas and yolk sac tumors that develop in fetuses and infants and are classified as nonseminomas, (2) adult testicular cancer, both seminomas and nonseminomas, which appear in men aged between 20 and 40 years, and (3) spermatocytic cancer, which affects elderly men [20]. The first two types have characteristics in common with mouse carcinomas. Teratomas and yolk sac tumors that arise early in human life are the most similar to the teratocarcinomas described in mice, even if normal karyotypes are present, and probably also originate from PGCs. With respect to adult human testicular tumors, the cellular origin of these tumors is carcinoma *in situ*, which is considered to develop early from the germ line due to the similarity to PGCs and gonocytes. Between these similarities, there is the expression of specific membrane markers, such as Kit or TNAP; stem and early germ cell genes, such as Nanog or Vasa; and genomic imprinting [23]. These cells can remain nonpathological until adult life and later develop into teratomas. Unlike the previous type, these cancer cells show chromosomal abnormalities, such as isochromosome of the short arm of the 12th chromosome (iso-12p) [24].

As in the mouse, the PI3K/Akt pathway is related to teratoma formation in humans. AKT1 overexpression, Trp53 deficiency and PTEN inactivation, or KRAS and NRAS mutations, which lead to activation of this pathway, correlate with testicular cancer formation. The Kit signaling pathway can also be implicated in the origin of human ECCs. Both KIT and Kit ligand (stem cell factor) mutations are related to human teratomas [19].

In addition, typical reprogramming factors such as Oct4 and Sox2 [3] are related to *in vivo* germ cell reprogramming. The Oct4 expression level appears to affect germ cell reprogramming *in vivo*, given its reported overexpression in both teratomas and adult testicular cancers [25]. On the other hand, Sox2 is only expressed in ECC, but not in PGCs or other testicular cancer cells [26].

The appearance of human testicular cancer has also been shown to be related to disturbances in the environment of the germ cells in the embryo, and in diseases such as cryptorchidism, gonadal dysgenesis, or estrogen exposure during pregnancy [27–29]. Estrogen upregulates c-Kit in the genital ridges, which leads to an increased proliferation of PGCs and reprogramming [30, 31].

3. PGC reprogramming *in vitro*

EGCs are derived *in vitro* from PGCs when cultured with a specific cocktail of growth factors: stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF) [4, 5, 32]. EGCs are complete pluripotent cells, which are, like ESCs, able to give rise to all cell types in the organism and to fully contribute to blastocyst complementation, including contributions to the germline transmission [5, 32]. EGCs also share specific markers, such as stage-specific embryonic antigens (SSEAs), Oct4, Nanog, and TNAP, with other pluripotent stem cells [33].

EGCs were first derived from mice [4, 5] and then from various other animals [34]. EGCs have also been derived from human PGCs of around 5–10 weeks of gestation, providing a source of pluripotent stem cells and a good model of reprogramming [32].

Contrary to ECCs, EGCs are euploid and the primary difference between them and ESCs is the epigenetic state [7]. At the time of reprogramming, PGCs find themselves in different phases of the erasure and reestablishment of genomic imprints, and these epigenetic features are transmitted to the resulting EGCs. The epigenetic state of the PGCs is related both to the maintenance of the latent totipotency and to the inhibition of the stemness [35]. The manipulation of PGC epigenetics has proven the capability of reprogramming to EGCs (as we discuss later), showing how close these cells are to pluripotency.

Pluripotent stem cells can be found in various development states, with mouse ESCs representing the most undifferentiated, or naïve. This state is characterized by small, compact colonies; better survival when passaged as single cells; higher efficiency in chimera formation; shorter doubling time; and different culture condition requirements [36]. Human ESCs and mouse epiblast stem cells represent the most differentiated or primed state, characterized by larger flat colonies [37]. Human EGCs share some of their features with naïve stem cells, such

as colony shape and culture requirements, and other features related to a more differentiated state, such as the lack of teratoma formation when injected into mice and low efficiency when derived from subculture [38]. This outcome shows how close PGCs are to totipotency and how useful they can be as a study model. The only major problem for this model is the availability of PGCs, which are scarce at the time when they are able to reprogram and are difficult to expand, because they only survive approximately a week in culture [33].

4. Classical PGC reprogramming mechanism

As we previously explained, PGCs can be cultured with LIF and SCF, maintaining their phenotype and promoting their survival for approximately a week [11]. When basic fibroblast growth factor (bFGF) is added to the media, reprogramming of the PGCs is induced. The mechanisms involved in this process are still not fully understood. Several signaling pathways and genes have been shown to be implicated, such as Blimp1 downregulation, PTEN inactivation, Klf4, and c-Myc upregulation, PI3K/Akt signaling activation, and transforming growth factor (TGF) β signaling, among others [21, 39–41]. Most of these mechanisms are also involved in iPSC derivation, indicating a common regulatory network between the various reprogramming processes [19].

The dynamics of the transition from a unipotent germ cell to a pluripotent stem cell have been studied [42]. Three phases have been described, which are similar to those in iPSC derivation [43], based on the loss of germ cell characteristics and the expression of pluripotency genes: induction, preparation, and maintenance. This change in gene expression is a gradual process that takes approximately a week to complete. In the induction phase, along with the upregulation of Klf4 and embryonic stem cell-expressed ras (Eras), some germ cell markers, such as Dnd1 and Ddx4, start to be downregulated. Surprisingly, a large amount of PGCs that begin the reprogramming die in this first step. Some pluripotent factors, such as Klf4 and Eras, begin to be upregulated in the early phase, but they do not reach their higher expression until later phases, when most pluripotent factors, such as c-Myc and Nanog, are upregulated. In the preparation phase, some pluripotent markers, such as Klf4, Nanog, and Zfp42, reach their highest expression level, whereas others, such as Klf9 and Sox11, continue their gradual upregulation. It is also interesting to notice the high expression in this phase of the Meis family of transcription factors, a family related to the maintenance of hematopoietic stem cells.

