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Protein Kinases and Associated Pathways in Pluripotent State and Lineage Differentiation

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Abstract

Protein kinases (PKs) mediate the reversible conversion of substrate proteins to phosphorylated forms, a key process in controlling intracellular signaling transduction cascades. Pluripotency is, among others, characterized by specifically expressed PKs forming a highly interconnected regulatory network that culminates in a finely-balanced molecular switch. Current high-throughput phosphoproteomic approaches have shed light on the specific regulatory PKs and their function in controlling pluripotent states. Pluripotent cell-derived endothelial and hematopoietic developments represent an example of the importance of pluripotency in cancer therapeutics and organ regeneration. This review attempts to provide the hitherto known kinome profile and the individual characterization of PK-related pathways that regulate pluripotency. Elucidating the underlying intrinsic and extrinsic signals may improve our understanding of the different pluripotent states, the maintenance or induction of pluripotency, and the ability to tailor lineage differentiation, with a particular focus on endothelial cell differentiation for anti-cancer treatment, cell-based tissue engineering, and regenerative medicine strategies.

Keywords

Endothelial cells; phosphorylation; pluripotency; protein kinases; signaling pathways; stem cell

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

1. GENERAL INTRODUCTION TO PROTEIN KINASES AND PLURIPOTENCY

Protein kinases (PKs) play a central role in cell fate specification during embryonic development, identity maintenance and homeostasis of adult tissues by regulating key processes such as cell cycle, gene transcription, and metabolic switching [1-3]. In response to extrinsic and intrinsic signals, PKs promote the phosphorylation of substrate proteins by transferring phosphate groups from high-energy donors, usually adenosine triphosphate (ATP) or guanosine triphosphate (GTP), thus modulating the activity and availability of these substrate proteins [2]. The human kinome encodes overall 538 putative PKs with more than 500,000 potential phosphorylation sites and 25,000 phosphorylation events [2, 4]. However, a much smaller number of PKs might be sufficient for the specification of cellular identity [1], and assuming that an individual cellular phenotype is linked to a specific kinome profile, it is very likely that various permutations of cell type-restricted PKs contribute to the generation of cellular diversity in the human body.

In recent years, much attention has been directed towards PKs and signaling transduction networks [5, 6] as part of the molecular signature of cellular pluripotency or “stemness” [7-9]. Pluripotency typically represents the undifferentiated cellular state derived from the inner cell mass of blastocyst-stage embryos [10-12]. Pluripotent cells (PCs) are characterized by self-renewal capacity [13-15], clonogenicity [16] and in most cases, totipotency or multipotency [17]. Their hallmark features also encompass positivity for alkaline phosphatase (ALP) and specific surface markers, ability to differentiate into all three germ layers, discrete transcriptional and epigenetic signatures and teratoma formation in immunodeficient mice [18]. Since the isolation of embryonal carcinoma cells (ECCs), the first PCs to be characterized [11, 12], numerous PC types including embryonic stem cells (ESCs) [19, 20], embryonic germ cells (EGCs) as derived from primordial germ cells (PGCs) [21, 22] and epiblast stem cells (epiSCs) [23, 24] have been identified. There are also induced pluripotent stem cells (iPSCs) that are generated from human or mouse somatic cells through the process of reprogramming, with the introduction of specific transcription factors, such as Octamer Binding Protein 3/4 (Oct3/4), Sex Determining Region-Y Box-2 (Sox2), Kruppel-like factor 4 (Klf4), c-Myc, homeo domain protein Nanog, and Lin28 [25-31]. Recent studies also highlight the role of small molecule compounds [32-37] and PK-related cytoskeletal remodeling in somatic cell reprogramming [38]. Despite the revealed differences in terms of morphology, expression profile and developmental potential, amenability to homologous recombination and culture requirements, all these stem cell (SC) types share in common the fact that they do not undergo crisis or senescence, are not subjected to contact inhibition or anchorage dependence and that they all retain a diploid karyotype [7, 9].

The PC environment, or SC niche, regulates cellular behavior by providing appropriate signals that act directly on self-renewal, maintenance and differentiation [39, 40]. Pluripotent state is analogous to a cellular macrostate, compatible with a wide variety of interchangeable molecular microstates, and is defined by patterns of gene/protein expression [41, 42]. Transcription regulators and kinases are consisted of many phosphorylated members and the activity of many of these proteins is involved in the self-renewal network and is tightly regulated by post-translational modifications (PTMs) [43-47]. To this view,

intracellular fluctuations in the expression and phosphorylation status of PKs and their upstream molecular effectors [e.g. leukemia inhibitory factor (LIF), bone morphogenetic protein 4 (BMP4), transforming growth factor-ß (TGF-ß), Activin A, insulin growth factor (IGF), fibroblast growth factor (FGF) and others], the so-called "extrinsic/external/exogenous regulators of pluripotency" [48, 49], in concert with genetic/epigenetic regulatory networks and miRNAs, the so-called "intrinsic/internal/endogenous regulators of pluripotency" [42, 50, 51], account for the "dynamic equilibrium" in which individual cells transit stochastically between distinct metastable states [52], while the overall molecular profile of the population remains unique and stable [53]. That being said, functional pluripotency emerges spontaneously from the dynamic variability intrinsic to the underlying molecular state, with PKs representing central contributors [1, 54-56]. Subsequently, active signal transduction cascades impact on the cell's ability to engage transcriptional programs that lead to phenotypic change [57]. Apparently, such fluctuations temporarily sensitize individual PCs to differentiation-inducing signals, transiently priming them for differentiation without, however, marking definitive commitment [58]. Lineage commitment comes with "permanent" stimuli and it is accompanied by a radical reorganization of the cellular proteome that extends far beyond signaling alone [59, 60].

The duality of the involved signaling networks and their auto- and cross-regulatory interactions allow for the transition from the naïve or ground state to the primed state of pluripotency through somatic cell reprogramming, and vice versa. The naïve or ground state has been described in mouse (mESCs) and human (hESCs) embryonic stem cells and is characterized by single cell clonal ability, rounded colony morphology, reduction in DNA methylation and H3K27me3 repressive chromatin mark deposition, and comparative refractoriness to differentiation towards PGCs [61-64]. The primed state, on the other hand, has been described in mouse epiblast stem cells (EpiSCs) and human induced pluripotent stem cells (hiPSCs), characterized by flattened colony morphology and insufficient clonal expansion, increase in DNA methylation, prominent deposition of H3K27me3 and poise for differentiation to PGCs [65, 66]. While LIF/STAT3 and BMP4 are considered to be salient naïve-related signaling pathways, they are dispensable in the maintenance of primed pluripotency or, more specifically, in triggering the differentiation of primed SCs into different lineages [7, 67]. Similarly, autoinductive stimulation of the FGF/Erk1/2, TGF-ß/Activin A/Smad2/3, and glycogen synthase kinase-3 (Gsk3) signaling supports the pluripotency of primed SCs and in contrast, propel the lineage differentiation of naïve SCs [7, 68, 69]. Instead, phosphoinositol 3 kinase (PI3K) and Wnt signaling appear to be two of the few identified signaling pathways that are implicated in the maintenance of both naïve and primed pluripotency [7, 70].

Despite the technical limitations related to PSC investigation, we have now a far better understanding of cell signaling networks in this cellular group. A number of proteomic and phosphoproteomic analyses have provided insights into the functional protein content and the phosphorylation events implicated in the generation and/or maintenance of pluripotency [3, 43-47, 71-79]. Van Hoof *et al.* [59], while monitoring the dynamic phosphoproteomic changes of hESCs at the onset of BMP4-induced differentiation, detected 3090 unique phosphopeptides with 2431 serines, 582 threonines, and 54 tyrosines phosphorylation sites. According to the suggested *in vivo* kinome for hESCs, a prominent role was attributed to

cyclin-dependent kinase (CDK1/2) given that it mediates 26% of the phosphorylation events in hESCs, while mitogen-activated protein kinase (MAPK) −8, −11, −14, TGF-ß, Gsk3ß, and nuclear extract kit 2 (NEK2) have overrepresented activity in hESCs, with many of them being potential mediators of Sox2 phosphorylation. The recent comparative receptor tyrosine kinase expression and phosphorylation profiling for hESCs and hiESCs performed by Son *et al.* [80] revealed up-regulation of EPHA1, ERBB-2/EGFR-2, FGFR-4 and VEGFR-2 and down-regulation of AXL, EPHA4, PDGFRß and TYRO3 as related to the maintenance of hESCs. As of yet, the basic PKs signaling framework described in PSCs is defined by Insulin/IGF/FGF/LIF signaling through PI3K [81, 82], TGF-ß/Activin A/Nodal signaling through Smad [83, 84], FGF signaling through the Ras/MAPK/Raf/Mek/Erk pathway [82, 85], and the canonical Wnt/Gsk3 signaling pathway [86, 87]. The JAK/STAT [88] and Src pathways [89] have also a significant impact on this process. Although not extensively investigated, signaling cascades through Aurora kinases [90], Bcr-Abl [91] and Hedgehog pathway [92, 93] or pathways influenced by neurotrophins (NTs) and tyrosine kinase receptors (TKRs) [94], have been implicated in the maintenance of pluripotency, whereas others, including the stem cell factor (SCF) / Kit pathway [95, 96], Tie2 [97] and Flk1 [98], are mostly involved in the lineage commitment of pre-differentiated PSCs. Protein kinase C (PKC) seems to be a requirement for the differentiation of hESCs [99, 100]. Its inhibition maintains mouse, rat and human pluripotency by upregulating Nanog expression in the presence of the Oct-Sox composite element [101] without the need of STAT3 activation or Erk/Gsk3 signaling pathway inhibition [102, 103].

