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Mechanisms of self-renewal in human embryonic stem cells

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ABSTRACT

Embryonic stem cells (ESCs) are the pluripotent cell population derived from the inner cell mass of pre-implantation embryos and are characterised by prolonged self-renewal and the potential to differentiate into cells representing all three germ layers both in vitro and in vivo. Preservation of the undifferentiated status of the ESC population requires the maintenance of self-renewal whilst inhibiting differentiation and regulating senescence and apoptosis. In this review, we discuss the intrinsic and extrinsic factors associated with self-renewal process, together with possible signalling pathway interactions and mechanisms of regulation.

1. Derivation and culture of hES

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of day 5–8 blastocysts[1–3] or morula stage embryos.[4] They are capable of karyotypically stable, prolonged self-renewal and are also characterised by their potential to differentiate into cells representing all three germ layers, both in vitro and in vivo. ESCs therefore hold great promise as a source of cells for transplantation therapy, development of drug discovery programmes, and as models for early embryonic development. First, the ICM is isolated from the trophectoderm by either immunosurgery[1,2,5,6] or mechanical isolation[7] and is then expanded in culture, most commonly on mitotically inactivated murine embryonic fibroblast (MEF) feeder layers (see Fig. 1). More recently, proliferative MEF feeder layers have been shown to provide efficient support for undifferentiated in vitro culture of human (h)ESC populations.[8] To reliably utilise hESCs in future therapeutic and medical applications, exposure to animal-derived feeder layers/culture supplements must be avoided to eliminate the risk of xenogeneic pathogen/viral transfer. There has recently been a flurry of reports documenting prolonged propagation of hESCs in feeder-free cell culture systems, using specific ECM components such as fibronectin, or basement membrane matrix such as Matrigel as ESC attachment substrates.[9–11] Unfortunately, many of these cell culture systems require medium conditioned by feeder layers in order to support and maintain undifferentiated growth of ESCs, again introducing possible xenogeneic or allogeneic pathogen transfer (see Fig. 1). It would therefore be extremely beneficial to identify the factors present in MEF-conditioned medium (CM), which promote self-renewal and proliferation without destabilising the ESC karyotype. Recently, Stojkovic and colleagues[12] provided an alternative to feeder-free/CM culture systems by successfully culturing hESCs on feeder layers derived from the ESC population itself (autogenic feeder layers). The same investigators have also recently reported on the maintenance of undifferentiated

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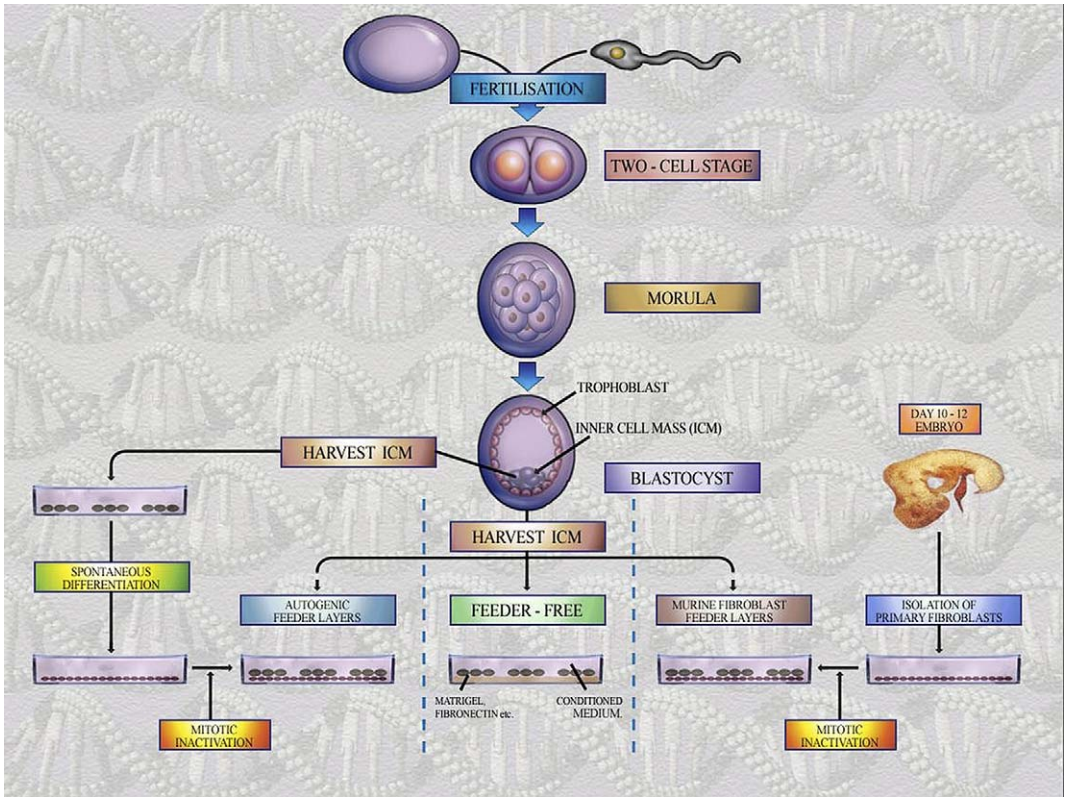

Fig. 1 – Derivation and culture of hESCs: The inner cell mass (ICM) of the blastocyst is isolated from the trophectoderm by either immunosurgery[1,2,5,6] or mechanical isolation[7] and is then expanded in culture on murine embryonic fibroblast (MEF) feeder layers, human autogenic (ESC-derived) feeder layers,[12] or under feeder-free conditions on basement membrane matrix such as Matrigel.[9–11]

hESCs on human serum, using medium conditioned by autogenic feeder layers[13] (see Fig. 1). It is of prime interest to determine the factors, both intrinsic and extrinsic, that maintain and control undifferentiated propagation of ESCs. Understanding these self-renewal mechanisms will aid in the isolation of specific factors and molecules, which will be integral in establishing animal-free in vitro expansion of hESCs.

2. Intrinsic self-renewal factors and markers of pluripotency in ES cells

Several pluripotency markers and self-renewal factors associated with ESCs are listed in Table 1. The principal categories of these markers are outlined below.