Compared with PGC reprogramming, the traditional iPSC derivation process is far longer, taking approximately 3 weeks to complete. One primary difference between the EGC and iPSC derivation processes is that in the latter, a mesenchymal-to-epithelial transition takes place in an early phase. The lack of that event in PGC reprogramming could be due to the lack of the inverse process (epithelial-to-mesenchymal transition) during PGC specification, which is contrary to most somatic cells [8]. Moreover, the activation of genes already expressed in PGCs, such as SSEAs or TNAP, takes place in this first phase. The preparation phase is characterized by the upregulation of Nanog (as in EGC derivation), Sall4, and Esrrb. Other genes reactivated after these are Rex1, Lin28, and finally, Stella, Dppa4, or Pecam, among others. Between the transduced factors, endogenous Oct4 is typical of the preparation phase, whereas endogenous Sox2 is necessary for the maintenance phase [8].

Inhibition of Blimp could be the primary mechanism of bFGF-mediated EGC derivation. Blimp is the key germ cell specification gene [44], which has a potent repressive function. Among its targets are c-Myc and Klf-4, two of the primary pluripotency transcription factors; thus, Blimp inhibition leads to their upregulation [39]. These factors are particularly important in the acquisition of pluripotency, and they are the factors not expressed naturally in PGCs. It has recently been reported that deletion of Blimp1 in PGCs is sufficient to cause the derivation of EGCs in culture without bFGF [45].

5. Non-classical PGC reprogramming

It has been shown that the PI3K/AKT signaling pathway is involved in PGC reprogramming *in vitro*. PI3K is activated not only by bFGF but also by LIF and SCF, the three factors needed for EGC derivation. One of its primary downstream effectors is Akt, also known as protein kinase B, which has been observed to improve EGC derivation efficiency when activated in PGCs, even allowing the reprogramming in late PGCs up to 14.5 dpc. [40]. The specific *in vivo* inactivation of the tumor suppressor PTEN in PGCs, which leads to PI3K/Akt signaling activation, and enhances both EGC derivation and testicular teratoma formation [21]. Among Akt targets is the tumor suppressor Trp53. Akt inhibits its transcriptional activity by preventing its phosphorylation and nuclear accumulation. Deletion of Trp53 increases the incidence of testicular teratomas in mice and is enough to cause PGC reprogramming in culture in the absence of bFGF [40]. Trp53 deletion has similar effects on iPSCs, enhancing their induction [46].

The cell proliferation rate also appears to be as important in PGC reprogramming as it is in iPSC derivation [47]. The time when PGCs have the highest potential to give rise to EGCs coincides with the moment of the highest proliferation *in vivo* [5]. The three growth factors typically used for EGC derivation are mitogens that can alone promote proliferation [48]. Also, bFGF can be replaced by other known mitogens, such as RA or forskolin [11, 49], which activates protein kinase A by increasing the intracellular cyclic AMP. These pro-mitogenic effects could be triggered both by MAPK signaling [50] and the PI3K/Akt pathway, which enhances proliferation through several downstream effectors. One is by the already mentioned inhibition of Trp53, but there are others, such as the activation of cyclin D and inhibition of cyclin-dependent protein kinase inhibitors (CDKIs) [51]. The effects of CDKI inactivation have been observed in the mutation of the CDKI inhibitor of CDK4 (INK4), which enhances teratoma formation in mice, along with Trp53 inhibition [22].

PGC reprogramming can also be achieved by inhibition of MAPK/extracellular signal-regulated kinase (ERK), and glycogen synthase kinase-3 (GSK3) signaling. The two inhibitors that have been used for this purpose have been PD0325901 (PD) and CHIR99021, respectively (named 2i), which in combination with LIF can replace both SCF and bFGF and give rise to EGCs [52]. The same inhibitors have been used to enhance iPSC derivation, thus obtaining a more undifferentiated phenotype [53]. The mechanism followed by 2i or by bFGF reprogramming differs, because bFGF activates MAPK signaling [54]. It has been proposed that MAPK/ERK inhibition can lead to pluripotency by promoting long-term self-renewal and inhibiting differentiation through downregulation of Lef1. In ESCs, Lef1 promotes differentiation by inducing lineage specific genes and suppressing pluripotency gene expression. On the other

hand, GSK3 inhibits the Wnt/ β -catenin pathway, being responsible for the Tcf3-mediated repression of other pluripotency-related genes, such as Sox2, Oct4, Nanog, and Esrrb [55, 56]. The difference between the mechanism triggered by bFGF and 2i is also revealed by the timing of the required compounds, whereas bFGF is only needed during the first 24 h [57, 58], continuous culture with 2i is required [52].

Recently, another glycogen synthase kinase 3 (GSK3) inhibitor has been found to achieve EGC derivation [41]: kenpaullone, which inhibits not only GSK3, but a wide spectrum of kinases such as CDKs, and is sufficient for late (13.5–14.5 dpc) PGC reprogramming. Also, TGF β R inhibition by SB431542 can promote reprogramming in 11.5 dpc PGCs. The fact that these treatments have no effect on early PGCs, contrary to bFGF and 2i treatments, underlines the differences between the mechanisms that can trigger PGC reprogramming. TGF β inhibition could induce reprogramming through promotion of proliferation [59], reducing MAPK activity [60], and directing induction of the pluripotency network. TGF β inhibition can replace Sox2 transduction in iPSC generation by inducing Nanog expression [61]. On the other hand, the effect of various treatments can be combined to enhance reprogramming efficiency, such as TGF β R inhibition with 2i, with an efficiency of 12% [42]; TGF- β inhibition + ERK inhibitor (PD) [62], or bFGF + 2i + RA + forskolin, with an efficiency of approximately 20% [48]. This outcome shows that various mechanisms can synergize their effects in the reprogramming of PGCs. It has also been reported that mutations in genes involved in PGC development, such as Dnd1, Pten, and Pgct1, improve EGC derivation efficiency [19].

