Gene Ther Mol Biol Vol 13, 130-143, 2009

Signaling and epigenetic mechanisms regulating stem cells

Review Article

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Keywords: stem cells, embryonic, somatic, pluripotency, multipotency, and mechanisms
Running Title: Mechanisms of pluripotency and multipotency

Received: 8 May 2009; Revised: 15 May 2009;
Accepted: 18 May 2009; electronically published: 2 June 2009

Summary

The enormous potential of stem cells in human therapeutics heightens the relevance of studies addressing the cellular mechanisms, which control their proliferation and fate choice decisions. While embryonic stem cells retain the ability to differentiate into most if not all the cell types of the adult, somatic stem cells retain a restricted potential to differentiate into most or all the mature cell types of the tissue that they are derived from. In this report we review the literature and concepts related to cytoplasmic and nuclear regulatory mechanisms, which confer the properties of pluripotency or multipotency on stem cells. The recent flurry of activity related to the induction of pluripotency in fibroblasts and other relatively mature cells has given us pause in considering the actual nature of the two extremes of terminal differentiation and 'stemness', and the apparent reversibility of both phenomena. An analysis of the current literature on mechanisms of pluripotency and multipotency could lead to a better understanding of the possibilities by which a cell may be maintained in a preferred state of differentiation or de-differentiation at will.

I. Introduction

While stem cells provide extremely interesting scientific platforms in which important questions in biology are being unraveled, they have achieved unparalleled notoriety because of the immense potential they present for human disease therapies. In this review we will attempt to highlight some of the mechanisms by which mammalian embryonic and somatic stem cells maintain their pluri- or multi-potential nature respectively. A concerted effort is being directed towards configuring methods for the differentiation of these cells to specific mature phenotypes. It is also of practical importance to determine the cellular pathways and mechanisms, which allow these cells to maintain their capacity to differentiate along a few to several lineages, depending on the pedigree of the stem cell in question.

The processes of maintenance and differentiation of a stem cell may be considered a continuum wherein several individual and overlapping events define the status that the cell is in at any instant. The mechanisms dictating this continuum could be based on a constant crosstalk between competing or augmenting events (including signaling complexes in the cytoplasm and nucleus), the results of which become progressively more irreversible as the stem cell heads towards commitment and differentiation into a mature fate. Intrinsic to this definition is the idea that the greater the potential of the cell to differentiate into various fates, the greater is its capacity to respond to various stimuli that could direct it to differentiate along particular lineages. This enhanced responsiveness in a stem cell could be due to the expression of a greater cohort of proteins enabling the cell to respond to various diverse stimuli, which gets culled down to a much more defined set of proteins as commitment occurs and the cell acquires the capacity to respond only to a specific set of stimuli. This reduction in global gene expression could be brought about by epigenetic modifications, which exert control of gene expression by regulating the accessibility of gene promoters through modifications to chromatin structure. Thus the signaling and epigenetic states of the stem/progenitor/mature cell define the context in which signals provided by the environment are received and interpreted, making the environment or 'niche' in which the cell is maintained yet another facet of this complex continuum. The recent observations relating to the induction of pluripotency in mature cells seem to complete the circle of the so-called continuum where given the correct set of stimuli 'mature' cells may be rendered seemingly pluripotent again.

Stem cells are generally defined by their dual properties of pluripotency/multipotency and self-renewal. Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst stage of a mammalian embryo (Evans MJ and Kaufman MH, 1981), and somatic stem cells (SSCs) are derived from various developing and mature tissues in which they are resident. While ESCs are pluripotent as defined by their property of multilineage differentiation into any cell type in the body, SSCs are generally more restricted in their potential and give rise to all or some of the cells characteristic of their tissue of origin, termed multipotency. An understanding of the intracellular signaling pathways involved in maintaining pluripotency of ESCs will help in the more effective utilisation of ESCs for cellular therapy and regenerative medicine as this could lead to the design of more reliable methods to establish ESC cultures. Also it would enable the development of better strategies for directed differentiation of ESCs to cell populations of our interest. In this review, we look into the various cellular molecules that are involved in controlling the self renewal and pluripotency of ESCs, which thereby facilitate ways to harness the therapeutic potential of ESCs in transplantation therapies and drug discovery (McNeish J, 2004). The ultimate effect of signaling and epigenetic interactions is realized at the level of gene expression, wherein distinct transcription factors and epigenetic modifications play critical roles in determining the gene expression profiles and other characteristic properties of ESCs (Boyer LA et al., 2005, Loh YH et al., 2006).

Epigenetic mechanisms have been implicated in the regulation of gene activation and silencing at the level of transcription. They regulate the manner in which genomic DNA is packaged along with histones into chromatin. ESCs have a distinct gene expression profile as compared to differentiated cells, and have potential to activate all, or most, of the gene expression programs that are found in embryonic and adult cell lineages (Chambers I and Smith A, 2004). Transcriptional profiling studies have been performed to understand the molecular mechanisms of stemness and pluripotency of ESCs (Armstrong L et al., 2006, Ramalho-Santos M et al., 2002).

II. Some mechanisms of pluripotence in ESCs

A. Cytoplasmic signaling and transcription factors

ESCs are generated, maintained and used in culture. As a result most maintenance and differentiation protocols arise by the manipulation of the culture environment, which includes the addition of growth and differentiation factors and modification of the substratum and the gases in the culture milieu. Extensive effort is being directed towards understanding the intracellular signaling pathways which are activated downstream of these extracellular stimuli, and towards understanding the kinases and transcription factors which act as important modulators of the final outcome. The most successful of these studies permit the manipulation of specific intracellular intermediates, providing very specific and sometimes beneficial effects, potentially facilitating a means of bypassing the necessity of activating signaling pathways at the level of ligand/receptor interactions at the cell surface.