Owing to the explosion of interest in applying chemical approaches to stem cell biology and regenerative medicine [104-106], many compounds that regulate cell fate have been identified and characterized in recent years. Since the first reports that TGF-ß receptor inhibitors directly enhance reprogramming [34, 37], a large number of modulators or biological interventions seem to be able to modify these processes [32-37]. The coexistence of LIF with the dual molecular inhibition of Erk1/2 and Gsk3ß signaling (termed 2i/LIF conditions) support a totipotent state comparable to early embryonic cells [107]. A recent kinase inhibitor screen identifying small molecules that enhance, or prevent, reprogramming [90] further supports the hypothesis that kinases would likely play pivotal role in inducing pluripotency and determining cell fate during differentiation. This kinase inhibitor screening performed by Li and Rana [90] revealed that p38, inositol triphosphate 3-kinase and Aurora A kinase inhibitors, or equivalent knockdown of these target kinases, also enhance the induction of PSCs. Actually, iPSCs derived from these inhibitor-treated somatic cells are capable of reaching a fully reprogrammed state and subsequently, of differentiating into specific lineages *in vitro* and *in vivo* [90]. In addition, short hairpin RNA screen targeting 104 ESC-associated phosphoregulators identified Aurora A kinase as an essential kinase in PSC because depletion of this kinase severely affects self-renewal and differentiation [108]. Sakurai *et al.* [38], through a kinome-wide RNAi screen, uncovered the critical role of cytoskeletal remodeling in iPSC generation and identified two key serine/threonine kinases, testicular protein kinase 1 (TESK1) and LIM kinase 2 (LIMK2), which specifically phosphorylate the actin-binding protein COFILIN (COF) and modulate reorganization of the actin cytoskeleton during reprogramming. Their results showed that knockdown of TESK1 or LIMK2 in mouse embryonic fibroblasts promoted mesenchymal-to-epithelial (MET)

transition, decreased COF phosphorylation, and disrupted the actin cytoskeleton during reprogramming. Inversely, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) pathways are suggested to favor maintenance of undifferentiated hESCs because inhibition of their corresponding receptors results in hESC differentiation [43]. A pro-cardiogenic effect of PP2 was revealed due to inhibition of adhesion- and PDGFß-induced focal adhesion kinase (FAK) activation [109]. EGF(R) signaling also triggers a protein kinase C/Ca^{2+} influx/Erk1/2 cascade that leads to DNA synthesis in mESCs [110]. Being also responsible for helping the ESCs meet the high metabolic demands of the actively dividing cells [111], inhibition of EGFR signaling in ESCs induces selective apoptosis and suppresses growth rates of certain differentiated cell types [112]. Nevertheless, single growth factors have been shown to be necessary but not sufficient to sustain these processes indicating that the combined action of multiple growth factors is required to maintain undifferentiated hESCs [7]. c-Jun N-terminal kinases (JNK) activation, which is induced by LIF withdrawal, plays a negative role in reprogramming to PSCs by suppressing Klf4 activity [113], whereas the specific inhibition of AXL is significantly advantageous in maintaining undifferentiated hESCs and hi-ESCs and for the overall efficiency and kinetics of hiESC generation [80]. Even the oncogenic transformation of differentiated cells “resembles” cellular reprogramming with the *de novo* acquisition of unlimited self-renewal potential, a feature shared with PCs. At a molecular level, this parallelism is supported at by “facilitators” (eg. several reprogramming TFs represent *bona fide* oncogenes) and “barriers” (eg. genes that act as barriers to reprogramming correspond to known tumor suppressors) shared between these processes [114]. Similarly, PKs play essential role as modulators of reprogramming and at the same time, they have been considered hallmark of oncogenesis [114, 115].

2. PLURIPOTENCY AND ENDOTHELIAL CELL FUNCTIONING

Vascular formation is coordinated in a number of steps, which include differentiation and proliferation of endothelial cells (ECs) towards *de novo* blood vessels formation (vasculogenesis), blood vessel sprouting and branching from preexisting, remodeled and refined ones (angiogenesis), and differentiation and migration of vascular smooth muscle cells to cover vessel tube (arteriogenesis) [116]. The monolayered endothelium lines up the luminal surface of all blood and lymphatic vessels. A variety of angiogenic signals induce ECs to adopt an activated phenotype: detachment from their neighbor cells, growth modulation, reactivity of the underlying smooth muscle, control of the interaction of vessel wall with circulating blood elements, regulation of vascular responses to hemodynamic forces, sprouting towards gradients of proangiogenic factors, proliferation to form provisional tubes, perivascular cells recruitment and, finally, remodeling to form a functional network while becoming phenotypically specialized towards arterial, venous, lymphatic and hemogenic fates [117]. Perturbations of the molecular mechanisms that govern these events during development often result in embryonic lethality, whereas defects in these processes postnatally contribute to the development of prevalent vascular, lymphatic, and hematopoietic pathologies.

Although a ‘vascular stem cell’ population has not been identified, vascular endothelial and mural cells can be derived from currently known endothelial progenitor cells (EPCs) and

PSC sources, including hESCs and iPSCs, mesenchymal stem cells (MSCs), cancer stem cells (CSCs) and mouse germline-derived PSCs [118]. Isolated from human bone marrow, peripheral blood, adipose tissue, skeletal muscle, and various vascular beds, EPCs encompass a number of cell types that are generally thought to function as immediate precursors to vascular endothelial and/or mural cells, with a limited capacity to differentiate into other lineages [119-121]. They express among others CD31, CD34 and VEGFR-2, together with vWF and/or endothelial nitric oxide synthase (eNOS) and have significant roles in vascular homeostasis and diseases [119-121]. Although it is still debatable as of whether EPCs can drive vascular repair [122-125], they are thought to be critical for vascular homeostasis given that dysfunctional and/or low levels of circulating EPCs can contribute to various diseases [126-130]. Endothelial cells derived from hESC represent a cellular population isolated from embryoid bodies grown either in the presence of EC growth factors [131], or by co-culturing hESCs on monolayers of OP9 feeder cells (murine bone marrow stromal cells) [132]. They express surface markers consistent with primordial ECs (i.e. CD31, VE-cadherin and vWF) and form tubes when expanded *in vitro* under the effect of fibronectin or other extracellular matrices [133, 134]. Despite the lack of human clinical trials utilizing hESC-ECs, *in vivo* data indicates the formation of vascular networks and the improvement of cardiac functioning in animal models of ischemic heart disease [135]. Yamanaka's breakthrough discovery of human iPSCs provided a potential inexhaustible source of vascular cells, as it has been shown that hiPSCs can also be differentiated into ECs [136], a process that can be regulated by induced microRNA-21 and subsequently, TGF-β pathways [137]. In fact, iPSC-ECs were found to highly express EC markers and to be capable of "forming vascular networks and increasing blood perfusion of the hindlimbs of SCID mice" [137, 138]. Samuel *et al.* [139] reported a unique approach for the derivation of endothelial precursor cells from human iPSCs using a triple combination of selection markers—CD34, neuropilin 1(NRP1), and human kinase insert domain-containing receptor (KDR)—and an efficient 2D culture system. This approach allows for the generation of a large number of *bona fide* ECs endowed with *in vivo* potential of functional vessel formation. The successful generation of ECs from type I diabetic patient-derived human iPS cell lines and the *in vivo* blood vessel formation is an important milestone towards the clinical translation of this approach [140]. Mesenchymal stem cells, on the other hand, represent non-hematopoieticprecursor cells. They reside in the bone marrow (BM), contribute to the maintenance and regeneration of connective tissue and are recognized by the expression of adhesion molecules and stromal cell markers, such as CD73, CD105, and CD44 in the absence of hematopoietic markers, and the endothelial marker CD31 [141]. There is evidence indicating that "BM-derived mesenchymal cells could contribute to tumor angiogenesis by providing a supportive role as carcinoma-associated fibroblasts" [142]. Recently however, Pederson *et al.* [143] showed that a quiescent phenotype of ECs was generated by adding a low percentage of MSCs to the culture system. Biomarkers for vascular maturation and angiogenesis were both regulated by MSCs, which were also found to be a potent producer of VEGFA, an essential growth factor in vascular development. Both ECs and ECs/MSCs constructs demonstrated a higher vascular density when compared to empty control scaffolds, but the highest capillary density was generated in constructs comprising both ECs and MSCs [143]. Overall, once ECs are differentiated and coalesced into a vascular plexus, their proliferation must be tightly regulated to achieve proper

remodeling of an expanding circulatory network. This regulation involves the coordination of multiple signaling pathways that either promote or inhibit EC cycle progression. Five major pathways regulating EC proliferation and vascular growth have received enormous attention: VEGF/VEGFR, PDGF-β/PDGFR-ß, EGF/EGFR, angiopoietin and Tie2 receptor, and DII 4–Notch 1 pathways [144, 145].

With regard to the SC-derived ECs, there are considerable differences between hiPSC-ECs and hESC-ECs especially in terms of their proliferation capability [146]. In the study reported by Li *et al.* [146], a comparative analysis of gene expression between hiPSC-EC and hESC-EC revealed in hiPSC-ECs higher expression of proliferation inhibiting genes. Precisely, EC proliferation is compromised in hiPSC-ECs by a significant up regulation of PF4 as well as NOTCH4, DLL1, JAG2, DTX4, HEY2, HES7, NOTCH1, and APH1A, all genes related to Notch signaling pathway. Instead, SNCA, HGF, PFN2 and KLF2 genes, which play a positive modulatory role in the proliferative, migratory, and angiogenic capabilities of endothelial progenitor cells, were ranked as highly downregulated genes in hiPSC-ECs. Similarly, VEGFA and VEGFC, which also induce EC proliferation and migration, were also downregulated in hiPSC-ECs [146]. Despite this gene expression heterogeneity, ECs derived from both hESC and hiESC are very similar and resemble the primary derived ECs with lower however levels of NOS3 and vWF, which might be indicative of incomplete maturation. Another comparison of the transcriptomes [147] revealed limited gene expression variability between multiple lines of hiESC-derived ECs or between lines of hESC- and hiESC-derived ECs, and while there are some iPS-specific upregulated genes, most of the gene expression differences were mainly related to cell cycle and adhesion [147].