6. PGCs and hypoxia reprogramming

Recently, we have shown the reprogramming of PGCs cultured in hypoxic conditions without bFGF. The EGCs obtained had proven to be pluripotent, even if they were not completely reprogrammed, as shown by their limited proliferation [6].

The positive effects of hypoxia in enhancing reprogramming have been reported in iPSCs [63]. These hypoxia effects could be related to the idea of cancer stem cells arising *in vivo* from differentiated cells [64], due to environmental causes.

Hypoxia induces a change in the cell's energetic metabolism, from oxidative phosphorylation to glycolysis. This switch in the metabolism has been shown to be required for reprogramming of somatic cells to iPSCs. It has also been observed that the closest the somatic cell metabolism is to an ESC, the more efficient the reprogramming [65]. This metabolic change is an active process at the beginning of the reprogramming process; it has been shown that the expression of glycolytic genes, such as glucose transporter (Glut)1, Hxk2, Pfkfb3, and lactate dehydrogenase A (Ldha), is previous to pluripotency genes [66]. The relationship between stemness and glycolytic metabolism has been widely described in the Warburg effect, in which cancer cells in normoxia change their metabolism from oxidative phosphorylation to glycolysis [67]. It is still not clear whether the stem cell program triggers the metabolic change or whether it is the metabolic shift that activates the stem cell program; however, it has been demonstrated that these two processes are correlated. Our PGC data clearly supports the second hypothesis [6];

one described mechanism of this hypothesis is the positive feedback between glycolysis and the oncogene NF- κ B [68].

The induction of PGC pluripotency by hypoxia has been demonstrated to be mediated by hypoxia-inducible factors (HIFs), given the inhibition of their degradation by dimethyloxaloylglycine mimics the effect of hypoxia in the EGC derivation. This agrees with the improved reprogramming efficiency observed in iPSC derivation when a prolonged expression of HIF1 is forced [69].

HIFs are transcription factors that regulate a large number of downstream effectors under hypoxic conditions. Among genes regulated by HIFs are pluripotency genes such as Oct4 and c-Myc, regulated by HIF2 or Notch, and ETS-1, regulated by HIF1 [70]. The metabolic switch can also be provoked by HIF activation. HIF regulates several metabolism-related genes, promoting the expression of glycolytic proteins such as GLUT1 & 3, LDHA, ENO1, aldolase A, phosphoglycerate kinase 1 (PGK1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hexokinase 1 & 2, phosphofructokinase-2 (PFK2), and phosphofructokinase, liver type (PFKL)[65, 71].

These effects in the metabolism coincide with those provoked by Akt signaling, which is consistent with the mechanisms proposed for bFGF-mediated reprogramming of PGCs. Akt promotes glycolysis by the inhibition of FoxO transcription factors [51]. In somatic cell reprogramming, FoxO1 Akt-dependent phosphorylation enhances both glycolysis and iPSC derivation [72].

The mechanism by which HIFs provoke dedifferentiation in PGCs is partially known. It has been shown that the deregulation of Oct4 mediated by HIFs could be directly responsible for hypoxia-induced reprogramming [6]. Oct4 is one of the primary components of the pluripotency network; small changes in its expression levels result in great effects on stem cell development, promoting both undifferentiated and differentiated states, depending on the context [73]. Thus, in addition to the effect of its transduction on iPSC derivation, high levels of Oct4 can lead to differentiation and low levels can lead to pluripotency entry, provoking a dose-dependent induction of differentiation between mesoderm and trophoctoderm in ESCs.

However, pluripotent hypoxia-derived EGCs cannot be passaged long-term, probably due to a lack of upregulation of c-Myc and Klf4. Gene expression analysis of these cells suggests that they have not reached the stabilization phase of cell reprogramming. Comparing the gene expression of these phases with those of hypoxia-derived EGCs has shown that the genes typical from initiation and maturation are upregulated; however, those belonging to the stabilization phase, such as Dppa3, Dppa4, Utf1, Eras, Lin28, Sox2, and Dnmt3l, are not [6].

As in 2i-mediated derivation of EGCs, and unlike bFGF, hypoxia is needed continuously to provoke PGC reprogramming. This need suggests a mechanism closer to that triggered by 2i. The relationship between hypoxia and GSK3 inhibition has been demonstrated by the fact that HIF1 α stabilization depends on an inactive GSK3 β pathway [74]. Under long hypoxia exposure, HIF1 is downregulated though activation of GSK3 β . It has also been reported that Akt, which inhibits GSK3 β , can upregulate HIFs through mammalian target of rapamycin (mTOR) activation [51]. This correlation supports the hypothesis of these two methods of PGC reprogramming, hypoxia and 2i, being connected.

7. Primordial germ cell reprogramming and energetic metabolism

Energetic metabolism has deep implications in germ cell development. As evidence, human PGCs show lipid droplets and glycogen accumulations on their cytoplasm in order to obtain energy [75].