2.1. *Cell surface markers*

Undifferentiated hESCs can be characterised by the expression of cell surface markers such as stage specific embryonic antigen (SSEA)-3 and -4, tumour rejection antigen (TRA)-1-60, and -81, germ cell tumour marker (GCTM)-2 and by the absence of SSEA-1. The expression profile of murine (m)ESCs is the converse in that they express high levels of SSEA-1 when undifferentiated, but do not express other SSEA antigens, TRA-1-60/81 or GCTM2.[14–18] A common feature of both hESCs and mESCs is a high expression level of alkaline phosphatase, which is detectable by TRA-2-49/54 and by enzymatic reactions respectively.[19,20]

Table 1 – Pluripotency markers and self-renewal factors involved in the maintenance of undifferentiated mESCs and hESCs

Antigen	hESC	mESC	References
SSEA-1	–	+	[14,15,17]
SSEA-3	+	–	[1,2,14–16]
SSEA-4	+	–	[1,2,14,15]
TRA-1–60	+	–	[1,2,14,15]
TRA-1–81	+	–	[1,2,14,15]
TRA-2–49	+	–	[20]
TRA-2–54	+	–	[14,20]
GCTM-2	+	–	[2,198]
Alkaline phosphatase	+	+	[19,20]
Telomerase	+	+	[48,64]
TRF-1	+	+	[199]
TRF-2	+	+	[199]
Nanog	+	+	[21,22]
Oct3/4	+	+	[32,33]
Sox2	+	+	[34,39]
Rex1	+ (variable)	+	[200,201]
Foxd3	+ (variable)	+	[199,202,203]
Dppa5/Esg1	+	+	[204,205]
FGF4	+	+	[206]

2.2. *Nanog, Oct3/4 and Sox2*

Nanog (named after Tir nan Og, the mythical Celtic land of the ever young,[21]) is a homeobox-containing transcription factor, essential in maintaining the ICM in vivo and ESCs in vitro.[21,22] *Nanog* is present in the ICM,[23] and in undifferentiated mESC and hESC populations, and is down-regulated during differentiation.[21,22,24] In the mouse, removal of *Nanog* results in primitive endoderm differentiation,[21,22] induced by the up-regulation of transcription factors *Gata4*[25] and *Gata6*.[26] *Nanog* knockdown in hESC populations produces a similar increase in *Gata4* and *Gata6* expression, also signifying primitive endoderm differentiation. An increase in expression of *Cdx2* is also observed in hESCs,[27] which is indicative of trophectoderm differentiation.[28] This reciprocal expression pattern of *Nanog* and *Gata4/Gata6/Cdx2* suggests that *Nanog* may maintain pluripotency by suppressing the expression of these transcription factors, thereby inhibiting extraembryonic differentiation.[29] The presence of *Nanog* binding domains within the enhancer region of *Gata6* implies a direct mechanism of inhibition.[22] The mechanisms involved in the regulation of *Nanog* are not fully understood, although recent reports suggest a pivotal role for the transcription factors *Oct3/4* and *Sox2*, which have adjacent binding sites within the *Nanog* promoter region.[30,31] *Oct3/4* is a POU domain transcription factor which regulates downstream genes by binding to the octamer repeat sequence AGTCAAAT within the promoter region.[32,33] *Oct3/4* acts in conjunction with *Sox2*, a member of the Sox family of HMG box transcription factors,[34] which binds to sites adjacent to the Oct3/4 octamer repeats.[35] Both factors have an essential role in self-renewal and are expressed at high levels in the majority of ESC lines.[36] Even discrete modulations in expression have an effect on the pluripotent status of the ESC population both in vitro and in vivo. Increases in *Oct3/4* expression promote mesoderm and endoderm formation whereas down-regulation of either factor results in trophectoderm differentiation.[37–39] Loss of *Sox2* also contributes to extra-embryonic endoderm development.[39] *Nanog* is not the only gene to be regulated by the Oct–Sox complex. Binding sites for both transcription factors have also been identified within the promoter regions of *Fgf-4*,[40,41] *Utf-1*[42] and *Fbx-15*.[43]

DNA microarrays,[44] serial analysis of gene expression (SAGE),[45] and massively parallel signature sequencing (MPSS)[46] are powerful tools for transcriptome profiling and have proven instrumental in the identification of novel hESC markers, such as the RNA-binding protein Lin28, the embryonic DNA methyltransferase DNMT3b[47] and the homeobox-expressed transcription factor HESX1,[45] which are expressed in undifferentiated hESCs and downregulated during hESC differentiation. Characterisation of these markers may further our understanding of ESC self-renewal and pluripotency mechanisms.

2.3. *Telomerase*

Telomeres are DNA complexes that cap the ends of eukaryotic chromosomes thereby maintaining chromosomal stability and preventing end-to-end fusions and chromosome degradation.[48] Telomeres are composed of hexanucleotide TTAGGG double-stranded tandem repeats, with a 150–200 base 3′ overhang of the G-rich strand,[49,50] which folds back into the double stranded telomere forming the "T-loop".[50–52] In somatic cells, telomeres shorten with each cell division, as DNA polymerases cannot replicate the distal end of the lagging strand (otherwise known as the "end replication problem").[53,54] Eventually, telomeres reach a critical length[55] and cells either senesce[54,56,57] or, if telomere shortening continues, enter "cellular crisis" which leads to extensive apoptosis.[58,59] Cells and tissues with a capacity for self-renewal and rapid proliferation overcome this problem of critical telomere shortening by expressing the holoenzyme telomerase, a ribonucleoprotein complex which functions to extend and stabilise telomeric DNA.[60] Telomerase is comprised of two essential subunits, the telomerase reverse transcriptase component (TERT) and the telomerase RNA component (TR) which holds the RNA template required for the generation of new TTAGGG DNA repeats[61,62] (see Fig. 2). Although telomerase activity can be reconstituted in vitro using recombinant TERT and TR,[61] telomerase associated proteins such as TEP-1, Dyskerin, p23 and HSP90 are also required for telomerase activity in vivo.[63] These proteins, together with hTR are constitutively expressed in somatic tissues, unlike TERT which is the regulated, rate-limiting element of telomerase activity.[63] ESCs display high levels of TERT expression and telomerase activity, both of which are rapidly down-regulated during differentiation.[64] Telomerase activity or expression of TERT can therefore be regarded as a marker of undifferentiated ESC populations. Although down-regulation of TERT is a consequence of differentiation, it is not necessarily the driving force behind loss of pluripotency. mESC populations showing mutations in mTERT or mTR retain an undifferentiated expression profile[65,66] whereas over-expression of mTERT promotes proliferation, confers protection from oxidative stress and apoptosis, and enhances haematopoietic differentiation.[67,68]

A minority of immortalised mammalian cell lines are devoid of telomerase activity yet remain able to overcome the end replication problem and maintain telomere length during prolonged self-renewal. This is accomplished using recombination-mediated mechanisms, known as ALT (alternative lengthening of telomeres) pathways,[69–72] ALT+ cells are characterised by significant variations in telomere length within individual cells,[71,73] and often display mutations or imperfect DNA repair. For this reason, telomerase-mediated mechanisms may be considered more advantageous for the maintenance of telomere length.