Of great investigational importance is also how cancer development manipulates the endothelial stemness to mediate tumor-associated neovasculature and consequently tumor growth, progression, invasiveness and metastasis. The net angiogenic activity depends on the balance between positive and negative modulators. In healthy tissues, the vasculature remains quiescent due to the dominance of negative regulators of angiogenesis. The tumor angiogenic endothelium though, undergoes the so called “angiogenic switch” through downregulation of the negative regulators as well as a shift towards positive regulators, which are mainly released by neoplastic and inflammatory cells [148, 149]. The ECs that constitute the vascular tumor bed show a dramatically increased proliferation rate compared to normal ECs, resulting in a structurally aberrant and functionally defective vasculature [144]. Compared to those isolated from normal adjacent tissue, EC isolated from human tumors show “enhanced angiogenic capabilities and increased survival, adhesion to tumor cells, motility, distinct markers and chemoresistance” [150]. The consequent vascular phenotype is usually associated with increased permeability that allows the traffic of tumor cells into the circulation. Neoangiogenesis, vascular co-option, mosaicism, vasculogenic mimicry, and postnatal vasculogenesis have been described as potential mechanisms contributing to the formation of tumor-related vasculature [151]. The combination of stimulatory signals within the tumor microenvironment prompt changes in multiple cell types, including perivascular cells, platelets, inflammatory and tumor cells. Endothelial cells actively recruit bone marrow–derived cells [endothelial progenitor cells (EPCs) and myeloid cells] in an angiocrine manner by producing and releasing multiple growth factors or Notch

ligands that attract circulating stem and progenitor cells [152, 153]. Endothelial progenitor cells-mediated vasculogenesis represents an important component, especially at the early stage of tumor growth - when EPCs are critical for promoting the “angiogenic switch”, and during metastasis, when EPCs promote the transition from micro- to macro-metastases [151]. To achieve this, tumor-associated ECs release angiopoietin-2, which recruits a population of Tie2-expressing monocytes into the tumor microenvironment; there, they associate with angiogenic blood vessels and prompt the ECs to release additional factors that escalate the angiogenic response [154]. Cancer stem cells can differentiate to become bona fide ECs, or tumor cells can physically participate in the formation of new vessels through vascular mimicry [144]. A paradigm to this cellular interaction is the communication between vascular ECs and glioma cells in order to promote the properties of glioma stem cells [155]. Specifically, ECs seem to promote the appearance of CSC-like glioma cells when co-cultured with glioma cells, as demonstrated by the increase in tumorigenicity and the expression of stemness genes, such as Sox2, Olig2, Bmi1 and CD133. In addition, ECs provide Shh in the tumor microenvironment to activate Hh signaling pathway in glioma cells, thereby promoting glioma CSC-like phenotype formation and glioma propagation [155].

3. SPECIFIC PROTEIN KINASES AND ASSOCIATED SIGNALING PATHWAYS IN PLURIPOTENCY AND ENDOTHELIAL CELL DEVELOPMENT

3a. PI3K/Akt Pathway

Translocation of PI3K to the cell membrane confers an increase in the phospholipid byproducts phosphatidylinositol (3,4)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which serve as ligands for the Pleckstrin Homology (PH) domains of several signal transducers, including the serine/threonine kinases, phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB)/Akt [156]. Coordinated localization of these lipid kinases modulates the activity of key regulators involved in metabolism, cell cycle, cell proliferation, and tumorogenesis [156, 157]. The main cascade uses PKB/Akt to relay intracellular signals generated by growth factors such as insulin, IGF, EGF and FGF-family members, heregulin, nerve growth factor (NGF) and neurotrophins (NTs) and the cytokine LIF in mESCs [158, 159]. Upon activation, PKB/Akt signals to other downstream effectors by stimulating the mammalian target of rapamycin (mTOR) and ribosomal protein S6 kinase (S6K) and by inhibiting Gsk3 with a consequent stabilization of c-Myc [160]. The effects of PI3K/Akt signaling are counteracted by the tumor suppressor PTEN, a lipid phosphatase that dephosphorylates PIP2 and PIP3 [161, 162].

It is well documented that PI3K-dependent signaling is one of the few signal transduction pathways that play pivotal role in maintaining the self-renewal property of both mESCs and hESCs [81, 82, 163, 164]. Previously, PI3K signaling was found to dramatically enhance the yield of pluripotent hybrid colonies after cell fusions between ESCs and somatic cells and its LIF-dependent activation was shown to regulate pluripotency in mESCs [165]. Further studies suggested that the active myristolated form of Akt maintained mESC self-renewal independently of LIF, BMP4 or Wnt/ß-catenin signaling [81], whereas inhibition of this pathway could skew the self-renewal balance resulting in differentiation, regardless of the

presence of Nanog [166] or LIF [82, 165]. In mESCs, post-translation modifications (PTMs) of Oct4 form a positive feedback loop, which promotes PKB/Akt activation and maintains the pluripotent gene expression signature [167]. In the absence of Oct4 phosphorylation, a negative feedback loop is formed that inactivates PKB/Akt and initiates the DNA damage response [167]. It has also been shown that PI3K signaling pathway maintains pluripotency by increasing Nanog and c-Myc expression [164, 165, 168], while PKB/Akt directly phosphorylates Sox2 at Thr118 and promotes its stabilization [169]. In hESCs, pluripotency can be also maintained by PI3K/Akt activation through the interaction of various NTs with the tyrosine kinase receptors (TKRs) B and C [94]. Besides, PTEN loss is correlated with PKB/Akt activation and subsequent increase in self-renewal potential and proliferation [170]. PTEN knockdown confers a higher percentage of hESCs expressing pluripotency markers and is consistent with a significant increase in the expression of pluripotency network genes including Oct4, Nanog, TDGF, DPPA5, DPPA2, UTF, and DNMT3B [161].

There are complex cross-talks between PI3K/Akt pathway and other signaling pathways. In hESCs, PI3K/Akt modulates the threshold level of Smad2/3 activity, suppresses Ras/MAPK/Raf/Mek/Erk activity, which subsequently reaches over to the canonical Wnt pathway by inhibiting Gsk3ß [7, 65, 171]. In turn, Gsk3ß acts as a central switch to the canonical Wnt/β-catenin, PI3K/Akt, and Ras/MAPK/Raf/Mek/Erk cascades, permitting the activation of a specific subset of target genes required for self-renewal [7, 65, 171]. A recent study supports the notion that PI3K signaling by itself is sufficient for the maintenance of pluripotency regardless of the canonical Wnt signal transduction [172]. In the absence of PI3K/Akt signaling, Smad2/3 signaling is enhanced and Erk is activated, and when coupled with an enabled Wnt signaling, they promote Gsk3ß and ß-catenin activation with opposite outcome [7, 65, 171]. Although PI3K/Akt activation results in Gsk3ß inhibition in hESCs, this pathway also preserves a separate supply of Gsk3ß in its active form for the suppression of Wnt/ß-catenin signaling [171]. This active pool of Gsk3ß could antagonize the differentiating ß-catenin stimulus in hESCs [171]. In mESCs, Gsk3ß is directly inactivated by LIF-activating PI3K/Akt phosphorylation. Gsk3ß phosphorylation regulates the shuttling of Gsk3ß between nucleus and cytoplasm through the formation of a complex with the Gsk3ß-interacting protein Frat, which carries it out of the nucleus, thereby impeding its access to the pluripotency factor c-Myc [7, 65, 171].

With regard to EC development, sustained activation of Akt leads to recapitulation of the complex structural and functional abnormalities of tumor blood vessels [173]. This may be partially attributed to the fact that PI3K/Akt/eNOS pathway plays a pivotal role (a) in the process of endothelial progenitor cells (EPCs) mobilization, migration and homing [174]; (b) in promoting, together with the MAPK/Erk pathway, the differentiation of mesenchymal stem cells (MSCs) towards ECs when miR-126 is overexpressed [175, 176]; (c) in the differentiation of cardiac SCs into vascular ECs in the presence of VEGF [177]. It is also reported that endothelial lineage differentiation from iPSCs is regulated by miR-21, which targets the PTEN/Akt pathway [137]. Precisely, miR-21 inhibits PTEN and induces tumor angiogenesis through Akt and Erk activation and HIF expression [137]. Functional loss of PTEN, which is associated with activation of Akt as well as VEGFA, exhibits enhanced angiogenesis. In contrast, PI3K inhibitors counteract this activation by suppressing angiogenesis in PTEN mutants [178].

Addition of PI3K-specific inhibitors or removal of PI3K-specific activators leads to loss of PSC colonies in low-density dissociation assays [179]. Inhibition of PI3K by the pharmacological inhibitor LY294002 in mESCs leads to mTOR suppression, enhanced basal LIF-stimulated phosphorylation of Erk1/2 and Gsk3α/ß, and downregulation of important pluripotency-related transcription factors such as Klf2, Klf4, Nanog, c-Myc, Esrrb, Tbx3, and Zfp42, enabling mESCs to exit from their pluripotent state and exhibit differentiated phenotypes [180]. Treatment of hESCs with LY294002 causes upregulation of endodermal and mesendodermal markers (eg. Brachyury, Eomes, Goosecoid, and MixL1) as they exit the pluripotent state [181, 182]. Similarly, mTOR inhibitors inhibit Akt through a feedback mechanism that triggers the differentiation of mESCs [183]. This is in line with the fact that mTOR has been found to stabilize the core pluripotency factors—Oct4, Nanog and Sox2, promoting self-renewal and suppressing lineage differentiation [184, 185]. Rapamycin, an mTOR inhibitor, is known to have an antiangiogenic effect on ECs in pathological settings [186, 187] and have shown efficacy in the treatment of complicated vascular malformations [188]. Dual PI3K/mTOR inhibitors have been found to have a substantial antivascular response, consistent with significantly reduced vascular density and increased mean vessel size or loss of small functional vessels [189]. Pharmacological inhibitors of TKRs or neutralizing antibodies to NTs also decrease the clonal survival of hESCs in a dose-dependent manner through suppression of the PI3K pathway [94].

3b. TGF-ß/Activin A/Nodal/Smad Canonical Pathway

Despite the initial assumption for a direct involvement of this pathway in differentiating cell fate decisions [190-192], an indispensable connection has been reported between TGF-ß/Smad signaling and the core pluripotent transcription machinery [158, 193, 194]. Apparently, this function duality is attributed to the fact that Smads activate different sets of target genes in pluripotent and differentiating cells, depending on its level of activation and the availability of co-factors [83]. Indeed, TGF-ß superfamily ligands, including TGF-ß isoforms, Activin, Nodal, growth and differentiation factors (GDFs), anti-Müllerian hormone (AMH), and BMPs, bind to and activate type I (1-7) and II (1-5) receptor serine/threonine kinases, which in turn, together with activin-receptor like kinases (Alk) (1-7), phosphorylate the regulatory Smad proteins (R-Smad 1, 2, 3, 5, 8) [195]. TGF-ß/Activin/Nodal signaling molecules can phosphorylate RSmad2/3 by activating Alks4/5/7 and BMP ligands can phosphorylate R-Smad1,5,8 downstream of Alk1/2/3/6 [195]. Following phosphorylation, R-Smads bind to co-Smad (Smad4) and translocate into the nucleus, wherein they act as transcription factors to regulate the expression of target genes [195]. In contrast, inhibitory Smads (I-Smad6/7) inhibit the activation of R-Smads and thereby negatively regulate TGF-ß signaling [195].