One of the best established signaling mechanisms effecting pluripotency of murine ESCs (mESCs), acts through the janus associated tyrosine kinase (JAK), the direct target of which is the signal transducer and activator of transcription 3 (STAT3) protein. In mESCs, leukemia inhibitory factor (LIF) is indispensible for the self renewal and pluripotency, and mediates its effects via the JAK-STAT pathway (Smith AG et al., 1988). LIF belongs to the interleukin-6 cytokine family and binds to heterodimeric receptor, which includes LIF receptor β and gp130, whereby it activates the pathway. LIF can also activate the mitogen activated protein kinase (MAPK) or ERK (Extracellular signal regulated kinase) pathway, which has an opposing role to STAT3, i.e., it promotes differentiation (Burdon T et al., 1999) in mESCs. Thus LIF can regulate pluripotency of mESCs by striking a balance between JAK-STAT3 and MAPK effects. However, it has been shown that the maintenance of pluripotency of human ESCs (hESCs) is STAT3 independent (Humphrey RK et al., 2004) and does not require the presence of LIF. Intriguingly, the expressionof LIF receptor and signaling subunit gp130 has been shown in human ESCs, along with LIF mediated phosphorylation of STAT3 and nuclear translocation. Despite these, LIF does not seem to have any significant role in human ESC pluripotency. LIF is known to function in murine embryonic diapause, i.e., the temporary arrest of blastocyst development. This facilitates multiple and repetitive pregnancies in lactating female mice. In addition, LIF acts as an anti-differentiation factor for cells of the inner cell mass in mouse development (Daheron L et al., 2004). Absence of such functions for LIF in human embryonic development could account for its lack of primary involvement in human ESC maintenance. However, the exact difference in the cellular context in which the LIF stimulus is received by mouse and human ESCs is unclear. Among the various transcription factors implicated with the regulation of self renewal and pluripotency of ESCs, most of the important ones have either a direct or indirect link to LIF/STAT3 pathway. **Figure 1** summarizes some of these interactions.

Of the transcription factors which induce pluripotency, the POU family transcription factor, Oct3/4, NK-2 class homeobox transcription factor Nanog and Sox2, member of SOX (SRY-related HMG box) family are the best characterized. Oct3/4 is a master regulator of pluripotency, which blocks the ESC differentiation towards trophoectoderm by interacting with Cdx2 to form a repressor complex (Niwa H et al., 2005). However, the overexpression of Oct3/4 in ESCs leads them to endoderm lineage (Niwa H et al., 2000). Sox2 plays a significant role in governing pluripotency of ESCs, by blocking their differentiation into multiple lineages, including trophoectoderm (Ivanova N et al., 2006). Sox2 acts in concert with Oct3/4 in activating the Oct3/4 target genes (Yuan H et al., 1995). The self renewal genes, c-Myc, Klf4 and Nanog serve as direct downstream targets of STAT3 protein (Cartwright P et al., 2005, Li Y et al.,

2005, Suzuki A et al., 2006). c-Myc is a well characterized accelerator of cell cycle, which drives the G1-S transition by the transcriptional activation of cyclin E expression (Hooker CW and Hurlin PJ, 2006). JAK-STAT3 does not directly regulate Oct3/4 expression, however genes regulated by STAT3 have Oct3/4 binding sites (Rao M, 2004). The LIF/STAT3 pathway also targets the Nanog promoter region. Nanog is found to prevent ESC differentiation to Gata6 positive endoderm like cells (Mitsui K et al., 2003), block neuronal differentiation (Ying QL et al., 2003) and reverse mesoderm specification (Suzuki A et al., 2006) of ESCs. Recently it has been reported that Sall4 is another pivotal regulator of pluripotency in mouse ESCs. It forms a complex with Oct4, Sox2, Klf4 and c-Myc, the four genes which were employed in the generation of induced pluripotent stem cells (iPSC) from somatic cells (Takahashi K and Yamanaka S, 2006). Hence Sall4 is also implicated to be a key regulator for the cell reprogramming process, and is thought to be regulated by STAT3 at its promoter (Yang J et al., 2008).

In contrast to mESCs, FGF2 (fibroblast growth factor) replaces LIF in hESCs, for the maintenance of their pluripotency. It acts by binding to FGF receptor (Ginis I et al., 2004, Sperger JM et al., 2003). In addition to its inherent tyrosine kinase activity, activated FGF receptor signals by phosphorylating the docking protein FRS2, which complexes with Grb2 proteins and the nucleotide exchange factor SOS. The FRS2-Grb2-SOS complexes inturn activate the Ras-Raf-MAPK signaling pathway. Apart from hESCs, it has been implicated as important for maintaining stemness of other tissue specific stem cells as well. (Dvorak P et al, 2006). A model was proposed by Bendall and co- workers, suggesting that FGF 2 may crosstalk with IGF2 pathway, in maintaining hESC self renewal (Bendall SC et al., 2007). To further augment the role of FGF in ESC pluripotency, it has been shown that Activin/Nodal signaling through Smad2/3 maintains human ESCs in pluripotent state, where FGF2 acts as a competence factor (Vallier L et al., 2005). Inhibition of the FGF signaling via the FGFR1 tyrosine kinase, represses Oct3/4 expression, suppresses downstream kinases and drives ESCs into differentiation (Bendall SC et al., 2007, Dvorak P et al., 2005). FGF4, another member of the FGF family has been implicated in embryogenesis of mouse and is found to have restricted expression in undifferentiated ESC and embryonal carcinoma (EC) cell lines. Notably, Fgf4 gene is found to be a direct target of Oct3 and Sox2 (Yuan H et al., 1995).

Members of TGFβ (transforming growth factor) family of transcription factors, particularly TGFβ, BMP (Bone morphogenetic proteins), Growth and differentiation factors (GDFs), Activin and Nodal are known to sustain self renewal and pluripotency of ESCs. TGFβ signaling involves the binding of the ligand and activation of the Type II receptor, which is a serine/threonine kinase, which then activates the Type I receptor and that in turn phosphorylates Smad (mothers against decapentaplegic related) proteins which regulate targeted gene expression. BMP binds to receptor-regulated Smads (R-Smads: Smad1, Smad5 and Smad8) and activates them, which then form heteromeric complexes with Smad4.