Gene expression analysis comparing PGCs and EGCs showed no relevant differences in expression of pluripotency factors, whereas glycolytic enzymes displayed elevated levels in EGCs. This link between metabolism and PGC reprogramming has also been reported in PGCs cultured in hypoxia. This process leads to the induction of pluripotency, which is dependent on HIF1 α stabilization and in turn provokes metabolic reprogramming and Oct4 deregulation [6]. Other studies have also observed that low Oct4 expression favors a robust pluripotent state in embryonic stem cells or acquisition of pluripotency, whereas high Oct4 levels relate to differentiation processes [76, 77]. In addition, Oct4, which remains active in PGCs, participates in metabolism regulation. In embryonic stem cells, Oct4 can induce hexokinase, pyruvate kinase, and pyruvate dehydrogenase (PDH) kinase expression, and the overexpression of these enzymes can prevent cell differentiation [78]. This has also been observed in PGCs [6]. In fact, when glycolysis is favored at low-oxygen concentrations, an increase in iPSC efficiency, and an enhancement of the expression of pluripotency factors via HIF expression are observed. Furthermore, iPSC derivation can be achieved in hypoxic conditions using only Oct4 and Klf4 [63, 79]. As mentioned for the comparison between PGCs and their *in vitro* pluripotent counterparts, EGCs, metabolism is also involved in the malignant transformation of PGCs into their pluripotent partners *in vivo*, ECCs. In particular, miRNA-regulated expression of enzymes involved in glycolytic metabolism contributes to the growth of germ cell tumors [80].

Oxygen levels are closely related to metabolism and potentiality. PGCs cultured in hypoxia are reprogrammed toward pluripotent cells and cause an increase in glycolytic genes, while they downregulate genes involved in oxidative phosphorylation (OXPHOS) [6]. Abundant evidence relates hypoxia to inhibition of oxidative metabolism. Among its effects, hypoxia inhibits cytochrome c oxidase and complex II of the electron transport chain. In fact, usage of the OXPHOS inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) induces an upregulation of Oct4, Nanog, and Sox2 in embryonic stem cells [81]. On the other hand, culture of these cells in normoxia upregulates genes involved in differentiation [82, 83].

As previously mentioned, PGCs cultured in hypoxia give rise to pluripotent cells, and this process takes place in parallel with a metabolic shift toward glycolysis, which is governed by HIF1. Consequently, HIF1 inhibition disrupts PGC reprogramming, and HIF stabilization induces reprogramming [6]. HIF1 acts as a link between oxygen levels and metabolic phenotype inducing the expression of several genes related to glycolysis, such as glucose transporters, hexokinase, pyruvate dehydrogenase kinase 1, and lactate dehydrogenase. In fact, PGCs reprogrammed through culture in hypoxia show pyruvate dehydrogenase inhibition and mitochondrial inactivation [6]. Recent work from our laboratory has shown that PDH needs to be inhibited to achieve PGC reprogramming. Once the glycolytic profile is established under hypoxia, an increase in glycolytic flux through PKM2 activation renders in a synergetic effect with hypoxia (Sainz de la Maza et al. [129]).

Hypoxia can also alter the metabolic profile through mitochondrial mass modification. Mitochondria in pre-migratory PGCs are globular and localized around the nucleus. During migration, mitochondria increase in number and, when they undergo differentiation, they increase their number and size even further, and they acquire a more ovoid shape, further developing their cristae [84].

HIF1 inhibits PGC1 β and induces mitophagy through Bnip3 upregulation [85]. In fact, autophagy is required in the early steps of cell reprogramming [86]. A shift has been reported in iPSC derivation from complex, active mitochondrial networks with developed cristae in fibroblasts to small, spherical, perinuclear, inactive mitochondria without cristae in pluripotent stem cells [66]. The results from our laboratory have shown that hypoxia-induced reprogramming causes a marked increase in Bnip3 expression, an essential gene involved in mitophagy. Autophagy takes place during reprogramming and is required for pluripotency acquisition (Sainz de la Maza et al. [129]).

Additionally, the Lin28/let7 pathway is crucial in glucose metabolism. In particular, Lin28 increases glucose uptake and metabolism through PI3K/mTOR activation and is involved in the translation of genes related to glycolysis, glucose metabolism, cellular carbohydrate metabolism, oxidative metabolism, and mitochondria in human embryonic stem cells [87–89].

Lin28 is also closely related to pluripotency, since it contributes to cell reprogramming and is present in the reprogramming cocktail, giving rise to iPSCs [90], and is also capable of activating the translation of Oct4 at the post-transcriptional level in human embryonic stem cells [91].

Primordial germ cells express Lin28 from 7.5 dpc in mouse PGCs and play a key role in Blimp1 expression. Lin28 is an RNA-binding protein that is able to bind the let7 precursor, impairing let7 processing. Therefore, miRNA let7 is not synthesized and is not able to inhibit Blimp1 translation; Lin28 indirectly stabilizes Blimp1 so PGCs can fulfill their germ cell specification [44, 92].

8. PGC reprogramming and epigenetics

PGCs undergo profound epigenetic reprogramming during their development [42]. Once specified, PGCs express Blimp1, which is the master regulator of PGCs responsible for somatic program repression and germ cell identity [44]. In order to maintain Blimp1 expression and establish germ cell fate, Lin28 acts as a negative regulator of let7 [92]. In human PGCs, Blimp1 is the effector that represses the somatic program, and Sox17 is the determinant transcription factor that establishes the germinal fate [93]. A recent study has shown a stable and elevated expression of Sox15 in early PGCs, which infers a possible role for this molecule in human PGCs as a master regulator, rather than Sox17 [94].

At the onset of specification, PGCs show several epigenetic marks, such as H3K4me2, H3K4me3, H3K9ac, H3K9me1, H3K9me2, H3K9me3, H3K27me2, and H3K27me3. However, these marks are shared at this time by neighboring, future somatic cells [95]. Once PGC

migration begins, PGCs reduce the repressive mark H3K9me2 due to downregulation of the enzymes Ehmt1 and Ehmt2. PGCs also reduce DNA methylation by downregulation of the DNA methyltransferases Dnmt1, Dnmt3a, and Dnmt3b [8, 96]. Prdm14, an essential partner of Blimp1 in mouse germ cell development, and Tcfap2c intervene in this demethylation process downregulating Ehmt1, Ehmt2, Dnmt3a, and Dnmt3b. This partnership is also involved in the reexpression of transcription factors related to pluripotency, such as Sox2 and Klf2. However, Prdm14 is not necessary for human PGC development [97, 98]. PGCs progressively increase the repressive mark H3K27me3 via polycomb repressive complex 2 (PRC2) [95].

