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Mechanisms of pluripotency and epigenetic reprogramming in primordial germ cells: lessons for the conversion of other cell types into the stem cell lineage

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Abstract: Primordial germ cells (PGCs) provide an excellent tool to better understand ancestor–descendent relationships as well as the efficiency and molecular mechanisms governing pluripotency in the reprogramming of somatic cells, since the latter type of cells have a relatively lower efficiency of conversion to pluripotent cells. This kind of comparison has gained credence from the commonalities regarding the expression of key transcription factors such as octamer-binding transcriptionhttp://en.wikipedia.org/wiki/Transcription_ factor factor-4 (Oct3/4), SRY-related HMG box (Sox2), myelocytomatosis (c-Myc), and Nanog, as well as redundancy in terms of Kruppel-like factor 2 (Klf2), Kruppel-like factor 5 (Klf5), estrogen-related receptor beta (Esrrb), and estrogen-related receptor gamma (Esrrg) compensating for the absence of Kruppel-like factor 4 (Klf4). However, the exogenous addition of any one of these factors was found to be important, thereby implying that the expression level is important. L-Myelocytomatosis (L-myc) was shown to improve reprogramming efficiency without affecting tumorigenic potential. Molecular aspects of epigenetic reprogramming during the acquisition of pluripotency, as well as tumorigenic potential, have also been discussed, thus providing an understanding of the factors that can improve the former without increasing the possibility of neoplastic transformation. An improved understanding of the molecular events would pave the way for the development and use of endogenous biomolecules as well as currently available chemical reprogrammers for improving the efficiency of conversion of PGCs into cells of the stem cell lineage. Such chemicals, when adequately tested, can possibly be an alternative to viral vectors, since the introduced transgenes can become oncogenic.

Key words: Primordial germ cells, stem cells, chemical/genetic reprogramming, pluripotency, transcription factors, epigenetic, induced pluripotent stem cells, embryonic germ cells, embryonic stem cells, epiblast stem cells

1. Introduction

Takahashi and Yamanaka short-listed 4 transcription factors (TFs), called "Yamanaka factors" (Oct3/4, Sox-2, Klf4, and c-Myc), to genetically reprogram mouse fibroblasts to acquire pluripotency (viral transduction). This implied that the 4 factors were involved in the induction of genes that would normally have been expressed in the embryonic state. This Nobel Prize-winning work is of immense significance, since the use of autologous somatic cells can resolve immunological rejection-based concerns. Furthermore, they can possibly be used as cell-based disease models (Takahashi and Yamanaka, 2006) and targeted redifferentiation can be done to produce autologous cells of the desired cell type (Ieda et al., 2010). However, the low efficiency of conversion, as well as the lack of a precise understanding of the molecular mechanisms governing pluripotency in somatic cells, has prompted work on other cell-based model systems (Hanna et al., 2010) like primordial germ cells (PGCs). Evidence for such approaches is discussed below. This review discusses the origin of PGCs (a unipotent cell type in mice and humans), this cell type's underlying pluripotent state, and the concomitant epigenetic changes. Furthermore, this review also elaborates upon chemical reprogramming strategies since they are potentially a better option in comparison with virally/episomally mediated introduction of the important canonical transcription factors.

Near the juxtaposed extraembryonic ectoderm in mice, PGCs are found within the proximal margin of the epiblast (6.5 days post coitum (d.p.c.)). One day later (7.5 d.p.c.), the alkaline phosphatase positive cells have migrated to the mesodermal region (extraembryonic) near the base of the allantois at the posterior end of the primitive streak. By 10.5 d.p.c., the cells have reached the genital ridges, their new and final destination after migrating through the mesentery, subsequent to their association with the hindgut endoderm and migration from the primitive streak. Over a 5-day period (8.5–13.5 d.p.c.), the cells proliferate about 167-fold. The cells in the female enter the meiotic cell cycle, while there is growth arrest in