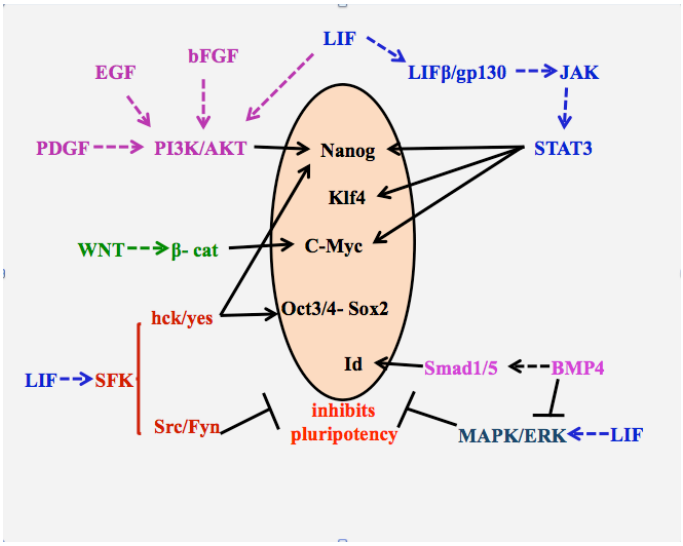

Figure 1: Signals regulating pluripotency in mouse ESCs.
The figure outlines some of the best-studied pathways regulating pluripotency. LIF acts through the JAK/STAT3 pathway to activate three important pluripotency factors, Nanog, Klf4 and c-Myc as shown. Further it can also channel into PI3K and SFK (Hck/Yes) pathways to promote pluripotency by activating Nanog and Oct3/4–Sox2. PI3 K activation by ligands such as EGF, bFGF, PDGF and LIF facilitate mES proliferation, cell survival and self-renewal by promoting Nanog expression. Wnts act through β-Catenin and cause targeted activation of c-Myc, which is also involved in maintaining pluripotency. BMP4, a member of TGFβ family, signals via Smad 1/5 to induce expression of Id genes, which inhibit differentiation of ES cells. BMP4 suppresses the blocking effects of the MAPK/ERK \pathway on mESC pluripotency. LIF appears to inhibit pluripotency by signaling via MAPK/ERK pathway and two other SFK members, Src/Fyn.

The Smad complexes translocate to the nucleus, and act as transcription factors. Bone morphogenetic protein 4 (BMP) is known to co-operate with LIF in maintaining the undifferentiated state of mESCs, in serum free conditions. BMP4 enhances the self renewal of mESCs by inducing the expression of members of Id (inhibitor of differentiation) gene family (Ying QL et al., 2003) and suppression of ERK signaling pathway in mESCs (Qi X et al., 2004). However in human ESCs, BMP4 induces mesodermal and ectodermal differentiation while BMP2 induces extraembryonic endoderm differentiation (Schuldiner M et al., 2000). TGFβ/Activin/Nodal signaling promotes pluripotency of human ESCs along with Wnt signaling, especially in the earlier stages of cell fate determination (James D et al., 2005).

Another major signal transduction pathway required for the self renewal of ESCs, particularly in mESCs, is the Wnt/ β-catenin/CBP pathway (Miyabayashi T et al., 2007). The cytoplasmic levels of β-catenin, an intermediate effector molecule of canonical Wnt pathway, is kept under control by a destruction complex, comprising of adenomatous polyposis coli gene (APC), Axin, and glycogen synthase kinase (GSK) 3β. Binding of Wnt to its receptors, Frizzled and LRP5/6 causes the inactivation of GSK3β, which results in the accumulation of β-catenin in the cytoplasm, and its nuclear translocation. Within the nucleus, it associates with transcription factors lymphoid enhancer factor (LEF)/Tcell factor (TCF). A pharmacological inhibitor of GSK3β, 6-bromoindirubin-3'-oxime (BIO), is reported to promote murine ESC pluripotency even in the absence of LIF (Sato N et al., 2004). Interestingly, BIO is also an effector of human ESC self renewal, and induces the expression of pluripotency markers, Oct3/4, Rex1, and Nanog. However the role of Wnt in maintenance of mESC pluripotency is a debatable issue as there is evidence that it promotes neural and mesodermal differentiation of ESCs (Yamaguchi TP et al., 1999). Wnt signaling is demonstrated to have a synergistic effect with LIF-STAT3 in maintenance of pluripotency of human and murine ESCs, as Wnt can upregulate STAT3 and also both Wnt and STAT3 tend to converge on c-Myc (Cartwright P et al., 2005, Sato N et al., 2004).

Phosphoinositide 3- kinase/AKT signaling is required for efficient self renewal of murine ESCs, by facilitating proliferation and survival of ESCs. This is mediated partly by the ability of PI3K signaling to maintain the expression of Nanog, a transcription factor which is indispensible for the maintenance of pluripotency of ESCs (Welham MJ et al., 2007). PI3K /AKT signaling participates in maintaining ESC pluripotency, upon activation by growth factors like LIF, bFGF, EGF and PDGF (Jirmanova L et al., 2002, Xu C et al., 2005). Class 1A PI3 kinases upon activation, generate second messenger phosphatidylinositol-3, 4, 5-tris-phosphate (PIP3). Akt1, which is a serine/threonine kinase, binds to PIP3 and translocates to the inner cell membrane, where it is activated by another serine/threonine kinase, PDK1. Inhibition of PI3 kinase activity in mouse ESCs affects the cell cycle progression from G1 to S phase, causing decreased cell proliferation (Jirmanova L et al., 2002). The deletion of Pten, a negative regulator of PI3K triggers ESC viability and proliferation (Sun H et al., 1999). Further it has been reported that artificial activation of AKT, can supplement the requirement of LIF, in governing pluripotency (Watanabe S et al., 2006).