TGF-ß signaling induces epithelial–mesenchymal transition (EMT), an important hallmark of embryonic development through canonical Smad, non-canonical Ras/MAPK/Raf/Mek/Erk and Rho signaling [196, 197]. The reversed process, mesenchymal–epithelial transition (MET), is a crucial early event in reprogramming to pluripotency. It can thus be anticipated that small molecules that block TGF-ß signaling or its downstream effectors facilitate MET and enhance reprogramming. Consistent with this idea, inhibitors of TGF-ß receptors, indeed, promote and accelerate the speed of reprogramming and can

replace Sox2 in the reprogramming of MEFs [34, 37]. The self-renewal capacity of this pathway was confirmed in mESCs when TGF-ß suppression by SB431542, in conjunction with inhibition by Mek inhibitor PD0325901, allowed for the highly efficient, reproducible generation of ground state miESCs from different refractory and non-permissive strains [198, 199]. This likely occurs by augmenting the BMP4 that cooperates to sustain self-renewal and preserve multilineage differentiation in serum-free mESC cultures [67, 200]. The critical contribution of BMP4 is to induce the expression of Id genes *via* the Smad1, 5, 8 pathways [67]. Forced expression of Id genes liberates ESCs from BMP4 or serum-dependence and allows for self-renewal in LIF alone [67]. Activated Smad1/5 have been shown recently to upregulate Erk-specific dual specificity phosphatase 9 (DUSP9) and thereby inhibit Erk, establishing a link between BMP and MAPK/Erk signaling, which sustains pluripotency [84]. Under self-renewal conditions, PI3K signaling also restricts the absolute levels of Smad2/3 phospho-activation and maintains it within a range compatible with self-renewal [82]. With PI3K signaling reduction, Smad2/3 phosphorylation increases, allowing the activation of target genes involved in early differentiation [82].

However, hESCs and mouse EpiSCs represent a primed PSC state that requires TGF-ß/Activin signaling to sustain pluripotency, while the presence of SB431542 rapidly deprives hESCs from their pluripotent state [201]. It was found that Smad2, but not Smad3, is required to maintain the undifferentiated pluripotent state by binding to regulatory promoter sequences to activate Nanog expression while in parallel repressing autocrine BMP signaling [201]. The microarray analysis of Xiao *et al.* [202] revealed that Activin A is necessary and sufficient for the maintenance of hESCs self-renewal by inducing the expression of Oct4, Nanog, Nodal, Wnt3, basic FGF, and FGF8 and suppressing the BMP signal. Indeed, increased autocrine BMP signaling caused by Smad2 down-regulation leads to cell differentiation toward the trophectoderm, mesoderm, and germ cell lineages [201]. This conundrum in the role of BMP4 in the maintenance of naïve versus primed pluripotency [201, 202] could be explained by the limited range of activated Smad2/3 that it is compatible with the self-renewal capability of hESCs, and the cross-talk of this pathway with pluripotency-associated PI3K, Erk1/2 and Wnt signaling [84].

This TGF-ß signaling pathway contributes to the control of EC proliferation in two important ways. First, by inducing the production and deposition of fibronectin, an extracellular matrix protein that not only promotes visceral endoderm survival and function that is necessary for VEGFA production, but also binds to integrins α5ß1(inhibitory effect) and αVß3 (stimulating effect) expressed on ECs to elicit opposing effects on cell cycle progression [203, 204]. TGF-ß signaling also controls EC cycle progression *via* the regulation of key cell cycle control genes [205]. Deletion or mutation of different members of the TGFß family have been shown to cause vascular remodeling defect and absence of mural cell formation, leading to embryonic lethality or severe vascular disorders [206]. Again, miR-21 overexpression increases TGF-ß mRNA and secreted protein level, consistent with the strong up-regulation of TGF-ß during iPSC differentiation to ECs. Indeed, treatment of iPSCs with TGF-ß induces EC marker expression and *in vitro* tube formation, whereas inhibition of SMAD3, a downstream effector of TGF-ß, strongly decreased *VE-cadherin* expression. Furthermore, TGF-ß neutralization and knockdown inhibits miR-21-induced EC marker expression [137]. All in all, depending on the levels

present, TGF can have both proangiogenic and antiangiogenic properties. Low levels contribute to angiogenesis by upregulating angiogenic factors and proteases, whereas high doses inhibit endothelial cell growth and stimulate basement membrane reformation.

3c. Ras/MAPK/Raf/Mek/Erk Pathway

Under steady-state conditions, Ras/MAPK/Raf/Mek/Erk pathway activity is generally restrained but can be transiently activated by growth factors, such as FGF through FGFR as well as IGF through IGFR and LIF through gp130 [207]. Activated receptors phosphorylate cytoplasmic SH2 domain–containing tyrosine phosphatase 2 (SHP2), which interacts with a complex containing the growth-factor-receptor-bound protein 2 (Grb2) adaptor and Sos guanine-nucleotide-exchange factor [207]. This interaction results in localization of Sos at the membrane and subsequent activation of the monomeric GTPase Ras [56]. SHP2 may be also associated with the scaffold protein Grb2-associated-binder protein 1 (GAB1), which recruits the lipid kinase PI3K [208]. Ras activation then triggers a cascade of transphosphorylations involving Raf and MAPK kinases that culminates in activation of Erk1/2. The latter phosphorylates cytoplasmic targets that undergo nuclear translocation, enabling them to modulate the activity of transcriptional regulators such as Elk, Ets, Myc and the serum response factor (SRF) [209].

Previous studies have shown elevated levels of FGF/FGFR signaling pathway components (FGFR1–FGFR4, and FGF2, 11 and 13) in undifferentiated hESCs compared with their differentiated progeny, human tissue and mESCs [191, 210]. Exogenously added bFGF binds to the FGF receptors and maintains pluripotency in hESCs through the induction of IGF-II [158, 211], while inhibiting the spontaneous differentiation towards extra-embryonic lineages [212]. As a matter of fact, low levels of Erk1/2 signaling, as maintained by a wide range of FGF2 concentrations, are compatible with self-renewal [213, 214]. While low FGF2 levels (<10 ng/ml) keep low Erk1/2 levels through mild activation of Erk1/2, higher levels (>50 ng/ml) activate the PI3K pathway, which prevents further signaling augmentation along the Ras/MAPK/Raf/Mek/Erk pathway [85]. Moreover, eliminating the SHP2-binding site from a chimaeric gp130 receptor in ESCs also blocks the Erk1/2 pathway but enhances the self-renewal response [56], partly due to the elimination of a negative feedback effect on JAK activity [21]. Isolated attenuation of Erk signaling – either by pharmacological MEK inhibition or by forced expression of Erk phosphatases – also facilitates self-renewal and prevents differentiation [215]. Myc/MAX complexes also suppress Erk activity by regulating the transcription of two members of the dual-specificity phosphatase (DUSP) family, which bind to and inactivate Erk1/2 by dephosphorylating residues required for its catalytic activity [216]. Ectopic DUSP2/7 expression severely delays differentiation, while loss of DUSP2/7 ectopically activates Erk, resulting in loss of pluripotency [216]. FGFR signaling also exhibits a pro-differentiating effect in hESCs towards ectodermal and mesodermal cells [217] as well as neural lineages [218, 219]. It is likely that the initiation of differentiation is accompanied by transcriptional up-regulation of all four FGFRs with unchanged levels of FGF2 [191]. In mESCs, FGFR signaling also appears to be crucial for early epithelial differentiation, as mESCs with disrupted FGFR signaling fail to complete the differentiation process, partly attributed to PI3K pathway involvement [220, 221]. Prolonged activation of Erk1/2 signaling achieves the pro-differentiation effect [222] by suppressing Nanog

expression. Paling *et al.* [165] showed that inhibition of PI3K signaling in mESCs enhances the activation of Erk1/2, which plays a functional role in the loss of pluripotency [82, 223]. Similarly, FGF4 (a direct target of Oct3/4 and Sox2 [224]) and its associated activation of the Erk1/2 signaling cascade induce mESCs to exit the self-renewal program [68]. Erk1/2 binds to the C-terminal domain of Klf4 and phosphorylates it at Ser123 residue in mESCs [225]. This phosphorylation downregulates the transcriptional activity of Klf4 and induces differentiation of mESCs whereas inhibition of Erk signaling enhances Klf4 activity and maintains the undifferentiated state of mESCs [225]. Paradoxically, Erk2 itself contributes to the destabilization of ESCs self-renewal by reducing expression of pluripotency genes such as Nanog, but it is not specifically required for the early stages of germ layer specification [226].

Suppression of FGF/Erk signaling pathway promotes self-renewal of mESCs, whereas inhibition of FGF/Erk signaling results in degeneration of mESC culture. Treating mESCs with FGFR inhibitors such as SU5402 prevents differentiation towards cardiomyocyte [190], while FGFR1-null EBs have disturbed hematopoietic development compared with wild-type SCs [227]. In contrast, inhibition of FGFR1 by addition of SU5402 to hESC cultures suppresses activation of downstream protein kinases, down-regulates Oct3/4 expression, up-regulates p27 and causes rapid cell differentiation [158, 191]. “Dual FGF4 and TGF-ß signaling pathway inhibitors, designated R2i, not only provide the ground state pluripotency in production and maintenance of naïve ESCs from blastocysts of different mouse strains, but also maintain ESCs with higher genomic integrity following long-term cultivation compared with the pharmacological inhibition of the FGF4 and Gsk3 pathways, known as 2i” [199], a finding likely attributed to augmented BMP4 signaling pathway.

FGF/FGFR and BMP4 are two key signaling components that are not only important for specification of mesoderm but also for its differentiation toward endothelial and hematopoietic cell fates. The FGF ligands are among the earliest angiogenic factors reported and are involved in promoting the proliferation, migration, and differentiation of vascular endothelial cells [145]. Experiments using mESCs suggest that BMP4 resides at the top of this signaling hierarchy, promoting mesoderm formation and initiating a FGF2-dependent program to regulate the specification of angioblasts, which are thought to function as endothelial progenitors. In contrast, differentiation studies in hESCs, which require bFGF for survival and growth even in an undifferentiated state, do not support a role for this factor in the commitment of mesodermal cells to the endothelial lineage. Rather, in hESCs, BMP4 is a critical regulator that functions downstream of Indian hedgehog (IHh) to promote endothelial cell differentiation [117].