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males (Ginsburg et al., 1990). Molecular lineage tracing experiments have provided strong evidence for the role of the B-lymphocyte-induced maturation protein (Blimp-1, also known as Prdm1, PRDI-BF1, and RIZ homology domain) in providing an important signal to repress the program in PGCs, followed by the adjacent somatic cells, thereby playing an important role in the specification of the 40 founder PGCs (Ohinata et al., 2005). Single-cell microarray technology has shown that Blimp-1-dependent pathways are involved in the repression of genes associated with the somatic cell lineage in mice (Kurimoto et al., 2008a). Apart from this molecular event in PGC germ-cell specification, the reacquisition of potential pluripotency and genomic reprogramming involves PRDM-14, another PR-domain-containing key transcriptional regulator (Kurimoto et al., 2008a, 2008b). Corroborative evidence for the role of this protein was evident from studies on PRDM-14 mutants in which the aforementioned 2 important events did not occur even though Blimp-1 was present (Yamaji et al., 2008). Earlier studies using a similar single-cell quantitative gene expression profiling approach have shown that a proportion of cells express Hox-1b and Sox-2. However, at a later stage, Hox-1b expression is repressed and Sox-2 is reactivated (Yabuto et al., 2006), and Blimp-1 plays a critical role in this process (Kurimoto et al., 2008a). A hierarchically ordered sequence of activation events is also involved in the final stages of germ-cell specification. These molecular events include the activation of Stella and Nanos3 and the repression of Hox genes. Most importantly, the coordinated induction of several specification genes with high specificity requires the presence of Blimp-1 and PRDM14 (Kurimoto et al., 2008a, 2008b).

Similar findings have been found linking human PGCs to the establishment of pluripotency as well as epigenetic reprogramming. This cell type from gonadal ridges and mesenteries, 5–7 days after fertilization, was able to form colonies of embryoid bodies resembling EG and pluripotent stem cells. This experiment involved culturing these cells on a feeder layer, in the presence of leukemia inhibitory factor as well as basic fibroblast growth factor (bFGF/FGF2) and forskolin, over a period of 7–21 days. These alkaline phosphatase-positive cells expressed markers that were indistinguishable from those found on embryonic germ cells (EGCs) as well as pluripotent stem cells, such as stage-specific embryonic antigen (SSEA-1, SSEA-3, SSEA-4) and tumor rejection antigen (TRA-1–60 and TRA-1–81). Last but not least, they were able to differentiate into 3 germ layers, which is one of the criteria for establishing pluripotency (Shamblott et al., 1998). This finding has been replicated by another research group in terms of the production of clones of EGCs, derived from human PGCs that resembled the EGCs produced by mouse PGCs (Li et al., 2002). While there are similarities between EGCs and PGCs, there are distinct differences in the transcriptional profiles, which may be cell line/strain-specific (Sharova et al., 2007). Distinct markers, indicated in parentheses, may help in segregating the unipotent PGCs (hemoglobin alpha 1, doublesex and mab-3 related transcription factor 1, sperm protein associated with the nucleus, X-linked, family member A1, and EH-domain containing 2 protein) from multipotent EGCs such as unique expression of importin 7, mediator complex subunit 7, RNA binding motif protein 26, heat shock 60 kDa protein 1, and Kirsten rat sarcoma viral oncogene homolog. There are other genes exclusively expressed in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) related to cellular metabolism, adhesion, and cell cycle whose gene products may be a distinguishing feature for these cell types in comparison with the EGCs, as well as help in better defining the germ line pluripotent state (Pashai et al., 2012). Furthermore, acquisition of the unipotent state involves several parallel as well as sequential epigenetic events (e.g., global demethylation) that are robust and redundant (Hackett et al., 2012). Alternative cell types (PGCs or ESCs from epiblast stem cells (EpiSCs)) can be produced in vitro by altering the signaling cues. Furthermore, differences have been reported between EGCs and EpiSCs, with the former cell type resembling ESCs more than the latter. These exciting findings provide an impetus to use molecular profiling-based approaches with PGCs to better understand transcriptional regulation in terms of lineage commitment (Hayashi and Surani, 2009) in comparison with the data from ESC and EpiSCs.

Under defined/different experimental conditions, endogenous expression of 3 out of the 4 key transcription factors (Oct 3/4, Sox-2, and c-Myc) is important, but not sufficient, for pluripotency. Furthermore, since these cells do not express Klf4, its absence could be compensated for by the expression of Klf2, Klf5, Esrrb, and Esrrg (Nagamatsu et al., 2013). Corroborative evidence is available for the role of L-Myc in establishing pluripotency and not tumor formation. This implies that a distinct domain in L-myc is involved in improving reprogramming efficiency (Nakagawa et al., 2010). Hence, sufficient evidence has been provided for the reader/scientist concerned to illustrate the importance of isolating and studying PGCs both in terms of lineage tracing and demonstrating links with pluripotency factors, including molecular similarities and differences in comparison with other cell types like EGCs, ESCs, and EpiSCs. This type of molecular information can be correlated with their epigenetic status and may also help in providing details regarding factors that can promote tumorigenicity. Furthermore, this calls for an improved understanding of the molecular factors regulating cell renewal versus differentiation, specifically the role of bFGF-2 as well as LIF/STAT3, apart from identifying back-up mechanisms in these processes.