The Src family of cytoplasmic protein-tyrosine kinases (SFKs) are another class of kinases, which play distinct roles in ES self renewal and differentiation pathways. It has been reported that regulation of self renewal property of mESCs by LIF requires the activation of these kinases (Meyn MA, 3rd et al., 2005). With regard to the SFKs, of the 7 members which are expressed in ESCs, Hck and Yes promote self renewal in ESCs by way of down-regulating the expression of orphan nuclear receptor Gcnf, which is known to repress Oct3/4 expression and by increasing Nanog expression (Anneren C et al., 2004, Blake RA et al., 2000, Meyn MA, 3rd et al., 2005). Two other SFK members, Src and Fyn play conflicting roles by fostering ESC differentiation (Meyn MA, 3rd et al., 2005).

Insulin like Growth Factor 1 Receptor (IGF1R), a tyrosine kinase, which is activated by IGF2 is relevant to hESC pluripotency (Bendall SC et al., 2007). The expression of IGF1R in hESCs correlates with the pluripotency markers Oct3/4, SSEA4 (Stage specific embryonic antigen4), TRA 1-60 (tumour recognition antigen 1-60) and TRA 1-81 expressions. The IGF2 pathway synergises with LIF to promote self renewal of rat ESCs (Takahashi A et al., 1995). The Stem Cell Factor (SCF) receptor cKit, a tyrosine kinase has been shown to participate in maintaining pluripotent state of mESCs (Lu M et al., 2007, Palmqvist L et al., 2005) and hence could be used as a possible marker for undifferentiated mESCs. The cKit expression is found to positively correlate with levels of pluripotent genes BMP4 and Nanog (Lu M et al., 2007). Expression of serine/threonine kinases, Pim-1 and Pim-3 is demonstrated to be upregulated by LIF/gp130-dependent signaling and the STAT3 transcription factor and thus they may be participating in the control of self renewal of mESCs (Aksoy I et al., 2007). Other pathways including NFκB have also been implicated in ESC renewal; however we have restricted our discussion only to the better described signaling pathways.

B. Epigenetic factors

Epigenetic regulation of the cellular genome includes post-translational modifications to the histones, DNA methylation of CpG nucleotides, and ATP-dependent chromatin remodeling. These events may be inherited along with the genomic sequence. Quantitative single cell in vivo imaging and biochemical analysis of endogenous proteins in undifferentiated mESCs have revealed that several major architectural histone proteins such as Histone 1 (H1), Histone 2b (H2B), Histone 3 (H3) and Histone P1 (HP1α) bind with less affinity to chromatin. This allows the chromatin to be more hyperdynamic in ESCs than in differentiated cells (Meshorer E and Misteli T, 2006) and remain open thus facilitating rapid genetic regulation, which may account for the pluripotency of mESCs. Heterochromatic DNA is highly methylated and condensed and not available for transcription, while

euchromatic DNA is lightly condensed and amenable for transcription.

Heterochromatic markers have been shown to be dispersed in mESC whereas they occur as more concentrated distinct foci in differentiated cells. Two of the most significant heterochromatic markers are increased trimethylated lysine 9 H3 (TriMeK9 H3), and decreased acetylated histones H3 and H4 (AcH3 and AcH4) (Kimura H et al., 2004, Lee JH et al., 2004). Both these conditions, when global, cause gene repression. Differentiation leads to a decrease in the euchromatic nature of the chromosome making it more condensed and heterochromatic leading to loss of pluripotency. The histone modifications and the chromatin patterns can spread over kilobase lengths of genomic DNA, which are then faithfully transmitted to daughter cells to maintain pluripotency and stemness giving rise to the concept of epigenetic inheritance (Cavalli G and Paro R, 1999, Hall IM et al., 2002).

Chromatin immunoprecipitation (ChIP) assays in mESCs have found large areas of chromosomes exhibiting methylation of Lysine 27 histone H3 (MeK27 H3) which represses transcription (Cao R and Zhang Y, 2004), alongside smaller regions of MeK4 H3 which is permissive for gene transcription (Bernstein BE et al., 2005, Schubeler D et al., 2004). These are present at highly conserved noncoding elements, which are associated with regions concentrated for genes encoding developmentally important transcription factors. These regions have been termed "bivalent" domains. They coincide with differentiation-associated transcription factor genes, expressed at extremely low levels in the ESC. Thus it is proposed that they act not only to silence such genes in ESCs in order to maintain pluripotency, but also to allow them to remain in standby mode for transcription so that they can be rapidly activated upon differentiation.

These changes in chromatin structure and its constituents at different stages of ESCs are brought about by several proteins. Some of these are discussed here very briefly (see **Table 1, Figure 2**). In mouse and human ESCs, Polycomb-Group (PcG) complex proteins mainly act to stabilize a repressive chromatin structure. PcG proteins comprise two functionally and biochemically distinct multimeric Polycomb repressive complexes called PRC1 and PRC2 (Levine SS et al., 2004). Polycomb repressive complex 2 (PRC2), which consists of EZH2, EED and SUZ12 in ESCs, functions as a histone methyltransferase that causes tri-methylation of lysine 27 (K27) of histone H3 (H3K27me3) (O'Carroll D et al., 2001). The complexes PRC1 and PRC2 co-occupy 512 genes, many of which encode transcription factors with important roles in development, indicating that repression of developmental pathways by Polycomb complexes may be required for maintaining ESC pluripotency and plasticity (Boyer LA et al., 2006, Lee TI et al., 2006).

Histone demethylases participate in complex interactions which lead to up-regulation or down-regulation of gene transcription. Among the histone demethylases, a member of the Jumonji protein family, JARID1, specifically catalyze the demethylation of H3K4me3 and H3K4me2 (Christensen J et al., 2007).

Table 1: Known epigenetic events in ESCs
The table provides a brief review of epigenetic events, which have been shown to occur in ESCs.