3. Extrinsic self-renewal factors in ES cells

3.1. *LIF signalling*

In 1981, Martin and colleagues[5] isolated the ICM from murine blastocysts and successfully cultured and maintained the pluripotent mESC population on STO/MEF feeder layers. However, when the cells were transferred to gelatin-coated dishes, differentiation occurred, suggesting that the feeder layers were secreting factors into the culture medium necessary for self-renewal and prolonged proliferation of mES cells. These findings were supported by Smith and colleagues in 1987[74] who successfully maintained undifferentiated mESCs in the absence of feeders by using medium

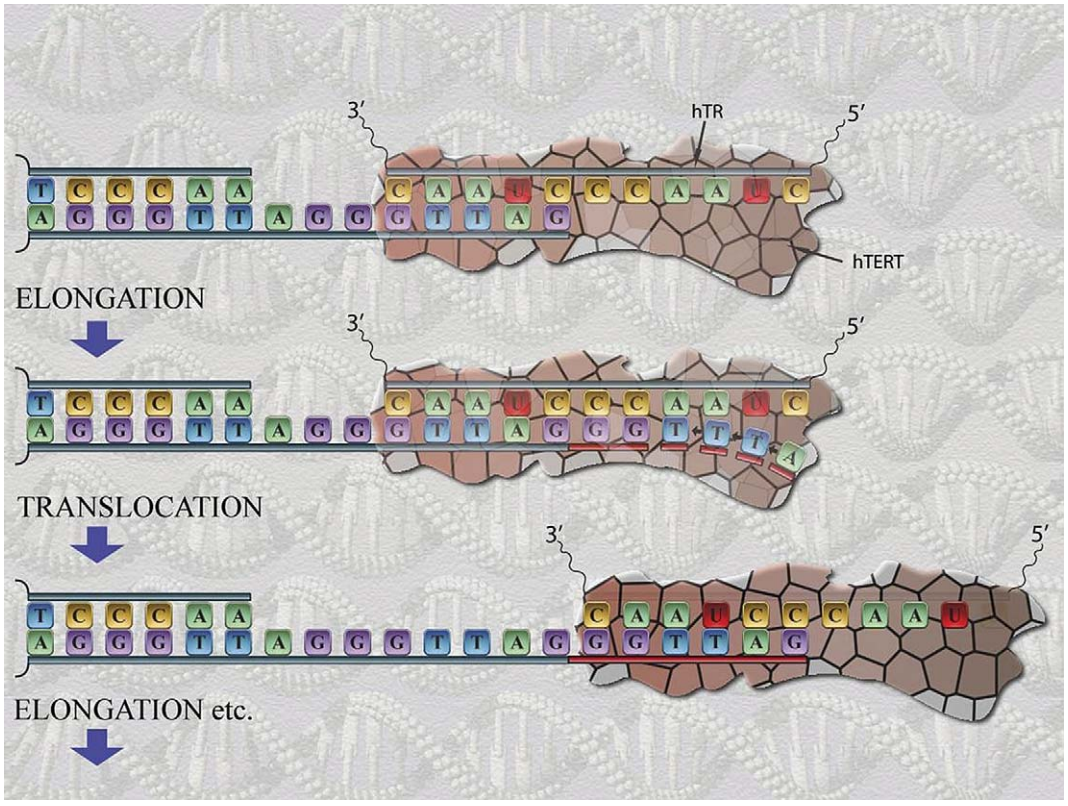

Fig. 2 – Telomere length maintenance by telomerase: Telomeres are composed of hexanucleotide TTAGGG double-stranded tandem repeats with a G-rich overhang.[49,50] Telomerase is a ribonucleoprotein complex comprised of the telomerase reverse transcriptase component (TERT) and the telomerase RNA component (TR), which holds the RNA template required for the generation of new TTAGGG DNA repeats.[61,62]

conditioned by Buffalo rat liver cells. Analysis of the conditioned medium identified the presence of a soluble 20–35 kDa polypeptide factor termed DIA (differentiation inhibiting activity). It was later discovered that DIA could be substituted in culture medium by the haematopoietic regulator, myeloid leukaemia inhibitory factor (LIF), which is similar in both structure and function to DIA.[75,76] LIF is member of IL6 family of cytokines and binds to a receptor complex consisting of two transmembrane proteins, LIFRβ and gp130 (the latter is common to all IL-6 cytokines). Binding of LIF to its receptor complex results in recruitment of JAK kinases which activate the STAT3 signalling pathway[77,78] and induces transcription of self-renewal genes (see Fig. 3). Several factors have been implicated in modulation of LIF pathway including the megakaryocytic growth factor thrombopoietin (Tpo) (which activates STAT3,[79]) IGF2[80,81] and CD9.[82] There is no substantial evidence to suggest that the LIF signaling pathway interacts with Nanog/Oct3/4/Sox2 and vice versa.[21,22,83] It is clear from experiments to date that LIF is crucial for maintaining mESCs in an undifferentiated state in vitro, although this can only be achieved in the presence of serum.[83] This implies the existence of other factors (e.g. BMPs, see later section), perhaps present in serum, which may be involved in maintaining mESCs and which contribute to self-renewal.[84,85] This notion is supported by reports that mESC lines have been successfully derived from LIFRnull and gp130null embryos.[86]

The binding of LIF to its receptor complex also initiates signalling pathways antagonistic to self-renewal, such as the ERK pathway, which promotes differentiation.[78] Following gp130 stimulation, receptor-bound SH2 homology containing tyrosine phosphatase 2 (SHP2) is phosphorylated and recruits a complex containing growth factor receptor binding protein 2 (Grb2) and SOS guanine–nucleotide-exchange factor. Localisation of SOS to the cell membrane activates G-protein Ras, which recruits Raf-1 from the cytosol to the cell membrane. Raf-1 is phosphorylated and activates a transphosphorylation cascade involving MAPK/ERK kinase (MEK) and extracellular-signal-regulated kinase (ERK),[87] which translocates to the nucleus and initiates transcription of genes associated with differentiation[88,89] (see Fig. 3). The balance between the antagonistic signalling pathways of LIF and ERK plays an important role in regulating self-renewal and determining the fate of undifferentiated mESCs.[90]

LIFRβ and gp130 are also expressed in hESC lines and functional activation of the STAT pathway by human LIF has been reported.[91] However, this is not sufficient to maintain pluripotency in vitro and stem cells markers such as Nanog, Oct3/4 and TRA-1–60 are down-regulated during gp130-dependent signalling.[92] Therefore, in contrast to mESCs, LIF is not considered integral in the self-renewal process of hESCs in vitro.[1,91] This disparity may be attributed to differences in the requirement for diapause, the process by which mouse embryos are arrested at the late blastocyst stage and