2. LIF, SCF, FGF2: self-renewal and differentiation

In the presence of FGF2, in vitro mouse PGCs have increased capabilities of proliferating and forming colonies of undifferentiated ESCs and can synergize with Steel factor and LIF (Matsui et al., 1992). In in vitro murine systems, it has been demonstrated that bFGF is a powerful mitogen for PGCs (Donovan et al., 1994; Resnick et al., 1998) and their presence can be correlated with the expression of its cognate receptors (Resnick et al., 1998). There is good evidence in the literature to indicate that there are time- and concentration-dependent effects mediated by FGF2 and the development of a subset with endogenous FGF2 and FGF-receptor-3 in the nucleus that precedes pluripotency (Durcova-Hills et al., 2006). Trichostatin A, an inhibitor of histone deacetylase, can substitute for FGF-2 in a cocktail containing LIF for the dedifferentiation of PGCs into EGCs (Durcova-Hills et al., 2008). This provided indirect evidence of the role of FGF-2 in this process, obviating the need to introduce exogenous transgenes. Using a genetically modified Zebrafish model system to prolong the life of mRNA of FGF-2 (gene construct with the Nanos3 3'UTR), it was demonstrated that FGF-2 significantly increased PGC numbers at 14 and 21 days after fertilization (Wong and Collidi, 2013).

Another study has documented that the combined actions of SCF and/or LIF with forskolin, an activator of adenylate cyclase, can activate PGC proliferation in vitro (Dolci et al., 1993), as well as inhibit apoptosis (Pesce et al., 1993), without the need for a feeder layer (Cheng et al., 1994). Furthermore, bFGF, LIF, and SCF (Kit ligand) are necessary and sufficient for PGC transformation into EGCs (De Felici et al., 2009). A cocktail of factors containing Kit ligand, LIF, bone morphogenetic factor, stromal cell-derived factor, bFGF, and certain compounds (N-acetyl-L-cysteine, forskolin, retinoic acid) can promote the survival and self-renewal of mouse PGCs in the absence of support from somatic cells (Farini et al., 2005). In mouse ESCs, Nanog has been associated with the undifferentiated pluripotent state; heterozygous cells (Nanog +/-) could be converted to multilineage descendants in the presence of LIF. These descendants could be made undifferentiated by the addition of Nanog (Hatano et al., 2005). There is evidence in the literature to indicate that inactivation of Akt signaling may be responsible for the differentiated state (Watanabe et al., 2006). Fairly convincing evidence using differentiation inhibitors (mitogen-activated protein kinase (Erk1/2) cascade and glycogen synthase kinase 3 inhibitor-2i cocktail) has supported the theory that a combination of blockade of differentiation and a concomitant upregulation of LIF-mediated self-renewal mechanisms can produce EGCs without the need for FGF or SCF (Leitch et al., 2010). In fact, the LIF/STAT3 pathway has been implicated in the process of conversion of PGCs from a unipotent to a pluripotent state (Leitch et al., 2013b). Recent lineage tracing and cocktail standardization-based experiments again point to the combinatorial effects of bFGF as well as LIF and SCF in the formation of EGCs (Nagamatsu and Suda, 2013), thereby providing an impetus for studies involving other cell signaling pathways.

A recent paper has documented the biphasic nature of Wnt signaling; the downstream TFs (Tcf1/Lef1 and Tcf3/Tcf4) behave differently depending on the stage of the reprogramming process (Ho et al., 2013). Earlier on, activation of Wnt signaling can, via Tcf1/Lef1, upregulate target genes that interfere with the early reprogramming. At that stage of reprogramming, Tcf3/Tcf4 can activate, among other genes, Tcf1/Lef1, which can act as transcriptional repressors and thereby promote reprogramming. At a later stage, Tcf3/Tcf4 can modulate the Tcf1/Lef1 responses and convert them to transcriptional activators of targets that interfere with reprogramming. In other words, at a later stage, depletion of Tcf3/Tcf4 can enhance reprogramming. Evidence is available for the involvement of Wnt3a converting Tcf1/Lef1 into activators and preventing them from becoming repressors (Ho et al., 2013).