Epigenetic modifications	Enzymes	Modifications	Function	Reference
1) Histone modifications	a) Methyltransferases Polycomb-Group (PcG) of proteins	Trimehylation on H3K27me3	Transcriptional repression	O'Carroll, et al., 2001
	b) Histone demethylases Jarid1a	Demethylation of H3K4me1/2	Transcriptional repression when complexed with PcG	Pasini, et al., 2007
	Jmjd1& Jmjd2c	Demethylation of H3K9me1 at Nanog promoter	Prevent transcriptional repression of Nanog	Loh, et al., 2007
	c) Acetyltransferases p300	Binding at promoters of Oct4, Nanog	Transcriptional activation	Chen, et al., 2008 Jin & Zhong, 2009
	d) Histone deacetylases Nucleosome Remodeling Deacetylase (NuRD) complexes	ATP-dependent chromatin remodeling and histone deacetylation	Entire complex required for deactylation which leads to repression	Kaji, et al., 2006
2) DNA modifications	DNA methyl transferases Dnmt3a Dnmt3b	Methylation of cytosine of CpG islands in promoter regions	Transcriptional repression	Li, et al., 2007

The PRC2 complex recruits JARID1 to PcG target genes in order to enhance PcG mediated gene repression during ESC differentiation (Pasini D et al., 2008). Other reports suggest that JARID1b enhances ESC proliferation by a similar repressive effect (Dey BK et al., 2008). Jmjd1a and Jmjd2c, members of the Jumonji protein family, act in concert with the pluripotency factors Oct-4 and Nanog to regulate expression of genes that encode chromatin-remodeling enzymes. Depletion of *Jmjd1a* results in cellular differentiation, decreased expression of the pluripotency factors like *Tcl1, Tcfcp2l1*, and *Zfp57*, and induction of lineage specific genes. JMJD2c is required to inhibit H3K9 methylation at the Nanog promoter and prevent the transcriptional repressors HP1 and KAP1 from binding to it (Loh YH et al., 2007).

Histone acetyltransferases (HAT) and deacetylases (HDAC) also regulate ESC pluripotency. HAT, p300, was shown to be recruited to sites which bind multiple pluripotency factors including Nanog, Oct4, and Sox2 (Chen X et al., 2008). Epigenetic modification of histone acetylation at the distal regulatory region of Nanog was found to be dependent on the presence of p300 (Zhong X and Jin Y, 2009). It was shown that ESCs depleted of the HAT Tip60-p400 subunits, which are involved in Histone H4 acetylation, exhibit altered morphology and are impaired in their ability to self renew and fully differentiate (Fazzio TG et al., 2008). Components of the Nucleosome Remodeling Deacetylase (NuRD) complexes play important roles in gene regulation and are uniquely characterized by the possession of both ATP dependent chromatin remodeling and histone deacetylase properties. Methyl DNA-binding protein MBD3, a component of NuRD, is shown to be required for pluripotency because ESCs lacking MBD3 exhibit a growth defect and cannot commit to developmental lineages (Kaji K et al., 2006).

DNA methyltransferases (Dnmts) are a family of proteins involved in the establishment and maintenance of DNA methylation and are separated into two functional classes: the *de novo* and the maintenance methyltransferases. DNMT3a and DNMT3b are *de novo* methyltransferases responsible for remethylation in postimplantation mouse embryos and in germ cells (Okano M et al., 1999). The maintenance methyltransferase DNMT1 (Bestor T et al., 1988) is required for maintaining maternal and paternal methylation imprints in the early embryo. Inactivation of both Dnmt3a and Dnmt3b in mouse ESCs results in progressive loss of methylation in various repetitive sequences and single-copy genes. In embryonic stem cells, Dnmt3a and Dnmt3b are shown to be stably associated with each other (Li JY et al., 2007). These two enzymes directly interact and function to methylate the promoters of the Oct4 and Nanog genes. Inadequate methylation caused by ablating DNMT3a and DNMT3b is associated with dysregulated expression of Oct4 and Nanog during the differentiation of pluripotent cells and mouse embryonic development (Li JY et al., 2007). DNA methylation, induced by Dnmt3a and Dnmt3b, has also been shown to be important for the methylation of the X-linked homeobox gene cluster *Rhox* in ESCs (Oda M et al., 2006).

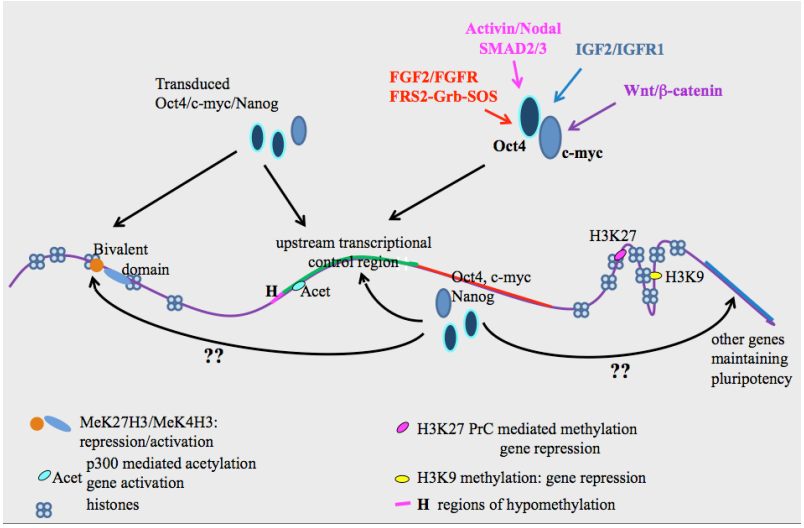

Figure 2: Cytoplasmic and epigenetic signals regulating pluripotency in ESCs.
The cartoon focuses on epigenetic events in ESCs. The chromatin in ESC is more euchromatic than heterochromatic in nature. In this schematic Oct4, Myc, Nanog genes, the markers of pluripotency, are represented within the nucleus of the ESC by the green (promoter region) and red (coding region) coloured DNA strand. Increased cellular levels of these transcription factors may regulate several events within the cell to promote pluripotency. They could regulate expression of themselves and other pluripotency markers, regulate other genes including epigenetic enzymes and miRNAs which promote pluripotency indirectly, and regulate the bivalent domains (such as ME K27 H3/MeK4H3) which would repress differentiation associated genes and increase expression of pluripotency genes.
Gene repression is also achieved by modifications on histones such as methylation by the polycomb group of proteins (H3K27), and other methylation events on H3K9. These two modifications are represented in heterochromatic regions, which are not easily accessible to transcription. Transcription may be selectively enhanced by acetylation, which is brought about by acetyltransferases such as p300. DNA methylation also plays a very important role in maintaining pluripotency of ESCs. Hypomethylation at promoters of the pluripotency genes and methylation of promoters of developmentally important genes is the hallmark of ESCs.
OCT4 levels in the cell may be increased either by cytoplasmic signals mediated by FGF2, Activin/Nodal, IGF2 and Wnts (largely shown in human ESCs), or by the introduction of these genes/proteins by transduction/transfection methods.