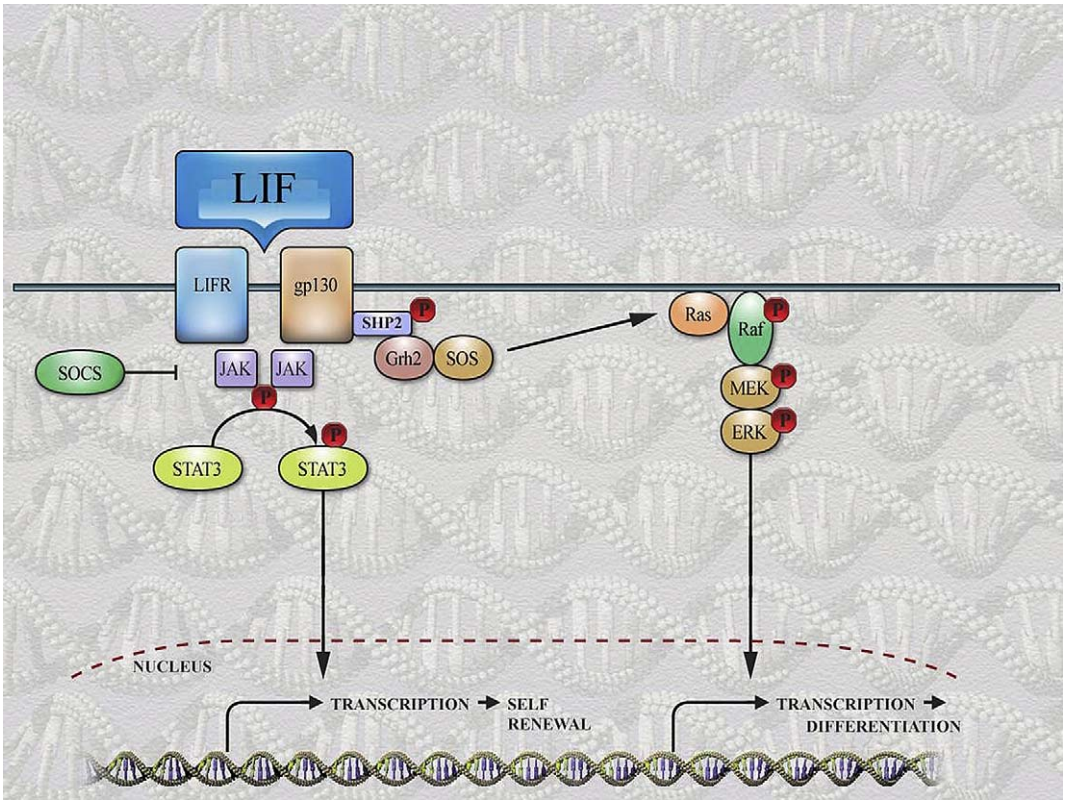

Fig. 3 – The LIF/ERK signalling pathway: Binding of LIF to its receptor complex results in recruitment of JAK kinases which activate the STAT3 signalling pathway[77,78] and induces transcription of self-renewal genes. LIF–LIFR complex binding also initiates the ERK pathway, which promotes differentiation.[78] Following gp130 stimulation, receptor-bound SH2 homology containing tyrosine phosphatase 2 (SHP2) is phosphorylated and recruits a complex containing growth factor receptor binding protein 2 (Grb2) and SOS guanine–nucleotide-exchange factor. Localisation of SOS to the cell membrane activates G-protein Ras, which recruits Raf-1 from the cytosol to the cell membrane. Raf-1 is phosphorylated and activates a transphosphorylation cascade involving MAPK/ERK kinase (MEK) and extracellular-signal-regulated kinase (ERK),[87] which translocates to the nucleus and initiates transcription of genes associated with differentiation.[88,89]

prevented from implanting in the uterus until the current suckling litter has been weaned, thereby avoiding competition for maternal nutrients.[93,94] As gp130 is essential in maintaining the blastocyst during this process, the insignificance of LIF signalling in humans may be explained by apparent irrelevance of diapause during early human embryonic development.[95]

3.2. *TGFβ signalling*

TGFβ signalling is involved in a wide range of cell fate decisions and cellular processes (e.g. cell proliferation, differentiation and apoptosis) in both embryo and adult. There are two branches of TGFβ signalling implicated in the self-renewal process in ESCs, the TGFβ/nodal/activin branch and the BMP signalling branch, both of which are discussed below.

3.2.1. *TGFβ/nodal/activin signalling*

The TGFβ/nodal/activin branch of TGFβ signalling involves the activation of intracellular Smad2/3, which becomes phosphorylated and complexes with co-Smad4 before being translocated to the nucleus. Negative regulation is provided by inhibitory Smads such as Smad7[96] and Smad anchor for receptor activation (SARA)[97] (see Fig. 4). The importance of Smad2/3 activation in hESC self-renewal has recently been demonstrated using the synthetic compound SB431542. Application of this compound inhibits Smad2/3 phosphorylation[98] which results in the down-regulation of hESC pluripotency markers such as Oct3/4.[96] In contrast to these findings, mESCs fail to display a loss of stem cell markers following exposure to SB431542,[96] suggesting that Smad2/3 activation in mESCs is not required for maintenance of pluripotency in vitro. However, it cannot be assumed that these findings recapitulate the processes occurring in vivo. Indeed, recent studies report that loss of Smad 2/3 during murine embryogenesis results in reduced epiblast cell populations and a decrease in levels of Oct3/4,[85] implying a reduction in stem cell self-renewal and loss of undifferentiated phenotype.

3.2.1.1. *Nodal*. In vertebrates, nodal is a mesoderm and endoderm inducer[99] and is involved in left-right axis determination in the mouse, frog and chick embryo.[99–101] Overexpression of nodal promotes mesoderm development in chick,[102] Xenopus,[103] zebrafish,[101] and in mouse[104] embryos. It is therefore surprising that several investigations have implicated an additional role for nodal in both mouse and