Later, once PGCs have reached the future gonads, they remove imprinting tags, increase H4R3me2 mark, and reactivate the inactive X chromosome in female PGCs [95, 99]. H4R3me2 modification, which takes place on arginine residues, is catalyzed by Prmt5, an epigenetic modulator that binds Blimp1, and exerts its epigenetic modifications until it is translocated back to the cytoplasm after E11.5 [100]. Another important event that occurs at this stage is major global DNA demethylation. It has been proposed that the vast extent of this DNA modification infers an active process and expression of the hydroxylase Tet1, the cytidine deaminase AID, and genes involved in base excision repair response (BER) have been detected. Nucleotide excision repair (NER) does not appear to be involved in PGC demethylation because no upregulation in molecules that take part in NER has been detected [101]. Finally, PGCs lose histone H1, increase nuclear size, lose chromocenters, reduce the epigenetic marks H3K9me3, H3K9me2, H3K27me2, H3K27me3, H3K9ac, and H4R3me2, and also lose nucleosomal, noncanonical histone H2a.Z [102, 103]. Blimp1 also induces the expression of Jmjd3 [104], responsible for removing the repressive epigenetic marks H3K9me2 and H3K9me3. In B cells, Blimp1 can also interact with chromatin modifier enzymes such as Kdm1a, a histone lysine transferase that catalyzes removal of methyl groups from lysines 4 and 9 of histone 3 [105]. In contrast to Blimp1, which appears to be essential in the repression of the somatic program, its effector Prdm14 contributes to establishing potential pluripotency through Sox2 upregulation in mouse PGCs, given that Sox2 is absent in human PGCs [97, 106]. Kdm6a is the histone demethylase that catalyzes the removal of the mark H3K27me3 during this second wave of reprogramming. The disruption of this process leads to an aberrant epigenetic reprogramming and loss of pluripotency markers Nanog, Oct4, and SSEA1 [107].

Sex determination begins at approximately E12.5. At this moment, DNA methylation is very low both in male and female PGCs [108, 109]. However, settlement of the epigenetic signature through DNA methylation occurs differently in male and female germlines. In males, *de novo* DNA methylation starts at around E13.5 and is accomplished before birth. Once methylation has been completed, gonocytes undergo vast proliferation and then they enter meiosis at the onset of gametogenesis. A disruption of methylation in male germ cells renders infertility [110]. In females, *de novo* methylation starts after birth and is not fulfilled until approximately P21 [111]. Oocytes undergo cell cycle arrest in meiotic prophase I, and it is not until ovulation that they re-enter meiosis. This coincides with the inability of PGCs to become reprogrammed.

Few studies have been performed on human PGCs, and the majority has focused on later stages of epigenetic reprogramming. Among the data available, early gonadal PGCs in humans (6–8 weeks of gestation) show low H3K9me2 and high H3K27me3 epigenetic markers [112]. Studies

on pigs have also reported that changes in H3K9me2 and H3K27me3 markers are previous to DNA methylation, as observed in mouse PGC development [113]. Human PGCs also display active H3K4me1 and H3K4me3 marks and a peak in H3K9ac from 10 to 13 weeks of gestation. DNA showed a hypomethylated status and a loss of imprinting marks from gestation week 10 onwards. It is noteworthy that Blimp1 is restricted to the nucleus from week 7 to week 12, whereas Prmt5 is located in the cytoplasm, showing that there is no Blimp1/Prmt5 association in human PGCs [112].

Demethylated human PGCs later recover a methylated status, from week 13 of gestation to birth [114]. In addition, human postnatal gonocytes show a different epigenetic pattern from E13.5 mouse PGCs. Low levels of H3K9me2 are shared with mouse PGCs, but H3K27me3 shows low levels in post-migratory PGCs [112]. Also in contrast to mouse PGCs, the H3K9me3 repressive mark is observed in human gonocytes, as well as increased levels of H3K9ac active marks [115, 116]. Mouse PGCs show the lowest methylation at E13.5, when less than 10% of cytosines located at CpG islands are methylated [109]. Analyses of global methylation status from early stages of development have led to the observation that the ICM maintains its methylation status and, consequently, to the hypothesis that the germline is responsible for most of the DNA demethylation that takes place during development [117]. Specifically, the pattern appears to be a global demethylation during PGC migration, in which specific methylation marks in CpG islands (CGIs) of PGC-specific genes, CGIs on the X chromosome and differentially methylated regions in imprinted genes are conserved. Secondly, a new demethylation wave occurs when PGCs reach the genital ridges, affecting the previously mentioned sequences with epigenetic memory. This process involves both active and passive demethylation pathways [109]. Specifically, mouse PGCs fall from a 78% of global methylation at the epiblast stage to 5% at E11.5 (**Figure 1**). In human PGCs, demethylation takes place during the first 12 weeks of development, falling to 7% of methylation [118].

EGCs and ESCs can be fused to B-cells in order to originate a tetraploid hybrid. Methylation analysis has shown that the generated hybrids from EGCs showed lower methylation levels than those generated from ESCs, probably showing some sort of epigenetic memory in which EGCs resemble the low methylation status of their precedent PGCs. These hybrids eliminate the imprints that were present in ESC cells, suggesting that epigenetic reprogramming in EGCs is dominant over ESCs [119]. As previously mentioned, PGCs eliminate their imprinting marks at approximately E11-5. EGCs obtained from E11-5 or later PGCs also show demethylated imprinting marks, which cause serious alterations in developing chimeras [120, 121]. However, EGCs originated from previous PGCs show a less profound imprinting erasure and a more hypomethylated status regulated by Prdm14 when EGCs are cultured in the presence of GSK3 β and MEK (2i) inhibitors [122, 123]. In fact, EGCs eliminate imprinting marks in the genes *Igf2*, *Igf2rr*, *Dlk1*, and *H19*, among others, which are established shortly after PGC specification [124, 125]. EGCs and ESCs also share as a common feature the activation of both X chromosomes in female cells [126].