Although DNA methylation has been studied extensively, hypomethylation patterns cannot be designated as sole master switches for controlling gene expression and the maintenance of pluripotency in mESC. However, an increase in DNA methylation has been noted in selected CpG islands, in a few hESC lines during long-term passages (Allegrucci C et al., 2007). Comparison of promoter DNA methylation in mESCs with histone modifications, binding of transcription factors such as Oct4, Nanog, and polycomb group of proteins on gene promoters, has been performed to analyse if epigenetic regulators act independently or in concert with each other. Consequently, it was found that promoter DNA methylation is the only marker found on more than 30% of genes, many of which are silenced in mESCs (Meissner A et al., 2008). Genome-wide analysis of DNA methylation of promoters in mESCs and pMEFs (primary mouse embryonic fibroblasts) showed differences in methylation involving 69 gene promoters predicted to be hypomethylated in ESCs, and methylated in pMEFs (Farthing CR et al., 2008). These studies suggest that methylation patterns in ESCs may be distinctly different from differentiated somatic cells. Some of the interactions described in the above section are unique to ESCs and are possibly instrumental in maintaining the stem cell state. Of the cytoplasmic signals, the Activin/Nodal/FGF2 pathways are thought to maintain hESCs, while LIF/STAT3 is necessary for mESCs. Epigenetically the repression of gene promoters related to differentiation in spite of the chromosomes being largely euchromatic seems to be a characteristic. This along with expression of relatively high levels of proteins related to pluripotence including Oct4, c-Myc and Nanog are hallmarks of the undifferentiated state of ESCs.

III. Lessons from somatic stem cells

Unlike embryonic stem cells, somatic stem cells are restricted in their potential and reside in tissues of developing or mature organs. Among the best studied of the SSCs are hematopoietic stem cells (HSCs), neural stem cells (NSCs), and mesenchymal stem cells (MSCs). While HSCs reside in bone marrow NSCs as the name suggests, reside largely in the nervous system. Stem cells may also be identified in other tissues including epidermis, intestine, breast, and retinal tissue, and potentially in muscle and pancreas.

As discussed in the previous section ESCs retain the potential to differentiate into most, if not all, the tissues which constitute the adult organism, hence pluripotent. SSCs on the other hand are multipotent and have restricted differentiation potential. They usually retain the capacity to differentiate into some, or all, of the cell types, which constitute the tissue that they reside within. Typically they reside in 'niches' within the tissue which provide the necessary environment to maintain a regulated number of stem cells in a state of quiescence, and which can be effectively mobilized when appropriately stimulated. Unraveling the factors and mechanisms which maintain niches will allow us to configure appropriate culture conditions and further the practical use of these cells. When one considers the various biological events, which might contribute to the multipotentiality of SSCs, they might include maintenance of quiescence, which could promote the maintenance of a stable stem-like phenotype in a mature tissue, cell proliferation, cell survival, and inhibition of commitment and differentiation.

The niche that SSCs reside in is largely comprised of some relevant cell types native to the region and their intrinsic and secreted principles, the blood vessels which carry soluble factors, and the extra cellular matrix which possibly contains immobilized cues (Alvarez-Buylla A and Lim DA, 2004, Kiel MJ and Morrison SJ, 2008). While there is a possibility that there are two niches for HSCs, one osteoblastic and the other vascular, it is also possible that these two niches actually serve to maintain distinct functions of the HSCs during its life as a stem cell. The osteoblastic niche may serve to maintain the cell at its peak of quiescence, while the vascular niches might play a greater role during proliferation and mobilization of these cells as and when required. The Tie-2 receptor along with its ligand angiopoietin is thought to be one of the major regulators of quiescence of HSCs (Arai F and Suda T, 2007, Fukuhara S et al., 2008). This receptor has intrinsic tyrosine kinase activity and functions through the PI3kinase pathway to maintain p21 levels thus maintaining quiescence. Osteopontin also serves to inhibit differentiation in the niche (Nilsson SK et al., 2005, Stier S et al., 2005). The mammalian target of rapamycin (mTOR) pathway could mediate HSC quiescence and maintenance by regulating reactive oxygen species (Chen C et al., 2008). Wnt and Jagged are expressed on osteoblasts and react with their respective receptors Frizzled and Notch on HSCs and are involved in controlling the maintenance of HSCs (Fleming HE et al., 2008, Li L et al., 1998). Interestingly Notch 1 is down-regulated in HSCs lacking Smad 4, which could be a possible mechanism by which smad4 is one of the factors involved in the maintenance and self-renewal of HSCs (Karlsson G et al., 2007). The calcium calmodulin dependent kinase (CaM Kinase) present in HSCs appears to participate in their maintenance by regulating bcl-2 levels through phosphorylation of CREB and CBP (Kitsos CM et al., 2005). Other molecules which regulate the niche include sonic hedgehog (Shh), CXCR4/CXCL12, and SCF/c-Kit (Kiel MJ and Morrison SJ, 2008).