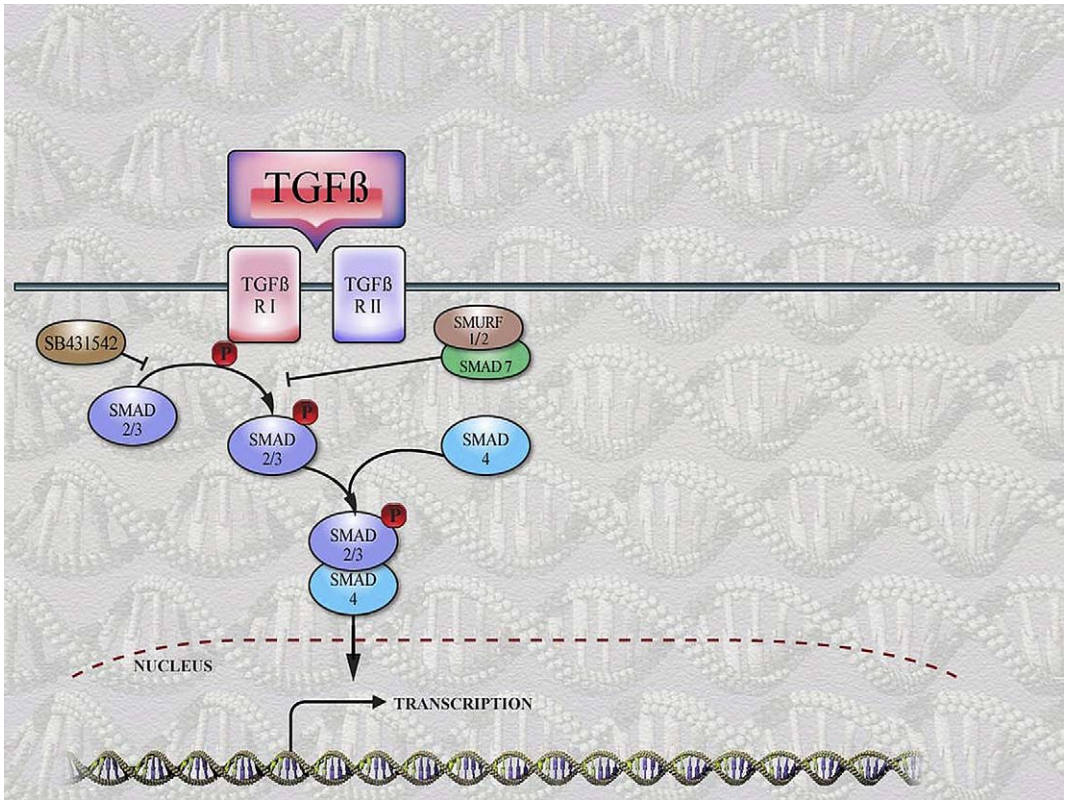

Fig. 4 – The TGFβ signalling pathway: TGFβ binds to TGFβRI and II, activating Smad2/3, which becomes phosphorylated and complexes with co-Smad4 before being translocated to the nucleus. Negative regulation is provided by inhibitory Smads such as Smad7[96] and Smad anchor for receptor activation (SARA).[97]

human ESC self-renewal. In the mouse, nodal is thought to play role in epiblast formation and expression of pluripotency markers; nodal null mice consistently show a reduction in the size of epiblast cell population, together with low levels of Oct3/4 expression, implying an attenuated ability to self-renew.[105–107] In hESC populations, nodal is expressed at high levels and becomes down-regulated during differentiation.[108] Over-expression either through application of recombinant nodal or constitutive expression inhibits differentiation of hESC into neuroectoderm, induces visceral endoderm formation and maintains markers of pluripotency.[107] It is unclear whether this is due to direct action of nodal, or to indirect action of nodal-induced extraembryonic differentiation.[107] The disparity between reports regarding the outcome of nodal over-expression can be explained in part by the fact that some experiments were conducted in vivo, whereas others were conducted in vitro in specific and defined culture conditions, which may lack certain co-factors/growth factors (e.g. BMP, Wnt, FGF) which would normally interact with nodal signalling in vivo. Care must be taken to consider this when assessing the role of individual factors on ESC self-renewal in vitro. Nodal activates the Smad2/3 signalling cascade by binding to type I (ALK4/7) and type II activin receptors[109–111] (see Fig. 5). Signalling is modulated by the EGF-CFC GPI-linked cofactor Cripto (also known as TDGF1),[107,112] by convertases which aid in the regulation of the signal response[99,113] and by the extracellular inhibitors lefty and Cerberus.[107]

3.2.1.2. Activin. Activins were first isolated in 1986 from porcine follicular fluid[114,115] and were considered to be gonadal proteins involved specifically in the synthesis and secretion of pituitary follicle-stimulating hormone (FSH). It is now known that the synthesis of activins is not restricted to ovaries and testes. In fact, activins are present in a wide range of tissues and organs including placenta, bone marrow, spleen and certain parts of the brain where they act as autocrine or paracrine growth factors/cytokines.[116,117] Activins have a wide range of biological functions including differentiation of endoderm from hESCs,[118] inhibition of neuronal differentiation in murine P19 embryonal carcinoma (EC) cells,[119] mesodermal differentiation in mESCs (in association with BMP4)[120] and β-cell differentiation from human pancreatic precursors (in association with betacellulin).[121] Activin concentration is thought to play a significant role in the cellular differentiation response.[122]

More recently, activins have been identified in MEF conditioned medium and have therefore been implicated in hESC self-renewal,[123] although the target genes of activin signalling have yet to be elucidated. Activins are homodimer TGFβ superfamily members which interact with activin receptors I (ALK4/7) and II and subsequently activate the Smad2/3 signalling pathway.[123] It is possible that activins may interact with other signalling pathways such as the Wnt pathway (discussed in a later section) which may explain the diverse and contrasting roles for activin as described above.