3d. JAK/STAT Pathway

The Janus tyrosine kinase (JAK)/ Signal transducer and activator of transcription (STAT) pathway starts from the cytoplasmic membrane-localized tyrosine kinases JAK1, JAK2, JAK3 and TYK2, by binding to the cytokine receptor *via* heterodimeric or homodimeric receptor complexes containing gp130 [228, 229]. LIF cytokine is a well-established triggering factor [230] that engages a complex consisting of two related cytokine receptors, LIF receptor (LIFR) and gp130 [231], and renders LIF/STAT signaling among the first

known signaling pathways involved in pluripotency [232]. Activated JAKs phosphorylate seven tyrosine residues on the intracellular portion of the gp130 receptor chain, which then act as docking sites for SH2-containing proteins [56, 229, 231]. In mESCs, the SH2-containing transcription factors STAT1 and STAT3 are recruited by phosphorylated LIFRs and also become phosphorylated by JAKs, which promote their dimerization and their translocation into the nucleus. There, STATs bind to STAT-binding elements in the promoter/enhancer regions of target genes, regulating self-renewal and maintaining mESCs in an undifferentiated state [56, 88, 229, 233]. Recruitment and activation of STAT3 in particular, targets the transcription factor c-Myc - which renders the cells undifferentiated and independent of LIF [232]; the transcription factor Klf4 - overexpression of which leads to increased Oct3/4 expression and mESCs self-renewal [234, 235]; the gastrulation brain homeobox 2 (Gbx2) – overexpression of which allows long-term expansion of undifferentiated mESCs in the absence of LIF/STAT3 signaling and enhances the reprogramming of mouse embryonic fibroblasts to iPSCs [236], as well as many genes that contain Oct3/4-binding sites [56]. Interestingly, c-Myc and Klf4 are two of the four factors (Oct3/4, Sox2, C-Myc, Klf4) that are implicated in the reprogramming of mouse and human somatic cells to become germline-competent iPS cells [27, 237]. Analysis of the mouse Nanog promoter region reveals a STAT3-binding site as well, and it has also been shown that STAT3 can bind this region *in vivo* [238, 239]. Studies using a chimeric STAT3 molecule that can be activated directly by estradiol indicate that STAT3 activation is not only necessary but might be sufficient to block differentiation even in the absence of LIF [232]. Phosphorylation of STAT3 on the Y705 site is absolutely required for STAT3-mediated mESCs self-renewal, but S727 phosphorylation, which is regulated directly by FGF/Erk signaling, is crucial in transitioning mESCs from pluripotency to neuronal commitment [240]. Loss of S727 phosphorylation results in significantly reduced neuronal differentiation potential, which could be recovered by a S727 phosphorylation mimic. Moreover, loss of pS727 sufficed LIF to reprogram EpiSCs to naïve pluripotency, suggesting a dynamic equilibrium of STAT3 pY705 and pS727 in the control of mES cell fate. All in all, the expression of an inhibitory STAT3 mutant or the protein inhibitor of activated STAT (PIAS)-induced inhibition of the transcriptional activity of STAT3 forces ESCs differentiation [241, 242]. Similar outcome is conferred by the inhibition or termination of LIF signaling, as achieved by SHP2-dephosphorylated phosphotyrosines, suppressors of cytokine signaling (SOCS) and/or gp130 modifications [241-243]. JAK2 and TYK2 are not involved in LIF-induced STAT3 activity and self-renewal of mESCs, but instead play a role in the early lineage decision of mESCs to various differentiated cell types [244]. A previous study indicated that the JAK2/STAT3 pathway is essential for the initial stages of cardiomyogenesis, as inhibition of JAK2 or STAT3 resulted in a complete loss of beating areas in EBs [245].

Although LIF sustains mESCs self-renewal through STAT3 [88] and BMP4 cooperation [67], whose activation is sufficient to prevent mESCs differentiation [233], a recent study has shown that by using the chemicals SU5402, PD184352, and CHIR99021 (called 3i), which suppress the FGF receptor (FGFR), MAPK kinase, and glycogen synthase kinase 3ß (Gsk3ß), respectively, STAT3-null mESCs could maintain self-renewal and a state of pluripotency that is indistinguishable from that of wild-type mESCs [56]. Neither is LIF/

STAT3 sufficient to maintain human pluripotency [230, 246]. A recent study of STAT3-null mESCs has revealed that transcription factor Tfcp2l1 is the most probable candidate factor among the STAT3 target genes that sustains pluripotency and connects the LIF signaling into the transcription factor core of naïve pluripotency [247, 248]. The forced expression of Tfcp2l1 could substitute LIF stimulation in mESCs and the transient expression of Tfcp2l1 was sufficient to reprogram EpiSCs into naive pluripotency without LIF stimulation [247, 248]. In addition, Zap70, a tyrosine kinase from the Syk family, has been found to negatively regulate the JAK/STAT3/c-MYC pathway and thus modulate the self-renewal capacity and differentiation ability of mESCs [249]. Zap70 interacts with SHP1 phosphatase and inhibits the phosphorylation of JAK, which in turn down-regulate the STAT3 dependent c-Myc induction [249]. The overall balance between activation and deactivation of STAT3 might determine the efficiency of the self-renewal of ESCs. As long as the balance is in favor of JAK1/STAT3 activation, self-renewal is sustained and differentiation is inhibited [56].

JAK2/STAT3/STAT5 signaling pathways also seem to mediate the regeneration of three-layer adult-like arterial wall when MSCs and ECs are co-cultured with BMP-2. This pathway was shown to account for the enhanced expression of HIF-1α and Id1 observed under these conditions [250]. Inversely, STAT3 inhibition through AZD1480 in tumor-associated myeloid cells leads to diminished myeloid cell-mediated angiogenesis, with additional direct inhibition of EC function *in vitro* and *in vivo*. In addition, AZD1480 reduced angiogenesis and metastasis in a human xenograft tumor model [251].

3e. Wnt/Gsk3/-catenin Signaling Pathway

The pleiotropic Wnt/Gsk3/β-catenin signaling pathway is one of the most potent pathways in maintaining PSCs and in promoting the differentiation of early mammalian cell lineages [252-255]. During embryogenesis, it regulates cell-to-cell interactions for early trophoblast lineage development, blastocyst activation, implantation, and chorioallantois fusion [256]. *In vivo* evidence also supports the role of Wnt around the time of gastrulation for germ layer formation [256]. Later on, Wnt signaling drives tissue-specific differentiation [252, 257]. This tightly regulated pathway in stem and progenitor cells is subverted in cancer cells to allow malignant progression [258]. Recently, a molecular link between Wnt/β-catenin signaling and cancer-associated telomerase activity has been reported [259].

The signal for this pathway is initiated by Wnt ligands, a family of glycoproteins with conserved cysteine residues, which are secreted from neighboring cells and modified by the retromer complex and complementary molecules (e.g. Porcupine and Wntless) in the secretory cells [260]. Wnt ligands bind to the Frizzled (Fzd) receptor (seven types) and LDL-receptor-related proteins 5 and 6 (LRP5/6) on target cells to activate downstream signaling [261]. In the canonical Wnt pathway, LRP receptors are phosphorylated by casein kinase 1γ (CK1γ) and Gsk3β [262], and Dishevelled (Dvl) molecules are recruited to the plasma membrane to interact with Fzd receptors and other Dvl molecules. Interaction of Axin with phosphorylated LRPs and the Dvl polymer inactivates the destruction complex, which confers cytosolic Gsk3-mediated protein ubiqutination [263]. The latter results in the cytoplasmic stabilization and nuclear translocation of β-catenin [264, 265]. In the nucleus, β-catenin forms a transcriptionally active complex with lymphoid enhancer factor (Lef)–T-

cell factor (Tcf) transcription factors, repressing genes that antagonize SC maintenance [266]. Apparently, the role of Dvl is not only the inhibition of Gsk3β in the cytoplasm but also the formation of a transcriptional complex with β-catenin and Tcf4 in the nucleus. It has been demonstrated that the disruption of the Dvl/β-catenin formation suppresses the canonical Wnt signaling pathway [267]. The non-canonical Wnt signaling essentially involves all Wnt-activated cell signaling pathways that do not specifically promote β-catenin stabilization. These include the planar-cell-polarity (PCP) pathway that guides cell movements during gastrulation [268] and the Wnt/Ca^{2+} pathway [269]. The classification into canonical and non-canonical Wnt signaling appears to depend primarily on the repertoire of Wnt receptors in a particular cell, as several Wnt proteins appear to have both canonical and noncanonical properties. Additionally, non-canonical Wnt signaling can apparently “antagonize” the canonical pathway [270].

The canonical Wnt signaling plays a rate-limiting role in regulating self-renewal and differentiation in mESCs [271]. PI3K/Akt activity is known to maintain self-renewal and restrains differentiation by suppressing the Raf/Mek/Erk and canonical Wnt signaling [65]. When PI3K/Akt signaling is low, Wnt effectors are activated and function in conjunction with Smad2/3 to promote differentiation [65]. In fact, differentiation triggers a burst of Wnt/β-catenin transcriptional activity that coincides with the disassembly of the complex and it is β-catenin that switches Smad2/3 from being a key element of the self-renewal machinery to an activator of genes required for early differentiation [84]. In addition, Wnt signaling targets the promoter of miR-302 that drives early embryonic development and somatic cell reprogramming [217]. This suppression occurs through β-catenin interaction with the Tcf/Lef binding sites of the promoter, preventing the miR-302 transcription and interference with the pluripotency factors Oct3/4, Sox2 and Nanog [272]. Moreover, the transcriptional and/or post-transcriptional down-regulation of Tcf3 represents a specific and primary response to Wnt activation in ESCs to regulate neuro-ectodermal lineage differentiation in mESCs [273]. Both the Wnt/β-catenin canonical and non-canonical pathways control differentiation to ectoderm, mesendoderm, endoderm and their derivatives [273], making Wnt signaling a very attractive target for small molecules in regenerative medicine.

Substantial evidence also points to Wnt signaling proteins as being involved in the self-renewal of mESCs [273] and the prevention of spontaneous differentiation of mESCs to epiPSCs [273]. The absence of canonical Wnt/β-catenin signaling is essential for maintenance of the undifferentiated state in mouse EpiSCs and in the epiblast of mouse embryos [273]. Attenuation of Wnt signaling with the small-molecule inhibitor XAV939 or deletion of the β-catenin gene blocked spontaneous differentiation of EpiSCs toward mesoderm and enhanced the expression of pluripotency factor genes, allowing propagation of EpiSCs as a homogenous population [273]. During self-renewal, there is negligible transcriptional activity of β-catenin due to its tight association with membranes as a β-catenin/Oct4/E-cadherin complex. Differentiation triggers a burst of Wnt/β-catenin transcriptional activity that coincides with the disassembly of the complex [273]. ten Berge *et al.* [273] found that Wnt proteins in combination with the cytokine LIF are sufficient to support ESC self-renewal in the absence of any undefined factors. Their results not only demonstrated that Wnt signals regulate the naive-to-primed pluripotency transition, but also identified Wnt as an essential and limiting ESC self-renewal factor.