While the NSC niche is in the process of being defined (Riquelme PA et al., 2008), the molecules that seem to be required for maintaining a pool of stem cells include Shh, Notch, Wnt, and FGF. GFAP positive neural stem cells were reduced in the sub-ventricular zone of Gli deficient animals, suggesting that Shh regulated the number of stem cells *in vivo*. In addition, the number of dividing cells as determined by BrdU staining, was also decreased (Palma V and Ruiz i Altaba A, 2004). Basic FGF (bFGF) is sufficient as a mitogen in monolayer cultures of NSCs, and is used in combination with EGF for neurosphere cultures (Johe KK et al., 1996, Reynolds BA and Weiss S, 1996). Both signal through the Ras/MAPK pathway, although EGF activates several other pathways in these cells including PI3kinase. In addition to these growth factor mediated effects, the polycomb protein Bmi1 is thought to function in the regulation of both the

HSC and NSC population in the adult (Molofsky AV et al., 2003, Park IK et al., 2003). Recently Wnts have been shown to be relevant to the self renewal of stem cells in the brain (Kalani MY et al., 2008). Notch could mediate possible density-related effects in NSC cultures (Kamakura S et al., 2004), while both Notch and BMPs cause differentiation effects, the latter of which are rather complex and yield three fates depending on the culture conditions: neurons, glia and smooth muscle (Rajan P et al., 2003). Manipulation of differentiation signals could also promote the maintenance of multipotency. Signals which activate neurogenin appear to cause neuronal fate choice, while we and others have shown that the activation of STAT3 is required for glial differentiation (Bonni A et al., 1997, Rajan P and McKay RD, 1998, Sun Y et al., 2001). Activation of STAT3, and glial differentiation, could be brought about by JAK downstream of CNTF/LIF, mTOR downstream of BMP, and possibly MAPK downstream of CNTF and EGF. Other studies have shown that p300 works cooperatively with STAT3 and Smad to cause glial differentiation under BMP treatment (Nakashima K et al., 1999). While several of the signaling studies have been performed on rodent systems for ease of work, some of these have been verified in human cells, particularly when there is relevance to cancer. Some of these pathways and their interactions have been recently reviewed (Rajan P and Snyder E, 2009).

MSCs were originally isolated and cultured from bone marrow, and are fibroblastic cells which have the potential for differentiation into adipocytic, chondrocytic and osteoblastic lineages, and also into muscle and tendons (Pittenger MF et al., 1999). MSC-like cells have subsequently been cultured from adipose tissue, umbilical cord, Wharton's jelly and placenta. They are arguably the stem cells which are the easiest to culture and manipulate, and several cell surface markers are used in the characterization of these cells (Majumdar MK et al., 1998). Stro1 is sometimes used as a prospective marker for these cells, although it is not unique to them, and recently other markers have been suggested for MSCs (Roche S et al., 2009, Simmons PJ et al., 1994). Although serum is the most commonly used culture additive, bFGF, LIF, HGF, Wnt, EGF and PDGF contribute to the maintenance of MSCs (Kolf CM et al., 2007).

Alternative approaches are yielding some windows into the complex methods, which are operational in SSCs and stem cells in general. Systems biology has proved to be an excellent tool for generating hypotheses relating to molecules which may be associated with, or characteristic of, a particular fate or event (Muller FJ et al., 2008, Ulloa-Montoya F et al., 2007). Some such studies have implicated molecules such as Gata2 as being instrumental in de-differentiation of mature somatic cells into a SSC like state (Huang TS et al., 2008). Other molecules which are associated with the somatic stem cell state include Angiopoietin1, Kit, Sox9, Timp3, and several genes which are also present in ESCs (Forsberg EC et al., 2005, Huang TS et al., 2008). Creative methods to activate specific receptor and downstream kinases have yielded interesting insights. Dimerisation of the thrombopoietin receptor, mpl1, by an artificial crosslinking method causes HSCs to proliferate for appreciable longer periods than is achieved in culture with known means, and certainly more than is obtained with receptor dimerisation with the natural ligand (Abdel-Azim H et al., 2008). The factors and active principles which maintain other stem cells including epidermal, intestinal and breast are in the process of being defined (Blanpain C and Fuchs E, 2009, Sato T et al., 2009, Scoville DH et al., 2008). Recently transcription factors such as Achaete-Scute have been implicated in intestinal stem cell maintenance (van der Flier LG et al., 2009).

While Oct 4, Nanog, Sox2, and other genes are largely associated with the pluripotent state of ESCs, they have also been seen to be expressed in NSCs, MSCs and HSCs to varying degrees. One study showed that the lack of Oct4 does not compromise the maintenance of somatic stem cells (Lengner CJ et al., 2007, Lengner CJ et al., 2008), while others showed that the suppression of Oct4 is a step in the generation of a NSC like cell from ESCs (Akamatsu W et al., 2009). Sox2 is a marker for NSCs and is thought to regulate the proliferation and maintenance of NSCs, and their differentiation into neurons (Episkopou V, 2005).

Thus, to recapitulate the above discussion of SSCs, the developmentally important molecules Shh, Notch, Wnt/b catenin, and the BMP family of receptors/ligands are important for maintaining self-renewal and the multipotency phenotype. In the case of NSCs and MSCs extensive culture work has revealed several soluble growth factors, which may be used to manipulate the stem-like, and differentiation end points. Due to limitations in the culture methods of HSCs the bulk of the experimental work is performed in *in vivo* models.

IV. Conclusions and projections

A circumspect study of the signals which mediate pluripotency and multipotency in ESCs and HSCs respectively could permit the design of reagents and protocols which will result in the stable differentiation of SSCs and mature cells from ESCs, and perhaps all other desired combinations. **Figure 1** and **Figure 2** summarize some of our thoughts on the salient cellular signals and events, which maintain the pluripotent state of mouse and human ESCs. It has recently been possible to de-differentiate cells which are thought to be of mature origin to cells which are reminiscent of ESCs by the induced expression of the transcription factors Oct4, Myc and Klf4 in combination with other transcription factors. It is possible that other procedures could use signaling intermediates and activated cytoplasmic and nuclear proteins.