PGCs can be reprogrammed into EGCs when cultured in the presence of trichostatin A (TSA), an inhibitor of histone deacetylase, in substitution for bFGF, stem cell factor and LIF [39]. In the case of bFGF, this growth factor needs to be added in the first

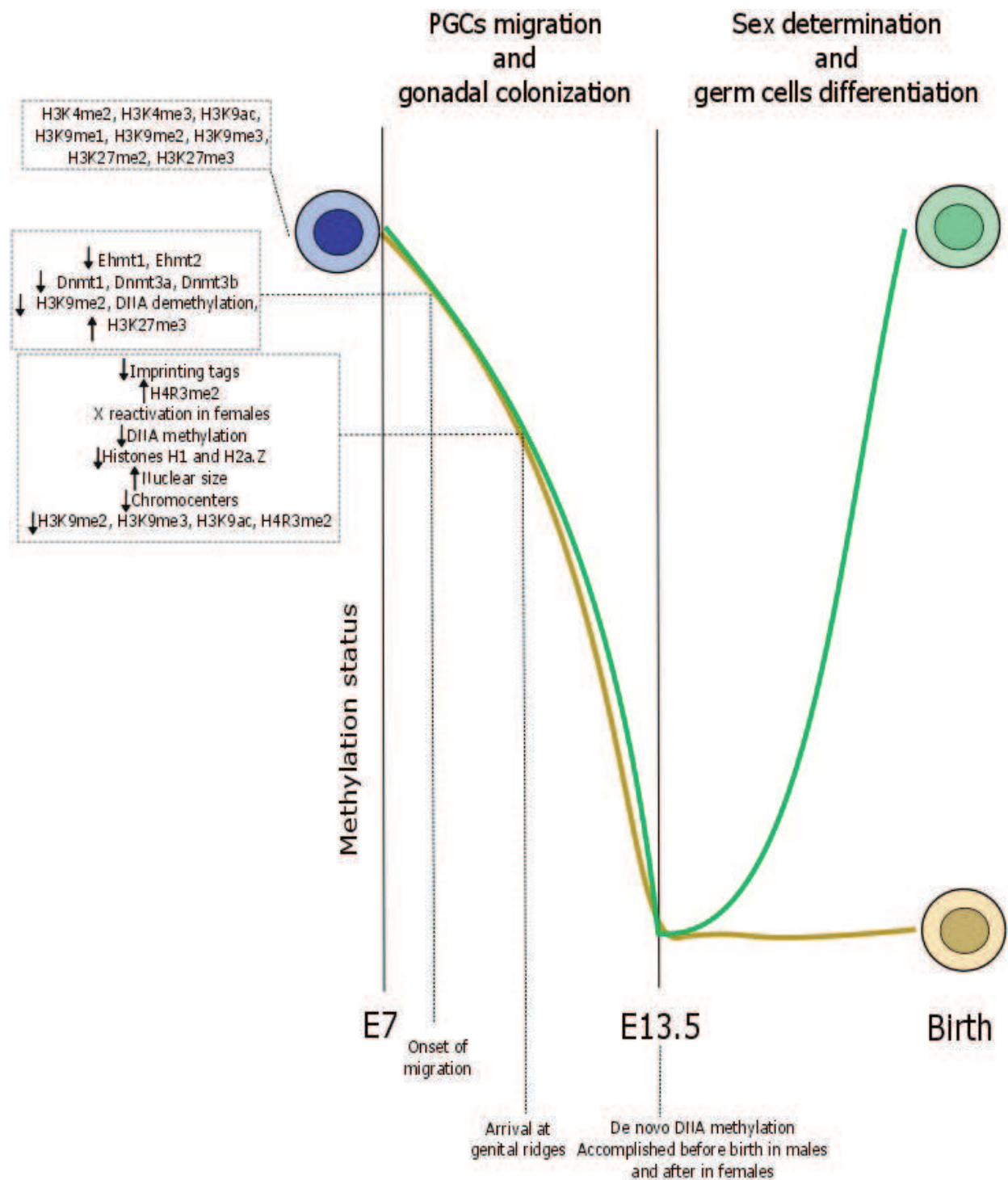


Figure 1. Modifications in DNA methylation during mouse PGC and gamete development. Both male and female germ cells share a first phase of epigenetic modifications, in which several processes lead to DNA demethylation and histone modifications. This phase corresponds to PGC migration and colonization of genital ridges in the first phase and development of gonads in the second. After sex determination, differential methylation is observed among male and female germ cells. Whereas male germ cells undergo *de novo* methylation at E13.5, female germ cells maintain hypomethylated status until birth.

24 h of culture. Substitution of bFGF by TSA accelerates the kinetics of EGC derivation, with a quicker downregulation of Blimp1 [39]. As stated in the classical PGC reprogramming section, bFGF-induced reprogramming of PGCs causes a downregulation of

Blimp1, which provokes an upregulation of Klf4, c-Myc, and Dhx38. This downregulation does not take place if bFGF is added later than 24 h of culture [39, 45, 127]. In EGC derivation by TSA, Blimp1 is absent in EGCs, whereas expression of Klf4, c-Myc, and Eras is detected. In bFGF-induced PGC reprogramming, Blimp1 disappears from PGC after 48 h [39].