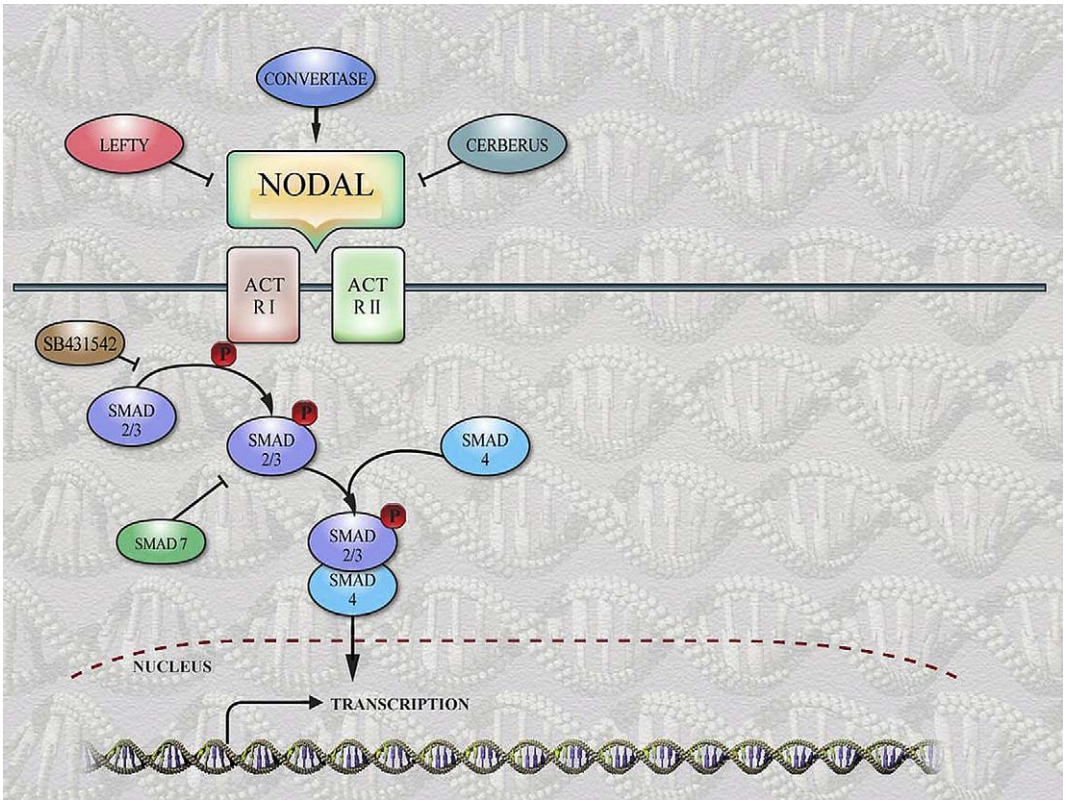

Fig. 5 – The Nodal signalling pathway: Nodal activates Smad2/3 by binding to type I (ALK4/7) and type II activin receptors.[109–111] Smad2/3 complexes with co-Smad4 prior to translocating to the nucleus. Signalling is modulated by the EGF-CFC GPI-linked cofactor Cripto (also known as TDGF1),[107,112] by convertases which aid in the regulation of the signal response[99,113] and by the extracellular inhibitors lefty and Cerberus.[107]

3.2.2. *BMP signalling*

Bone morphogenetic proteins (BMPs) are also members of the TGFβ superfamily but function via a different signalling pathway to that described for TGFβ/activin/nodal signalling.

The four BMP receptors, BMPIα (ALK2, ALK3), BMPIβ (ALK6) and BMPRII[124] form a variety of dimer combinations at the cell surface, each generating differing signalling effects and responses. The canonical BMP signalling pathway is initiated by the binding of BMP to heterodimers of BMPR1α and BMPRII.[83,125] This leads to activation of Smad1/5/8, which forms a heteromeric complex with Smad4 prior to translocation to the nucleus.[96,108,126] Suppressors of cytokine signalling (SOCS) and inhibitory Smads such as Smad7 provide negative feedback regulatory mechanisms.[83,86] BMP signalling results in the expression of members of the Id (inhibitor of differentiation) family of negative HLH factors[127,128] which interact with, and may functionally antagonise neurogenic bHLH transcription factors, e.g. Mash or non-bHLH transcription factors such as Pax 2, 5, and 8[129] (see Fig. 6).

BMP signalling has contrasting effects on mice and hESC cell populations. In mESCs, both LIF and BMP's (e.g. BMP2, BMP4 and GDF6) are required for efficient mESC self-renewal under serum-free conditions.[83] When acting individually, LIF and BMP induce neuronal and mesodermal differentiation, respectively.[83,86] BMP4 is known to inhibit neuronal differentiation, possibly by blocking the MEK/ERK signalling cascade,[130] and when combined with LIF in vitro, mESCs remain undifferentiated, even in the absence of serum. BMPs are the only TGFβ superfamily member involved in mESC self renewal.[83] In contrast to mESCs, BMP signalling does not contribute to self-renewal in hESCs and in fact promotes rapid down-regulation of Nanog and Oct3/4,[123] which subsequently induces trophoblast differentiation.[131] The low levels of activated Smad1/5/8 found in undifferentiated hESCs increase during differentiation and may compete with active Smad2/3 to complex with co-Smad4. In this way, BMP signalling in hESCs may act as a regulatory element of TGFβ/activin/nodal signalling.[108]

3.3. *FGF and PI3K signalling*

The FGF family of growth factors are capable of eliciting a wide range of cellular responses such as proliferation, migration,[132,133] differentiation,[134–136] cell cycle arrest,[134,137] and ESC self-renewal.[89,138,139] Such a diverse array of functions may be explained in part by the promiscuous nature of FGF ligand-receptor binding, and by the presence of various FGF receptor isoforms, which are produced by alternative splicing of *FGF genes 1–4*.[140,141] Cell type, cell maturity and interactions with other signalling pathways also seem to contribute to the FGF signal response.[142] FGF-2 (basic(b)FGF) in particular is an essential component for the maintenance of hESCs in vitro as withdrawal of bFGF results in ESC differentiation and loss of TRA-1-60 and Oct3/4.[138] Reports suggest that FGF promotes