The relatively recent progress in the creation of induced pluripotent stem cells (iPSCs) (Takahashi K et al., 2007, Takahashi K and Yamanaka S, 2006), and to a lesser extent nuclear reprogramming by cell fusion (Cowan CA et al., 2005), has challenged several of our notions regarding pluripotent, differentiating and mature differentiated states. It appears from the cell fusion studies that principles present in the cytoplasm of the ESC can reprogram the mature differentiated nucleus into an

undifferentiated 'pluripotent' one (Cowan CA et al., 2005). The surprising observation that the simple overexpression of about four genes, which are usually expressed in ESCs, has the capacity to reprogram a 'mature' cell has elicited great interest. The genes usually used for the induction of pluripotency are Oct4, Klf4, Sox2 and Myc, in the absence or presence of other genes such as Large-T and Nanog (Park IH et al., 2008, Yu J et al., 2007). The frequency with which iPS occurs is low, and it must be mentioned that the exact nature of the 'mature' cell which is being de-differentiated is not entirely clear. iPS seems to be much more efficient when performed in cells which are more stem-like than mature (Aasen T et al., 2008). Several refinements are now being rapidly reported where iPSCs are being generated with varying efficiencies using small molecules (Shi Y et al., 2008), micro RNAs (Judson RL et al., 2009), protein transductions (Zhou H et al., 2009), plasmid transfections (Okita K et al., 2008), adenoviral vectors (Stadtfeld M et al., 2008), transposons (Woltjen K et al., 2009) and other cell signaling manipulations (Feng B et al., 2009) instead of the original retro/lentiviral transductions. HDAC inhibitors such as valproate have been shown to greatly increase the efficiency of iPSC generation even with just 2 of the original reprogramming genes (Huangfu D et al., 2008), suggesting the involvement of epigenetic modifications in the regulation and/or maintenance of pluripotence. Lluis et al have shown that cyclic activation of Wnt/β catenin signaling can enhance the reprogramming of somatic cells upon fusion with ESCs, whereby the differentiated cells undergo the process of dedifferentiation (Lluis F et al., 2008). These supposedly 'safer' methods of generation could be an advance to potentially using these cells in autologous and allogeneic cellular transplantation therapies, as they are not induced to pluripotence with the aid of lentiviruses. While the promise of these cells in regenerative medicine still remains to be realized, their power in the establishment of *in vitro* models of disease and in screening models is obvious.

While the initial stimulus for iPSC formation is the forced expression of Oct4, Myc, Klf4, and other selected genes, the eventual transformation of the cell to its stem-like state is due to reprogramming of the nuclear architecture such that the cell starts to express endogenous Oct4, Myc, Nanog, etc. The exact mechanism by which Oct4, Myc, etc. brings about these epigenetic modifications to the cell, such that it is reprogrammed, is under study (**Figure 2**). While it has been recently possible to de-differentiate cells which are thought to be of mature origin to cells which are reminiscent of ESCs, the conversion of mature cells to SSCs remains to be achieved (**Figure 3**). One may also use the knowledge reviewed here to stably cause the differentiation of an ESC to an HSC or NSC (Bajpai R et al., 2009, Matsumoto K et al., 2009). This has been achieved by the selective addition of various growth factors and manipulation of tissue culture conditions. It may also be achieved by other more direct means including the manipulation of levels and interactions of specific transcription factors (as implied in the Gata2 study (Huang TS et al., 2008), or by directing the epigenetic modification of specific promoters to achieve the MSC, NSC or a pre-insulin producing β-cell state. Such manipulations would be of immense use in regenerative medicine, perhaps in the generation of specific cell types for regenerative medicine and for the generation of *in vitro* experimental models.

The recent literature would appear to suggest that differentiation of cells towards the mature end phenotype is not a one way phenomenon, and that the appropriate stimuli will cause ESCs, SSCs and mature differentiated cells to flip between states (**Figure 3**). As a case in point, iPSCs derived from skin fibroblasts have been differentiated into cells of the central nervous system (Dimos JT et al., 2008). While all these experiments have been performed in vitro, the possibilities of these types of dynamic shifts in a general sense, and in vivo, remain to be detected. The idea represented in **Figure 3** begs the question of whether the fully differentiated status of mature cellular phenotypes is actively maintained in the adult organism, as are stem cells.

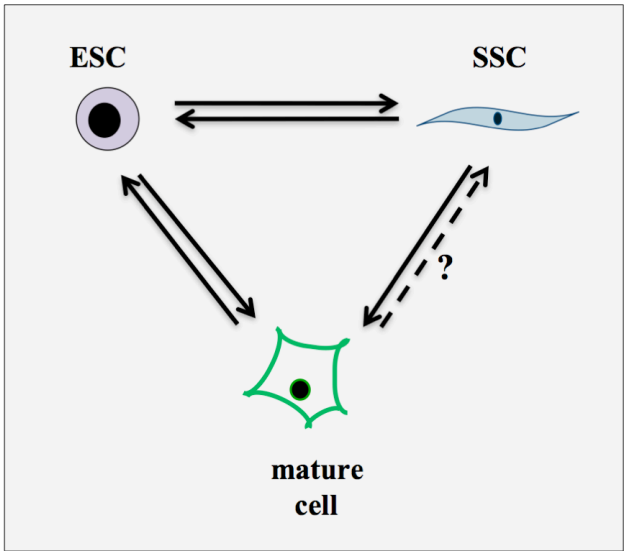

Figure 3: Are all cells in a dynamic equilibrium with respect to their 'fate choice' decisions?
The current status of ESC generation and differentiation suggests that ESCs, SSCs and mature differentiated cells may be inter-convertible given the correct stimuli. Somatic stem cells such as neural stem cells and keratinocyte precursors have been used successfully for iPSC generation. Mouse embryo fibroblast cultures, which are used for iPSC generation, are a heterogeneous population of cells, which may well contain MSC like cells. Adult fibroblasts and blood have also been used with varying levels of success for iPSC. By all recognizable criteria, iPSCs closely resemble ESCs. ESCs have been differentiated into NSC and HSCs. Of the other options possible in the continuum shown in the figure, ESCs and SSCs have been differentiated into the several types of mature cells. The successful de-differentiation of mature cells into their respective SSCs could be possible if the correct cohort of cytoplasmic, nuclear, and epigenetic signals were expressed in sufficient quantities, and with correct timing.

Acknowledgements

We gratefully acknowledge the financial support of the Department of Biotechnology of the Ministry of Science and Technology, India. We thank Dr. R.V. Shaji for commenting on the manuscript.

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