As far as Prmt5 is concerned, this epigenetic modifier stays in the nucleus up to 7 days of culture, when it translocates from the nucleus to the cytoplasm in EGC colonies. Since Blimp1 binds Prmt5 to repress gene expression through H2A/H4R3me2s, contributing to maintaining the germ cell phenotype, absence of Blimp1 again appears to be a crucial event in PGC reprogramming. Direct targets of this mentioned repression, such as Dhx38 or c-Myc, are detected a few days after a bFGF-induced reprogramming procedure. These events were also observed in TSA-induced reprogramming of PGCs. [39]. Prmt5 has also been linked to pluripotency in ESCs. Its loss disrupts pluripotency and causes differentiation of these cells, where it is located in their cytoplasm [128]. Prdm14 is also essential for PGC derivation into EGCs. As previously mentioned, Prdm14 is an effector of Blimp1 and is involved in the downregulation of methylation enzymes during the first demethylation wave and in the reexpression of pluripotency factors. PGCs deficient in Prdm14 are unable to reprogram into EGCs because they cannot downregulate the repressive marker H3K9me2 and upregulate the epigenetic marker H3K27me3 via PRC2 [97]. Recent studies from our laboratory have shown that PRC2 is not involved in hypoxia-induced PGC reprogramming, whereas addition of histone deacetylase inhibitor valproic acid (VPA) is capable of inducing PGC reprogramming (Sainz de la Maza et al. [129]).

It is not surprising that epigenetic modifications relate to potency. Pluripotent stem cells show an open conformation of chromatin and active chromatin markers, such as H3K4me and H3K9ac [130, 131]. On the other hand, differentiated cells display repressed chromatin markers, such as H3K27me. Partially differentiated cells show a bivalent chromatin, with both active and repressing markers. Akt, one of the primary factors related to the reprogramming of PGCs, as noted before, also promotes a more active chromatin, mainly by inhibition of Mbd3, a component of the nucleosome remodeling deacetylase complex. Mbd3 is important in heterochromatin formation, and its inhibition promotes reprogramming in both EGC and iPSC derivation [19].

Epigenetics is also involved in the derivation of iPSCs. Fibroblast reprogramming can be achieved exclusively using soluble factors. In the reprogramming cocktail used by Hou et al [131], some epigenetic modifiers were included, such as the histone deacetylase inhibitors (HDACi) sodium butyrate and VPA, and the Kdm1a inhibitor tranylcypromine [132]. HDACi are strongly related to pluripotency acquisition. VPA has been proven to enhance iPSC generation, and usage of HDACi can turn pluripotent colonies with fuzzy edges into typical, compact pluripotent colonies [133–135]. VPA can also eliminate the imprinting marks located at the Dlk1-Dio3 gene cluster [136]. Inhibition of the polycomb complex, responsible for gene repression through DNA and histone methylation, results in lower iPSC derivation efficiency, probably because this complex is essential to the repression of the somatic program [137]. However, inhibition of histone methyltransferases by using BIX-01294 or inhibition of DNA methyltransferases using 5-azacytidine has been reported to improve iPSC derivation. The usage of BIX-01294 on fibroblasts with induced expression of only Oct4 and Klf4 rendered a comparable efficiency to that of using the four factors [138, 139]. A deeper understanding of the impact of epigenetics in reprogramming is required in order to elucidate the role of chromatin and histone modifications in the acquisition

of pluripotency. Further research should be performed regarding the link between pluripotency and the germline program. For example, the induction of the expression of Prdm14, which is a Blimp1 effector, enhances iPSC derivation from mouse and human fibroblasts [140].

9. Concluding remarks and future directions

Various methods and soluble factors can be used for PGC reprogramming, including the classical bFGF [4, 5, 7, 141], the deletion of Trp53 [40], the addition of mitogens such as RA [11] or forskolin, [49], the inhibition of MAPK/ERK kinase and GSK3 signaling [52], and the addition of epigenetic modifiers such as TSA [39] and VPA (Sainz de la Maza et al. [129]). In addition, inducers of glycolysis such as hypoxia [6] and manipulation of cell metabolism are able to induce pluripotency (Table 1) and partial reprogramming, implicating many cytoplasmic and nuclear proteins (Figure 2).

All these data highlight the inherent potency of germ cells, allow for further and detailed characterization of the reprogramming process and are useful tools for the identification of genes involved in germ cell malignant transformation and the development of testicular tumors.

Method		Mechanism	Age (dpc)	Efficiency	Laboratory/year
bFGF		MAPK and PI3K/ Akt activation	8.5	10/80	Hogan [5] and McLaren-Surani [58]
Forskolin		cAMP agonist	11.5	14.5 ± 3.9/(1/2 genital ridge)	Nakatsuji [49]
Retinoic acid		Mitogen	11.5	12.5 ± 5.2/(1/2 genital ridge)	Nakatsuji 1996 [49]
Trichostatin A		HDAC inhibitor	8.5	25/80	McLaren-Surani [58]
Valproic acid		HDAC inhibitor	8.5	ND	De Miguel, submitted
2i	PD0325901	MAPK/ERK inhibitor	8.5	9.4%	Smith [52]
	CHIR99021	GSK3β inhibitor			
SB431542		TGFβR inhibition	11.5	2.08/10 ⁴	Nakano [41]
Kenpaullone		GSK3β and CDKs inhibitor	13.5	2.27/10 ⁴	Nakano [41]
Hypoxia		HIFs activation	8.5	6.9%	De Miguel [6]

Table 1. Comparison of different methods of PGC to EGC reprogramming.