Inhibition of Gsk3 has been implicated in the maintenance of mESC pluripotency *via* stimulation of Wnt signaling by β-catenin, stabilization of c-Myc protein and global de-inhibition of anabolic processes [273]. Gsk3 inhibition alleviates Tcf3 repression/regulation of the pluripotency network and increases ESCs resistance to differentiation [273]. β-catenin is not necessary for ESC identity or expansion, but its absence eliminates the self-renewal response to Gsk3 inhibition because β-catenin abrogates the repressive action of Tcf3 on core pluripotency genes. In rat ESCs, Gsk3 inhibition also leads to activation of differentiation-associated genes [274]. Lowered Gsk3 inhibition reduces differentiation and enhances clonogenicity and self-renewal [275]. The differential sensitivity of rat ESCs to Gsk3 inhibition is linked to elevated expression of the canonical Wnt pathway effector LEF1. These findings reveal that optimal Gsk3 inhibition for ESC propagation is influenced by the balance between TCF/LEF factors and can vary among species [276]. In another study using a β-catenin C-terminal truncation mutant or dominant-negative Tcf, β-catenin has been shown to act independently of Tcf/Lef to reinforce the pluripotent status of mESCs [277].

Activation of Wnt signaling either by addition of exogenous Wnt3a [278], pharmacological inhibitors of Gsk3β [271, 279], depletion of β-catenin [280, 281] and/or genetic ablation of Tcf3 [282], enhances mESC self-renewal. In contrast, inhibition of Wnt using soluble Frizzled (Fz8CRD) or the inhibitor IWP2, which interferes with Porcupine, inhibits the expansion of ESCs [283]. In hESCs, it was reported that activating the Wnt/β-catenin pathway with either Wnt3A or a Gsk3 inhibitor maintained the self-renewal of hESCs under feeder-free conditions [271]. Conversely, there have been reports where Wnt3a or Gsk3 inhibitors lead to differentiation of hESCs toward primitive streak and definitive endoderm lineages [284]. This is in agreement with the finding that Wnt/β-catenin signaling was inactive in the self-renewal of hESCs [280]. It has been speculated that different levels of endogenous Wnt signaling confer distinct lineage-specific differentiation properties to hESCs [285]. It has also been proposed that a spatially localized Wnt signal induces an oriented cell division that generates distinct cell fates at predictable positions relative to the Wnt source. Immobilization of Wnt proteins on beads and introduction of the consequent complexes to ESCs in culture produced an asymmetric distribution of Wnt signaling components, oriented the plane of mitotic division, and directed asymmetric inheritance of centrosomes generating a “Wnt-on” proximal daughter cell with high levels of nuclear β-catenin and pluripotency genes that maintain ES pluripotency and a “Wnt-off” distal cell that differentiates toward an EpiSC. Therefore, by orienting cell division, the Wnt signal positions the distal daughter cell out of its signaling range, leading to differentiation [286].

Wnt signaling has been implicated in the regulation and development of the vascular system, but the detailed mechanism of this process remains unclear. Wnt5a signaling in particular, is required for EC differentiation of ESCs and proper embryonic vascular development [287, 288]. Its downstream effectors β-catenin and PKC-a appear activated during mESC endothelial differentiation, while knockdown of either one of them results in inhibition of endothelial differentiation mediated by Wnt5a. Furthermore, consistent with the previous findings, the effects of endothelial differentiation in $Wnt5a^{-/-}$ mice could solely be reversed by transfection of both β-catenin and PKC-a [288]. Min *et al.* [289] reported that DKK1 and DKK2, originally known as Wnt antagonists, play opposite functions in regulating

angiogenesis; “DKK2 that was induced during EC morphogenesis promoted angiogenesis in cultured human ECs and in *in vivo* assays”. “Its structural homolog, DKK1, suppresses angiogenesis and is repressed upon induction of morphogenesis”. Importantly, local injection of DKK2 protein significantly improves tissue repair, with enhanced neovascularization in animal models of both hind limb ischemia and myocardial infarction.

3f. VEGF/VEGFR Pathway

The VEGF family is a key regulator of vasculogenesis and VEGFA is the most extensively studied among the five family members (VEGFA, VEGFB, VEGFC, VEGFD, VEGFE) [290]. The requirement for VEGFA is established early during vasculogenesis because heterozygous mutants are embryonic lethal due to the failed development of the vasculature [290]. VEGFA signals through its main tyrosine kinase receptors, VEGFR-1 (fms-related tyrosine kinase-1 [Flt-1]), VEGFR-2 (fetal liver kinase-1 [Flk-1] or kinase insert domain receptor) and VEGFR-3, and also interacts with the co-receptors neuropilin-1 and -2. Although Flk-1 has a lower affinity for VEGFA than Flt-1, it has stronger tyrosine kinase activity, and thus VEGFA responses in ECs and their precursors are usually attributed to Flk-1 activation. Binding of VEGFA to Flk-1 triggers its autophosphorylation, leading to complex formation with integrin αvβ3, an endothelial cell adhesion receptor, and αvβ3 autophosphorylation. Acting in a feedback loop, integrin αvβ3 is also capable of phosphorylating Flk-1, once it is bound by its ligand vitronectin. The cross-activation of these 2 receptors leads to the recruitment and activation of Src kinases, which mediate the mitogenic effects of VEGFA by activating the MAPK intracellular signaling pathway [291]. Mice lacking Flk-1 are embryonic lethal at E8.5 to 9.5 and lack blood island and vascular plexus development, despite normal formation of angioblasts. Consistent with this, *Flk-1*$^{-/-}$ mESCs can generate ECs; however, they fail to propagate *in vitro*. Similarly, VEGFA treatment of undifferentiated hESCs does not promote their differentiation toward an EC phenotype. Flt-1/VEGFR-1 also indirectly controls EC proliferation, by competing with Flk-1 for binding of VEGFA. *Flk-1*$^{-/-}$ mutants and zebrafish *flt-1* morphants exhibit EC hyperproliferation that leads to abnormal angiogenic development, suggesting that unlike Flk-1, Flt-1 plays a negative role in EC proliferation [291]. Di Bernardini *et al.* [292] have shown that VEGF induces functional differentiation of iPSCs toward EC lineage; precisely, stimulation of iPSCs with VEGF rapidly induced a marked change in the morphology of iPSCs, which lost their three-dimensional organization and displayed a flat adherent phenotype. Protein analysis and consequent gene expression analysis showed a consistent up-regulation of endothelial markers such as CD31, Flk1, and VE-cadherin, starting after 3 and 5 days of differentiation and peaking at day 7. Finally, the functionality of ECs derived from iPSCs was tested in an *in vitro* angiogenesis assay, showing a significant increase in tube-like structure formation ability upon cell differentiation, as compared with the cells grown in absence of VEGF. Efforts to target these pathways have resulted in the development of many VEGF/VEGFR inhibitors that are already utilized clinically.

3g. Angiopoietin (Ang) Pathway

Ang-1 and -2 signal through competitive binding to the endothelial membrane receptor Tie2 (tyrosine protein kinase receptor) to regulate distinct steps in vascular remodeling, vessel maturation and vascular inflammation [293]. Actually, Tie2 agonist, Ang-1, mainly induces

EC–EC and EC–mural cell adhesion mediating vascular maturation by stabilizing ECs in the quiescent G_0-phase, maintaining interactions between ECs, pericytes and the extracellular matrix, whereas the Tie2 antagonist, Ang-2, inhibits EC–mural cell adhesion destabilizing ECs and thereby, enabling the initiation of sprouting angiogenesis. Prolonged dissociation of mural cells from ECs mediated by Ang-2 also induces blood vessel regression [293-295]. Expression of endothelial specific Ang-1 and Ang-2 alters in the presence of MSCs, with an up- regulation of Ang-1 and down-regulation of Ang-2. MSCs induce EC quiescence and promote capillary formation. Tie2 mutations have been identified in an inherited form of venous malformation and 50% of sporadic venous malformation [296, 297]. Ang-2 released by tumor-associated ECs recruits a population of Tie2-expressing monocytes into the tumor microenvironment, where they associate with angiogenic blood vessels and prompt the ECs to release additional factors that escalate the angiogenic response [154]. A related study in a murine endothelioma tumor model has shown that preventing Ang-2 function either pharmacologically or *via* soluble Tie2 receptors inhibited growth of a tumor" [298].

3h. Notch Pathway

Evolutionarily conserved across diverse species, Notch-mediated signaling occurs over short distances and is commonly implicated in cell fate specification, self-renewal and differentiation [299]. Single-pass transmembrane Notch receptors are activated by engagement of ligands presented by neighboring cells through cell-to-cell interaction and are cleaved by metalloproteases that remove most of the receptor extracellular domain [300]. An enzymatic complex, known as γ-secretase, then cleaves the Notch transmembrane domain, releasing a Notch intracellular domain that is capable of translocating to the nucleus and forming complexes with other DNA-binding proteins [299, 301]. Notch activates numerous genes associated with differentiation and/or survival, including, the HES and HEY family of basic helix-loop-helix transcription factors, cyclin D1 and c-Myc [302-305]. Humans and mice express at least four Notch isoforms (*Notch1-4*). The outcome of Notch receptor/ligand signaling is highly dependent on the cellular context. In the vascular system, interaction of Notch receptors (especially Notch-1 and Notch-4) with their ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1and Jagged 2) directs the differentiation of ECs into vascular networks [306]. It initiates parallel death and survival pathways and exhibits a differential effect on endothelial survival depending on the apoptotic stimulus. Mice with targeted deletions of Notch-1 die due to severe defects in angiogenic vascular remodeling [307]. Although Notch-4 null mice develop a normal vasculature, Notch-1/Notch-4 double mutants reveal more severe effects than Notch-1 null mice. To this end, PI3K activity seems to regulate the expression of Slug, which is required for survival in Notch-activated ECs, while homocysteine blocks both PI3K activity and Slug expression in Notch-activated cells, leading to increased endothelial apoptosis [308]. Possibly, aberrant Notch expression provides tumor cells with endothelial-like properties including the formation of tubular networks [309]. The Notch-1 pathway was found to be active in human melanoma. *In vitro* studies demonstrated that Notch-1 signaling increased the metastatic capacity of primary melanoma cells, which was mediated by increasing β-catenin expression [310, 311].