3. Mechanisms of epigenetic reprogramming and pluripotency

As indicated in the introduction, Blimp-1 (also known as Prdm1), a known transcriptional repressor, is important for the development of the mouse germ cell lineage; its repression is a key early event in the differentiation of pluripotent stem cells (Durcova-Hills et al., 2008), even though it may not be absolutely mandatory for reprogramming (Bao et al., 2012). However, Blimp-1, Prdm14, and Tcfap2c play a combinatorial role in the suppression of genes responsible for the somatic cell fate and Prdm14 is necessary for the induction of epigenetic reprogramming in mice (Yamaji et al., 2008; Grabole et al., 2013). In this regard, the reported upregulation of Blimp-1 in mouse PGCs is a dominant and independent event in the repression of genes of the somatic lineage for germ-cell specification. PRDM14 was also shown to be involved in the reacquisition of potential pluripotency and epigenetic reprogramming. (Kurimoto et al., 2008a, 2008b). In this regard, the use of the aforementioned 2i cocktail (Ficz et al., 2013) by Nagamatsu et al. (2013) for global hypomethylation is necessary to mimic epigenetic reprogramming that may occur in vivo in PGCs. This cocktail is known to downregulate the de novo methyl transferases (DNMT3a and DNMT3b, and its regulator DNMT3L). Further downregulation of hydroxylases Tet1 and Tet2 and the PRDM14-mediated downregulation of DNMT3s induces the PGC-like pluripotent state in epigenetic terms (Leitch et al., 2013a). Hence, the inhibition of downstream differentiation processes,

involving both MEK and GSK-3beta, may be involved. The balance of hydroxyl-methylation and methylation of certain cytosine residues also ties in pluripotency (associated with the transient expression of the network of pluripotency factors in these cells) with epigenetic reprogramming (Seisenberger et al., 2012), while other genomic imprints remain intact (Ficz et al., 2011; Hackett et al., 2013). In this regard, Stella (one of the targets of Blimp-1) is associated with the protection of the maternal genome and the paternally imprinted genes from the wave of global demethylation (Wossidlo et al., 2011). This acquisition of pluripotency in mouse PGCs through synergistic passive and active global demethylation mechanisms, including deamination or oxidation of 5Mc (Mansour et al., 2012), is also potentially coupled with base excision repair and may be preceded by a demethylase (Utx)-induced removal of the H3K27me3 histone mark (histone modification) in PGCs and the transcriptional activation of some pluripotency genes (Mansour et al., 2012; Seisenberger et al., 2013). Methodologies that can be used, with high resolution, to identify critical epigenetic events that are involved in the transition from PGCs to pluripotent stem cells (Kobayashi et al., 2013) would pave the way for a better understanding of the complexities in the epigenetic changes (Nagamatsu et al., 2012a, 2012b). Such changes, including erasure and resetting of parental imprints, as per the Waddington canalization of energy-dependent pathways, play an important role in restricting development potency and decreasing capacity for germline transmission through chimera formation (Li et al., 2009). While studies have suggested that reprogramming is inextricably linked to pluripotency, striking similarities have been observed between reprogramming mechanisms and neoplastic transformation. Replacement of c-Myc by a Wnt pathway inhibitor and overexpression of 3 TFs can improve reprogramming efficiency (Kidder, 2014). Loss of the p53 tumor suppressor has been shown to increase reprogramming efficiency in a manner analogous to the cooperation between oncogenes in the conversion of normal cells to tumorigenic ones. Transient inhibition of the p53–p21 pathway, through the suppression of the Ink4/ARF pathway locus, may also be important in overcoming the barrier for reprogramming; this has also been reported for human fibroblasts with ARF4a being important for this cell type, unlike ARF for its murine counterpart (Hemberger et al., 2009). In the case of PGCs, PGC dedifferentiation is mediated by PI3K/Akt signaling via the inhibition of p53, a downstream molecule of the PI3K/Akt signal. Furthermore, these cells can be converted into ESCs under appropriate culture conditions, indicating that the 2 states are metastable. Further, these interconvertible states may be regulated by common epigenetic factors, warranting more studies with PGCs for an improved understanding of the factors governing reprogramming during the production of its derivatives (Yamano et al., 2010; Kimura and Nakano, 2011). Such approaches provide a logical and sound scientific approach to using the aforementioned key endogenous biomolecules as templates for the development of chemicals (experimental probes/chemical reprogrammers) (Masuda et al., 2013). This approach can possibly obviate the need for the virally mediated introduction of genes, which can become oncogenic, even though integration-free vectors are currently available (Yu et al., 2009; Okita et al., 2011).