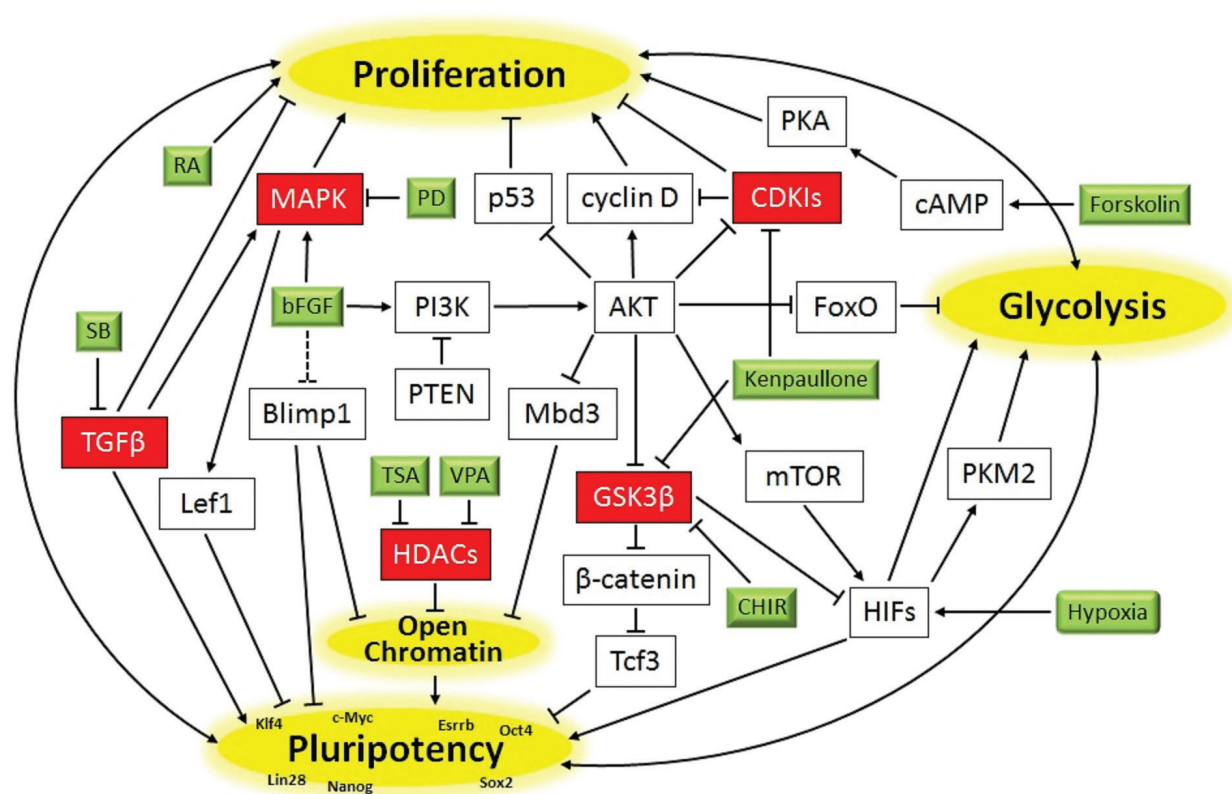


Figure 2. Schematic representation of the external factors (in green), signal transduction molecules (in red or white), and processes (in yellow) implicated in PGC reprogramming. Directly inhibited molecules in red. Arrows indicate induction and broken lines indicate repression of the pathway. PD: PD0325901; SB: SB431542; CHIR: CHIR99021; RA: retinoic acid; VPA: valproic acid; TSA: trichostatin A.

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Abbreviations

2i	two inhibitors (PD0325901 and CHIR99021)
BER	base excision repair response
bFGF	basic fibroblast growth factor
Blimp1	B lymphocyte-induced maturation protein-1
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CCCP	cyanide 3-chlorophenylhydrazone
CDK4	cyclin-dependent kinase 4

CDKI	cyclin-dependent protein kinase inhibitor
CpG	cytosine phosphate guanine
Ddx4	DEAD-Box Helicase 4
Dlk1	delta like non-canonical notch ligand 1
Dmrt1	doublesex-related transcription factor 1
Dnd1	DND microRNA-mediated repression inhibitor 1
Dnmt	DNA methyltransferase
dpc	days post coitum
Dppa	developmental pluripotency associated
ECC	embryonal carcinoma cells
EGC	embryonic germ cells
Ehmt	euchromatin histone methyltransferase
ENO1	enolase 1Embryonic stem cell-expressed Ras
ESC	embryonic stem cells
Esrrb	estrogen-related receptor beta
FoxO	forkhead box class O
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glut	glucose transporter
Hand1	heart and neural crest derivatives expressed 1
HDAC	histone deacetylase
HIF	hypoxia-inducible factor
Hxk2	hexokinase 2
ICM	inner cell mass of the blastocyst
iPSC	induced pluripotent stem cell
Jmjd	Jumonji domain containing
Kdm	lysine demethylase
Ldha	lactate dehydrogenase A
Lef1	lymphoid enhancer binding factor 1
Mbd3	methyl-CpG binding domain protein 3
MEF	mouse embryo fibroblast
Meis	myeloid ecotropic viral integration site
mTOR	mammalian target of rapamycin
NER	nucleotide excision repair
Oct	octamer-binding transcription factor
PARP	poly(ADP-ribose) polymerase
PDH	pyruvate dehydrogenase
Pecam	platelet endothelial cell adhesion molecule

PFK	phosphofructokinase
PGC	primordial germ cell
PGK1	phosphoglycerate Kinase 1
PKM	pyruvate kinase muscle
PRC2	polycomb repressive complex 2
Prmt	protein arginine methyltransferases
RA	retinoic acid
SCF	stem cell factor
SSEA	stage-specific embryonic antigen
Tbx3	T-box protein 3
Ter	teratoma
TGFβ	transforming growth factor β
TNAP	tissue non-specific alkaline phosphatase
TSA	trichostatin A
Utf	undifferentiated embryonic cell transcription factor
VPA	valproic acid
Zfp42	zinc finger protein

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