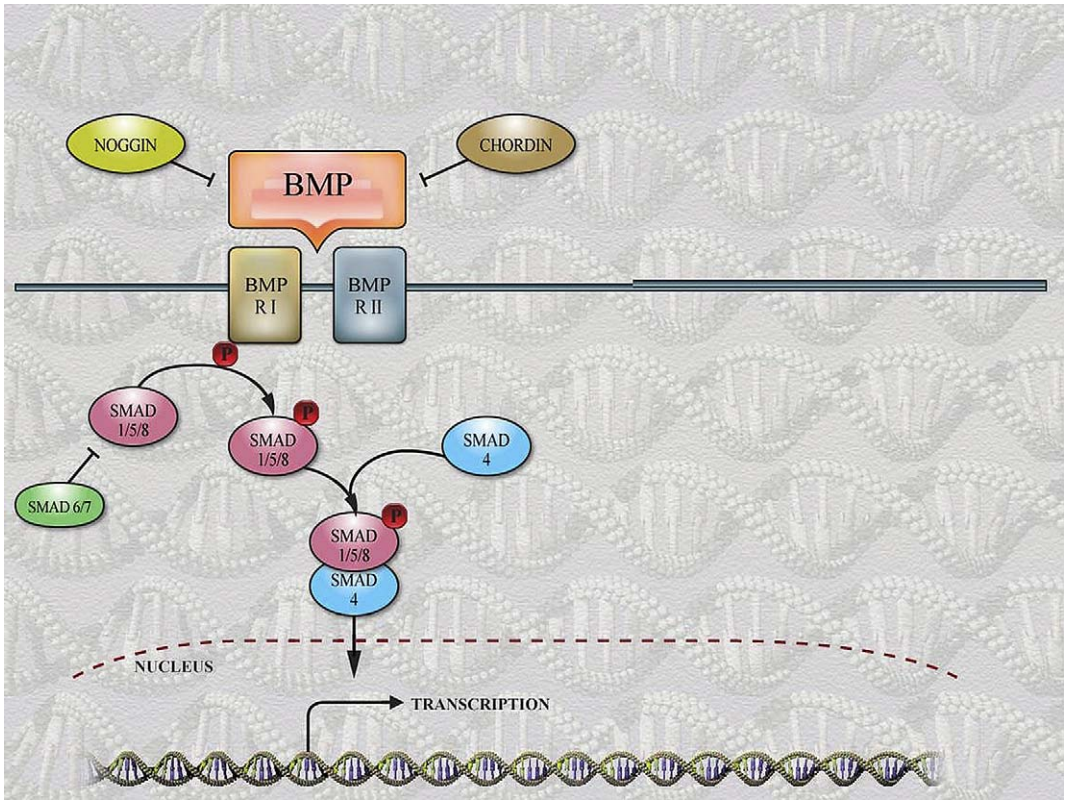

Fig. 6 – The BMP signalling pathway: The canonical BMP signalling pathway is initiated by the binding of BMP to heterodimers of BMPR1α and BMPRII.[83,125] This leads to activation of Smad1/5/8, which forms a heteromeric complex with Smad4 prior to translocation to the nucleus.[96,108,126] Suppressors of cytokine signalling (SOCS) and inhibitory Smads such as Smad7 provide negative feedback regulatory mechanisms.[83,86]

self-renewal in ESCs by antagonising the BMP pathway through Smad1 inhibition,[143,144] subsequently suppressing differentiation.[138] However, when FGF is replaced in vitro with BMP inhibitors such as noggin, differentiation ensues,[10,139] implying the existence of FGF-mediated pathways additional to BMP. One such candidate is the PI3K/AKT signalling pathway, which is activated by FGF binding[89,138] and promotes self-renewal in ESCs through inhibition of MEK/ERK signalling,[89,145] and/or up-regulation of ECM molecules,[138] which are essential for maintaining ESC pluripotency in vitro.[9,11,138] The possible antagonistic regulation of the BMP pathway by FGF may not be a unidirectional phenomenon. FGF-mediated PI3K/AKT signalling may be subject to regulation by BMP2, which is thought to inhibit the degradation of the tumour suppressor protein, PTEN,[146,147] a known negative effector of PI3K.[148]

3.4. *Canonical Wnt/β-catenin signalling*

The Wnt signalling cascade is highly regulated pathway involved in cell proliferation and fate determination. De-regulation results in carcinogenesis and is often implicated in colorectal cancer.[149] In the absence of a Wnt signal, the cytoplasmic protein β-catenin associates with a destruction complex containing a variety of proteins including axin, glycogen synthase kinase-3β (GSK3β) and adeomatosis polyposis coli (APC). On binding to this complex, β-catenin is phosphorylated and subsequently ubiquitinated, thereby marking it for proteosomal degradation.[150] The binding of Wnt to its surface receptor, Frizzled (Frz), results in the activation of the protein Dishevelled (Dsh) which inhibits the destruction complex.[151,152] β-catenin subsequently accumulates in the cytoplasm and translocates to the nucleus, where it acts as a transcriptional coactivator of T-cell factor (TCF, otherwise known as lymphoid enhancer factor, LEF)[153] (see Fig. 7). Wnt signalling is implicated in self-renewal of intestinal epithelial stem cells,[154,155] skin stem cells[156,157] and more recently in mESCs and hESCs. In 2004, Sato and colleagues[158] reported on the effect of the GSK inhibitor BIO (6-bromoindirubin-3-oxime), a synthetic derivative of Tyrian purple dye found in the Mediterranean mollusc *Hexaplex trunculus*.[159] BIO-mediated activation of the Wnt signalling cascade maintained the undifferentiated state of both mESCs and hESCs under feeder-free conditions,[158] possibly by Wnt-induced increases in expression of Nodal.[108] Recent reports suggest the PI3K/AKT pathway may contribute to β-catenin accumulation in the nucleus of cardiomyocytes[160] and intestine stem cells (ISCs),[161] possibly through PI3K/AKT-mediated inhibition of GSK3β.[160,162] Therefore, should similar pathway interactions occur in ESCs, BIO-mediated self-renewal of mESCs and hESCs may not necessarily act exclusively through Wnt signalling[158] (see Fig. 8).