4. Chemical reprogramming strategies

While there are a lot of published studies on chemical reprogramming strategies, data specifically related to chemical reprogramming in primordial germ cells are limited. Kimura et al. (2015) have shown that, in the absence of bFGF and SCF, LIF and a combination of chemicals (TGFβR inhibitor and/or Kempaullone-mimicking Sox-2 and Klf4, respectively) generated pluripotent stem cells using conventional ESC culturing procedures (Kimura et al., 2015). However, due to the several commonalities in signaling mechanisms among PGCs, EGCs, ESCs, and EpiSCs, data obtained from small molecule compounds and reprogramming in cell types other than PGCs can provide pointers for development of similar approaches to study and improve the efficiency of this process. Key studies published recently have been briefly reviewed. A novel chemical (CYT296) was shown to increase the efficiency of OSKM-mediated induction of iPSCs from mouse embryonic fibroblasts (MEFs) and this reprogramming was accompanied by chromatin remodeling (formation of a decondensed euchromatin-like structure, which is considered to be necessary for development into a pluripotent state) (Wei et al., 2014). In another study, transient folate deprivation with a new combination of small-molecule compounds (sodium butyrate, A-83-01, CHIR99021, and Y-27632 in place of Sox-2 and c-Myc) and Oct4 and Klf4 could reprogram MEFs at an accelerated rate in MEFs. The resultant cell lines resembled ESCs (Hu et al., 2014). Approaches such as these can help in dissecting the epigenetic processes acquired during reprogramming from the background epigenetic marks present in the somatic tissue of origin of these iPSCs (Vaskova et al., 2013).

Among the 4 major transcription factors, Oct4 has continued to be a challenge in terms of finding a chemical replacement. BIX-02194 (G9a methyltransferase inhibitor) has been shown to shift the epigenetic balance towards activation of endogenous Oct4 in reprogramming of mouse fetal neural progenitor cells. However, this approach required the viral transduction of the remaining 3 transcription factors (Shi et al., 2008). Subsequent work involved the systematic development of successive chemical screens. The first step involved development of molecules that would replace Sox-2, Klf4, and c-Myc. The

molecules were valproic acid (HDAC inhibitor), GSK3-β inhibitor CHIR99021, TGF-β inhibitor E-616542, and monoamine oxidase inhibitor tranylcypromine (VC6T) (Li et al., 2011). The second step involved chemicals that would replace Oct4; c-AMP agonist forskolin was identified for this purpose. In order to complete the reprogramming, other components identified were 3-deazaneplanocin A, an S-adenosylhomocysteine hydrolase inhibitor, as well as the 2i cocktail (MEK and GSK3-β inhibitor) (Ying et al., 2008). The work of Hou et al. (2013) represents a landmark approach in terms of providing a road map for the generation of iPSCs. Their approach involved the possible upregulation of endodermal-associated genes followed by the inhibition of the differentiation processes by the 2i cocktail (Hou et al., 2013). However, challenges remain in terms of translating this finding to the development of human iPSCs due to differences in the response of the 2i cocktail in mouse and human cells (De Los Angeles and Daley, 2013). In this regard, development of advanced methods, such as circular chromosome conformation capture-sequencing, to characterize the dynamic changes in chromatin would be useful. This methodology can complement the studies involving transcription factors and other epigenetic changes like DNA methylation and histone modification modulating gene expression. Using this method, key protein-like mediator and cohesin components have been identified that are involved in the reorganization of chromatin and specific subunits that play a role in differentiation as well as reprogramming. Rearrangement at the Nanog locus has been shown to precede transcriptional regulation reprogramming genes, thereby implying a possible causative, long-range, interaction-based mechanistic link (Apostolou et al., 2013; Ferrari et al., 2014). Epigenetic reprogramming of the unipotent PGCs into a pluripotent state involves a wave of hypomethylation. Differentiation of such cells is also associated with changes in the epigenome and is correlated with cell fate and lineage commitment (Lee et al., 2014). Detailed analysis of such dynamic and complex events would require application of current, state-of-the-art experimental and computational methods. Such methods, including the use of mathematical models, should take into account coordinate regulation and temporal variations at the subcellular, intercellular, and niche levels. Filling such knowledge gaps would help in better understanding not only PGCs and EGCs, but would also improve our understanding of pluripotency and epigenetic reprogramming of other cell types into the stem cell lineage (Wu and Tzanakakis, 2013) with obvious ramifications in cell-based therapies.

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