Notch directly stimulates the *Slug* promoter, resulting in the up-regulation of Slug and initiation of EMT in ECs [312]. In vertebrate embryos, genetic manipulation of Notch

signaling has demonstrated the importance of this pathway in driving arterial versus venous EC differentiation. Notch activation also results in morphological, phenotypic, and functional changes consistent with mesenchymal transformation. These changes include down-regulation of endothelial markers (VE-cadherin, Tie1, Tie2, platelet–endothelial cell adhesion molecule-1, and endothelial NO synthase), up-regulation of mesenchymal markers (α-SMA, fibronectin, and platelet-derived growth factor receptors), and migration. In EMT processes, Notch cross-talks with several transcription and growth factors relevant to EMT, including Snail, Slug, TGF-β, FGF, and PDGF [313]. With the use of transgenic zebrafish bearing a Notch-responsive reporter, it has been demonstrated that Notch is activated in endothelial progenitors during vasculogenesis prior to blood vessel morphogenesis and is maintained in arterial ECs throughout larval stages. Interestingly, some arterial ECs subsequently downregulate Notch signaling and then contribute to veins during vascular remodeling. Together, these findings demonstrate that Notch acts in distinct contexts to initiate and maintain artery identity during embryogenesis [314].

In immortalized ECs, Notch-1 indirectly represses E-cadherin gene expression through E-boxes in its promoter, inducing an EMT phenotype [315]. Notch also upregulates Snail and stabilizes it under hypoxic conditions in cancer cells [316]. A new connection between Notch and EMT has been reported through miR-200 [317]. Overexpression of miR-200 family members results in reduced Notch activity and slightly reduced Jagged-1 activity, with demonstrated rescue after miR-200 inhibition. Reduction in Notch signaling results in reduced proliferation, increased apoptotic susceptibility, and reduced tumorsphere formation. *In vivo*, miR-200 family members were increased in xenograft tumors after ZEB1 knockdown, and this correlated with reduced invasiveness and metastatic capacity [318]. The Notch and VEGF pathways are also closely interconnected and their interaction is indispensable for tumor angiogenesis induced by hypoxia [319]. Expression of the Notch ligand, delta-like ligand 4 (Dll4) by tip cells of growing vascular sprouts is VEGF-dependent. Thus, VEGFA provides an angiogenic stimulus that guides sprouting behavior and subsequent initiation of EC proliferation. Dll4 activates Notch signaling in adjacent ECs, reducing their ability to respond to VEGFA stimulation by downregulating Flk-1 expression. Similarly, inhibition of Notch signaling results in increased sprouting, increased expression of tip cell–associated genes, hyperproliferative ECs, and a denser and more highly interconnected vasculature. Interestingly, when both VEGFA and Notch signaling are impaired, excessive sprouting and tip cell formation are not observed. Taken together, VEGFA provides proangiogenic stimulus and induces Flk-1 expressing cells to sprout and form new vessels, while simultaneously activating Dll4-Notch pathway signaling to maintain the necessary balance between tip and stalk cell specification.

Inhibition of Notch signaling in EC using an inducible binary transgenic system limits VEGFA-driven tumor growth and causes endothelial dysfunction. In addition, biochemical and functional analysis reveals that endothelial nitric oxide production is decreased by Notch inhibition, suggesting that lack of functional vessels observed with Notch inhibition is secondary to inhibition of nitric oxide signaling. Co-culture and tumor growth assays reveal that Notch-mediated nitric oxide production in ECs requires VEGFA signaling [320].

3i. EGF/EGFR Pathway

The epidermal growth factor receptor (EGFR) family consists of four different tyrosine kinases (EGFR, ErbB-2, ErbB-3, and ErbB-4) that are activated following binding to epidermal growth factor (EGF)-like growth factors. Upon ligand binding, EGFR dimerization stimulates its intracellular protein tyrosine kinase activity, leading to autophosphorylation of several tyrosine residues in the *C*-terminal domain of the EGFR. This elicits downstream activation and signaling by several other proteins, including MAPK, PI3K, phospholipase C-γ, and c-Src, thus engaging in cross-talk with multiple pathways and thereby, regulating cell proliferation, survival, differentiation, migration, inflammation, and matrix homeostasis [321]. In addition to the classical pathway of activation, EGFR can be also activated through G-protein-coupled receptors (GPCR) without direct interaction with GPCR agonists, an event referred to as transactivation [322].

Multiple cell types within the vascular wall, including ECs, have the potential to express EGFR family members and EGF-like ligands. Both EGFR transactivation by GPCRs and classical activation by EGF-like ligands participate in EC function and consequently, in both physiologic and pathologic angiogenesis [323]. It has been shown that microvascular ECs express EGFR and their stimulation with EGF or TGF-α, cultured on collagen type I gels induces tube formation, whereas their treatment with gefitinib dose-dependently inhibits EGF-induced migration and tube formation [324]. Human umbilical vein ECs, on the other hand, express ErbB-2, ErbB-3 and ErbB-4, but do not express EGFR, and their stimulation with recombinant neuregulin (ligand for ErbB-3 and ErbB-4) induces rapid calcium fluxes, receptor tyrosine phosphorylation and cell proliferation [325], while their stimulation with betacellulin results in phosphorylation of the ErbB2–4 receptors and proliferation, migration and tube formation in collagen gels [326]. Multiple mechanisms exist whereby EGFR activation leads to VEGF signaling, including activation of PI3K and MAPK pathways and several transcription factors that regulate VEGF transcription, whereas EGFR inhibition decreases VEGF expression and vascularization [327]. This pathway might play a role in vessel maturation as well: heparin-binding EGF, which is produced by ECs and represents an ErbB-1 and -4 ligand, stimulates EGFR phosphorylation and migration of MSCs [328]. Of note, SC-based neovascularization following hind limb ischemia can be achieved using SCs that either express high levels of endogenous EGF or have been stimulated with exogenous EGF and similar outcomes have also been observed in the myocardium as well as the retina [323].

The activity of EGFR has been found abnormally elevated in most human solid tumors and has been associated with progression and poor prognosis [329]. Within the tumor microenvironment, the EGF/EGFR system is an important mediator of autocrine and paracrine circuits that result in enhanced tumor growth; such a circuit, is the synthesis and secretion of numerous angiogenic growth factors, including VEGF, IL-8, and FGF. Having said that, overexpression of ErbB-2 leads to increased expression of angiogenic growth factors, whereas treatment with anti-ErbB-2 agents produces a significant reduction of the synthesis of these proteins by cancer cells [330]. Actually, targeting the EGFR activity in blood vessels is sufficient to inhibit tumor growth and is accompanied by an increase in VEGFR-2 dependence in tumor ECs [331]. Endothelial cell-secreted EGF induced EMT *in*

vitro as well as the acquisition of a stem-like phenotype by head and neck tumor cells. *In vivo*, tumor xenografts vascularized with EGF-silenced ECs exhibited a smaller fraction of cancer stem-like cells (ALDH(+)CD44(+)) and were less invasive than tumors vascularized with control ECs [332]. However, it has to be taken into account that ECs isolated from different tumor tissues may vary in their expression EGFR levels.

3j. Hedgehog (Hh) Pathway

The Hedgehog (Hh) signaling pathway plays an important role in embryonic development and adult tissue homeostasis and repair by acting either as morphogens in the dose-dependent induction of distinct cell fates, or as mitogens regulating cell proliferation, or as inducing factors controlling the form of developing organs [333, 334]. Hh pathway is also involved in the proliferation and cell-fate specification of neural and neural crest stem cells [335]. There are three mammalian Hh ligands (Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog) and two transmembrane receptors, Patched1 (Ptch1) and Smoothened (Smo) [336]. To carry out signaling function, Hh ligands have to undergo autoproteolytic cleavage to generate a peptide with an N-terminal palmitoyl and a C-terminal cholesterol group. In the absence of Hh ligands, Ptch1 inhibits the membrane association and thus, the activity of Smo. Upon binding of Hh to Ptch1, the inhibition of Ptch1 to Smo is relieved, leading to Smo association with the cellular membrane *via* β-arrestin and activation of target gene transcription by regulating the Gli family of transcription factors. While Gli1 acts only as transcriptional activator, Gli2 and Gli3 are able to act as both activators and repressors [337]. Known target genes for Hh pathways include the D-type cyclins, *c-Myc*, *BCL2* and *SNAIL*, which regulate cellular differentiation, proliferation and survival [336]. Several kinases have been implicated in the regulation of Hh pathway and from this standpoint in SC development. In the absence of Hh signaling, protein kinase A (PKA), Gsk3 and casein kinase 1 (CK1) phosphorylate the Gli family proteins resulting in the degradation of Gli1 and Gli2 or formation of a repressor *via* proteolysis of Gli3. However, in the presence of Hh ligands, Smo translocates to the membrane and these kinases get inhibited, Gli proteins are allowed to translocate to the nucleus and activate the transcription of Hh signaling target genes [333, 336].

As anticipated from above, Hh controls a number of genes involved in cell fate determination and stemness features (i.e. self-renewal and pluri/multipotency). Hh signaling has been recently described to enhance the expression of Nanog, in concert with the loss of the oncosuppressor p53 [92]. Nanog is highly expressed in SCs from postnatal cerebellum, and acts as a critical mediator of Hh- driven self-renewal. Indeed the downstream effectors Gli1 and Gli2 bind to Nanog specific *cis*-regulatory sequences and activate gene transcription both in mouse and human neural SCs. Loss of p53, a key event promoting cellular stemness, activates Hh signaling, thereby contributing to Nanog upregulation [92]. As a developmental morphogen, Hh regulates the survival and maintenance of tissue progenitor and SCs in brain, hair follicles and hematopoietic system [338, 339]. During cerebellum development, sonic hedgehog (SHh) secreted by Purkinje cells supports the proliferation of granule cell precursors in the external granular layer by promoting the expression of several well-known SC and proliferative genes, including genes encoding Myc, cyclin D1, IGF-2 and BMI1 [340]. It is also involved in the injury-dependent

regeneration of many organs including pancreas, prostate and bladder. A significant cross-talk between Hh and other signaling pathways also exists. During bladder regeneration in mammals, cells within the urothelium secrete SHh to induce the expression of Wnt proteins in the adjacent stromal cell layer, which in turn signals back to the urothelium [341]. During vertebrate limb development, a set of feedback loops involving SHh, BMP and the BMP antagonist gremlin 1 (GREM1) are responsible for limb outgrowth and patterning [342]. SHh also upregulates TGF-β2 in bones to inhibit hypertrophic chondrocyte differentiation [343]. Last, in the developing cerebellum, BMP-2 and BMP-4 antagonize the proliferative function of SHh *via* downregulation of Smo and Gli1 expression [344].

The SHh pathway is often recruited to stimulate the growth of CSCs and to orchestrate the reprogramming of cancer cells *via* EMT [345, 346]. As a matter of fact, SHh pathway is highly activated in pancreatic CSCs and plays important role in maintaining stemness by regulating the expression of stemness genes [347]. In an *in vitro* model, human pancreatic CSCs derived spheres were significantly inhibited on treatment with sulforaphane (SFN), an SHh and Gli transcriptional activity blocker, suggesting the clonogenic depletion of the CSCs. SFN also inhibited downstream targets of Gli transcription by suppressing the expression of pluripotency maintaining factors (Nanog and Oct-4) as well as PDGFRα and Cyclin D1 [347]. Accordingly, inhibition of Smo or Gli1 expression using pharmacological agents or stable silencing results in a significant decrease in melanoma SC self-renewal *in vitro* [348]. In the squamous cell carcinoma of the lungs, protein kinase CI (PKCI) was found to phosphorylate Sox2 and recruit it to the Hh acyltransferase (*HHAT*) promoter for Hh ligand production and maintenance of a stem-like phenotype [349]. Although study showed minimal role of SHh pathway in maintaining pluripotency and regulating the proliferation of undifferentiated hESCs, Gli-responsive luciferase assay and target genes Ptch1 and Gli1 expression during differentiation with retinoic acid revealed that the SHh signaling pathway is highly activated [93]. Besides, addition of exogenous SHh to differentiating hESC as embryoid bodies increases the expression of neuroectodermal markers Nestin, SOX1, MAP2, MSI1, and MSX1, suggesting that SHh signaling is important during hESC differentiation toward the neuroectodermal lineage [93]. This comes in line with a previous report [350] that implicates the Hh pathway in a core developmental signaling network repressive for pluripotency, as revealed by a ChIP-chip dataset that regulates Yamanaka factors in iPSCs.

Hh signaling is also important for the development of the vascular system [351]. Hh signaling regulates the expression of VEGF in mouse embryos for endothelial tube formation and Notch-dependent arterial identity. However, overactivation of the Hh pathway through deletion of Ptch1 results in reduced vascular density [352]. In a study for the establishment of arterial and venous fates of vascular endothelial progenitor cells (angioblasts), Kohli *et al.* identified that the levels of VEGF or Hh homologs determine the arterial differentiation of the medial angioblasts [353]. In another study of embryonic endothelial-to-hematopoietic transition, Indian Hh ligand was shown to increase hematopoietic progenitors, whereas chemical inhibition of Hh signaling reduced hematopoietic progenitors without affecting the primitive streak mesoderm formation [354]. Moreover, myocardial FGF signaling triggers a wave of Hh activation that is essential for vascular endothelial growth factors VEGFA, VEGFB, VEGFC, and Ang2 expression. Hh

signaling is sufficient to promote coronary growth and to rescue coronary defects due to loss of FGF signaling [355]. It promotes the cardiomyocyte formation in zebrafish in dose-dependent and cell autonomous manner, making it an attractive target for manipulation of multipotent progenitor cells for cardiac regenerative medicine [356]. ECs in the tumor microenvironment provide Shh to activate Hh signaling pathway in glioma cells, thereby promoting GSC-like phenotype formation and glioma propagation [155].

3k. Src and SYK Family Pathway

Src family comprises nine non-receptor tyrosine kinases (SFKs) (c-Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk, and Yrk) implicated in diverse cellular processes, such as proliferation, survival, motility, adhesion, invasion and angiogenesis. Upon activation by various external stimuli (eg. growth factors, cytokines, extracellular matrices, and antigen receptors), this pathway becomes critical in generating an appropriate cellular response by triggering various interacting pathways [357, 358]. Precisely, SFKs cooperate with TKR-dependent activation of the Ras/Erk/MAPK pathway, enhancing DNA synthesis and cell proliferation, while they regulate P13K/Akt signaling to promote cell survival [359]. Acting through transcriptional factors, for instance STAT3, SFKs promote the transcription and secretion of proangiogenic growth factors and cytokines, including VEGF and IL-8 [360]. Having FAK as a critical downstream target, SFKs interact with p120 catenin to disrupt adherens junction, as well with $p130^{Cas}$, paxillin and Rho A to promote the complex formation with integrin molecules, important in interactions with the extracellular matrix [358]. At least seven SFKs are expressed in ESCs with many of them undergoing dynamic changes in transcriptional and post-transcriptional level during differentiation, suggesting distinct functions in the control of developmental fate. According to Meyn *et al.* [361] disparate SFK members may have opposing effects on ESCs self-renewal and differentiation *via* at least two independent pathways; one, including Hck and cYes, that promotes self-renewal, and another pathway, including cSrc and Fyn, that promotes growth and differentiation [53, 362]. cYes is highly expressed in mESCs and hESCs, and its activity is regulated by LIF/gp130 and various other serum components (eg. FGFs, Wnts, TGFβ etc) in the absence of LIF [89]. Induced expression of active c-Yes blocks the differentiation of ESCs to embryoid bodies by maintaining the expression of pluripotency genes [363]. LIF activates also the Src family member Hck [364], and induced expression of a kinase-active mutant of Hck maintains ESCs in an undifferentiated state when LIF concentrations are decreased but not absent [228, 365]. Downregulation of these SFKs is compatible with differentiation. Mouse ESCs transfected with vectors expressing Yes siRNA and a puromycin-resistance gene demonstrated inability to make stable clones in the presence of puromycin, confirming a specific role of Yes in self-renewal, proliferation or survival of mESCs [89]. This conclusion was further supported by the observation that the Yes siRNA induced a reduction in the mRNA levels of Nanog, whereas it increased the levels of GCNF, an orphan nuclear receptor that represses Oct3/4 [89]. Treating mESCs and hESCs with the selective SFK inhibitor SU6656, which is particularly active against Yes [366], decreases the expression of pluripotency marker genes such as AP, Oct3/4, FGF4 and Nanog, and thus causes ESCs to undergo linear differentiation in the presence of LIF [89, 361]. In line with the later, this inhibition modulates β-catenin relocalization leading to a population of simple epithelial cells (with upregulation of cytokeratins 18 and 8 (K18/K8)) that can further differentiate into

cells expressing keratinocyte or corneal-specific markers [367]. Similarly, rapid inhibition of tyrosine kinase activity of Hck and Lck as well as transcriptional silencing of Lyn and Lck has been observed during differentiation [89, 361]. In contrast, differentiation of ESCs neither affects protein expression nor kinase activity of Src or Fyn. Src inhibition in mESCs, in addition to MAP kinase and Gsk3 inhibition, retains their pluripotency independence of substrate elasticity and confers the formation of teratomas following transplantation of these mESCs [363, 368]. Contrary to selective SFK inhibitors, complete non-selective inhibition of SFK activity with either the PP2 or A-419259 ATP competitive inhibitors, or high levels of the 4-anilinoquinazoline SKI-1 inhibitor, decrease cell growth and prevent ESCs differentiation. Such divergent activity has been partly attributed to the SU6656-induced inhibition of Aurora kinases rather than SFKs [362]. Selective agonists or inhibitors of c-Src versus c-Yes activity may allow more precise manipulation of ESC fate and may have broader applications in other biological systems that express multiple Src family members, such as tumor cells [363].

Spleen tyrosine kinase (Syk), a non-receptor protein tyrosine kinase involved in coupling activated immunoreceptors to downstream signaling, is highly expressed in ECs and implicated in the proper development of the blood and lymphatic vascular system and the maintenance of its integrity [369, 370]. Syk-deficient mice reveal decreased number of ECs and abnormal morphogenesis of the existing ones under electron microscopy [370]. Consistently, adenovirus-mediated expression of Syk dominant negative mutants exhibit severely impaired proliferation and migration of human umbilical vein ECs [369]. It has been suggested that “Syk functions redundantly in an early progenitor to promote the migration of intersegmental vessel angioblasts and lymphangioblasts”, either downstream of, or in parallel to VEGFA [371]. Gene expression profiling of EPCs by oligonucleotide microarray analysis confirmed this assumption by including Syk as highly expressed in these cells [372]. Syk was identified as a major tyrosine kinase phosphorylating VEGFR-2 and thus promoting VEGFA-induced EC migration [373, 374]. However, activation of Syk can also take place independently of VEGF expression, as seen in Ang-2 stimulated angiogenesis *via* transactivation of the EGF receptor [375].

CONCLUSION

PKs and their signaling transduction networks represent a significant mechanism by which cellular pluripotency and lineage differentiation are regulated. The present review summarizes some of the current knowledge of the role of PK signaling in PSC maintenance, differentiation and iPSC reprogramming. When we study the kinome that regulates pluripotency, we first need to specify the pluripotent state we are considering, given the frequent contrasting expression and activity of PKs among these various states. In addition, although mESCs and hESCs share many common features, yet they have distinctive fundamental differences in the signaling pathways that underlie self-renewal and differentiation. The conserved pathways that regulate self-renewal in both mESCs and hESCs are the LIF-independent Wnt/Gsk3/β-catenin and the PI3K/Akt pathway, which is activated by LIF in mESCs and by NTs and IGF in hESCs. Besides, mESCs are maintained by activation of the LIF-related pathways with support from BMP4, whereas self-renewal and survival of hESCs is mediated by NTs and FGF2. Some downstream cytoplasmic

signaling pathways, such as Ras/MAPK/Raf/Mek/Erk and JAK/STAT, are also differently activated and play disparate roles in mESCs and hESCs. Targeting PK activity with the use of small synthetic modulators not only offers a powerful tool for manipulating cell fate to a desired outcome, but also provides the means for dissecting the underlying mechanisms. The clinical relevance of PKs in therapeutics and regenerative medicine can be clearly demonstrated in the paradigm of vascular development. An integrated understanding of how PK signaling pathways contribute to distinct phenotypes of endothelial stem cells would allow for future anticancer interventions and therapeutic organ regeneration programs.

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