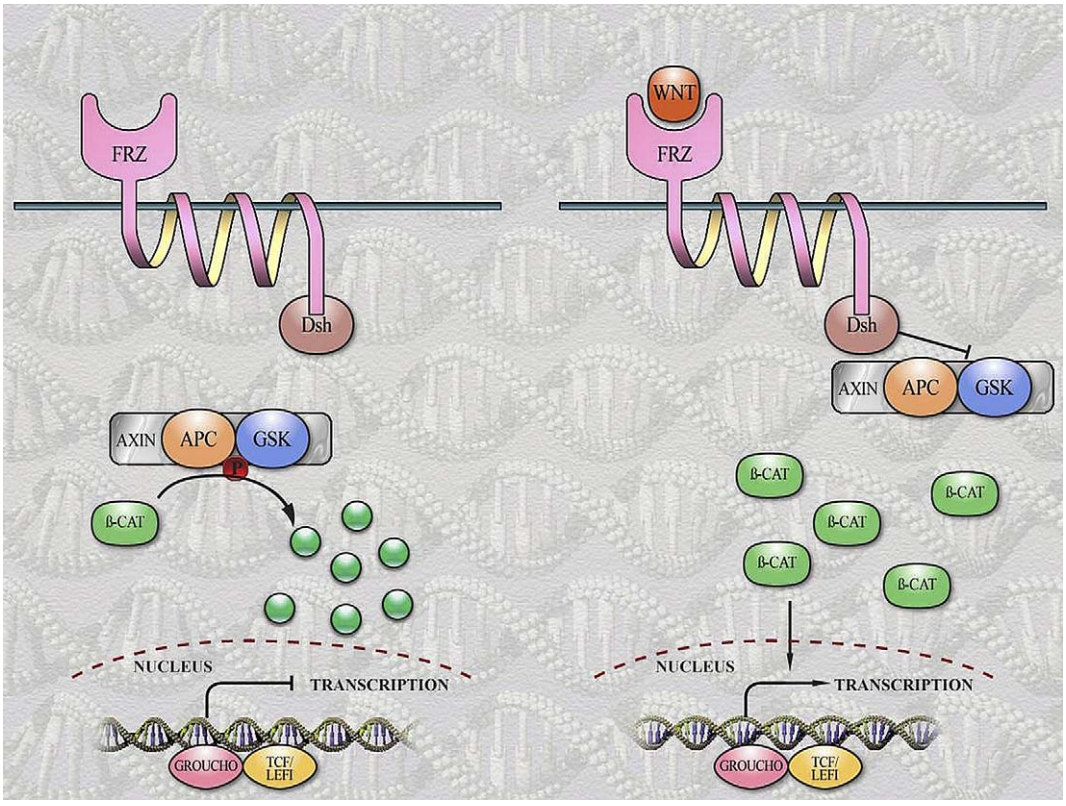

Fig. 7 – The Wnt signalling pathway: In the absence of a Wnt signal, the cytoplasmic protein β-catenin associates with a destruction complex containing a variety of proteins including axin, glycogen synthase kinase-3β (GSK3β) and adeomatosis polyposis coli (APC). On binding to this complex, β-catenin is phosphorylated and subsequently ubiquitinated, thereby marking it for proteosomal degradation.[150] The binding of Wnt to its surface receptor, Frizzled (Frz), results in the activation of the protein Dishevelled (Dsh) which inhibits the destruction complex.[151,152] β-catenin subsequently accumulates in the cytoplasm and translocates to the nucleus, where it acts as a transcriptional coactivator of T-cell factor (TCF, otherwise known as lymphoid enhancer factor, LEF).[153]

4. Chromatin structure and pluripotency

Intrinsic and extrinsic signalling do not act alone in governing transcriptional regulation of ESC self-renewal; epigenetic modifications such as DNA methylation (carried out by DNMT enzymes) and histone modifications (acetylation, phosphorylation, ubiquitination and methylation) also influence transcription factor-mediated cell fate decisions.[163,164] For example, the DNA methylation status of the Oct3/4 promoter region has proved significant in regulating the pluripotent status of ESC population. Hattori and colleagues[165] showed that murine trophoblast stem cells (normally Oct3/4 negative due to hypermethylation of the promoter) can be induced to express Oct3/4 following demethylation by 5-aza2-deoxycytidine and histone deactetylation inhibition by Trichostatin A.[166–168] Tsuji-Takayama and colleagues[169] also demonstrated a role for demethylation in reversal of differentiation; following exposure to 5-azacytidine, murine embryoid bodies (EBs) were shown to increase expression of the pluripotency markers SSEA1, alkaline phosphatase, Oct3/4, Nanog and Sox2.

Polycomb group (PcG) proteins have also been implicated with a role in chromatin remodelling and gene-specific transcriptional regulation of ESCs. These proteins form heterogeneous complexes 2–5 mDa in size at their target sites, and stably repress gene transcription through chromatin modifications.[170] *Bmi1*, *Mph1/Rae28*, and *Mel*-18 are known to regulate self-renewal of haematopoietic stem cells,[171] whereas *Rnf2* and *Ezh2* are required for embryonic development[172,173]; the latter is also essential for ESC derivation in vitro.[174]

5. Self-renewal and carcinogenesis

Elucidation of the mechanisms by which ESCs maintain pluripotency during prolonged proliferation may give insight to the mechanisms by which cancer cells self-renew, as it follows that both mechanisms would be similar, if not identical. In this respect, it is not surprising that several pathways and factors implicated in ESC self-renewal also play a role in tumour formation and maintenance. For example, Sonic hedgehog (Shh) and Notch signalling pathways are required for the self-renewal and maintenance of neural stem cells in the central nervous system,[175–177] although when these pathways become deregulated and when activation becomes aberrantly prolonged, medulloblastomas,[178] basal cell carcinomas[179] and T-cell leukaemias[180] may develop. Mutations in the Wnt signalling pathway also result in oncogenesis. Overactive β-catenin signalling is the major cause of many human colon carcinomas[181] and epidermal

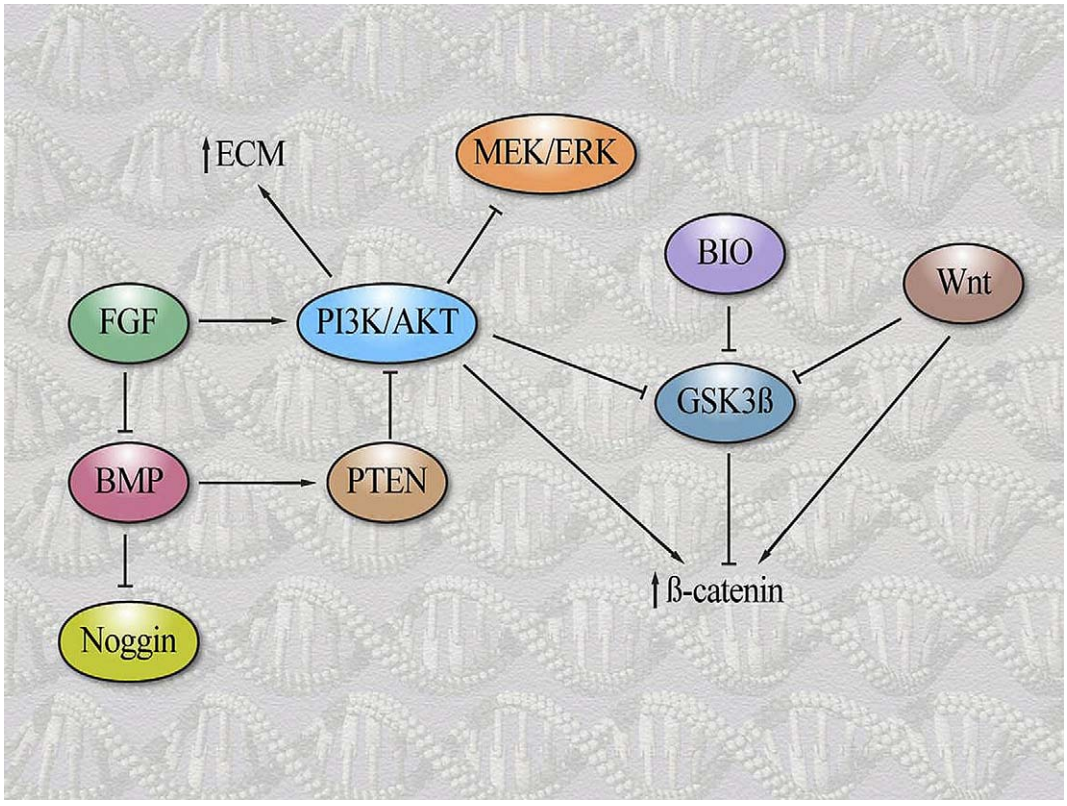

Fig. 8 – Possible crosstalk between BMP, FGF/PI3K/AKT and Wnt signalling pathways: FGF binding activates the PI3K/AKT signalling pathway[89,138] and promotes self-renewal in ESCs through inhibition of MEK/ERK signalling,[89,145] and/or up-regulation of ECM molecules,[138] which are essential for maintaining ESC pluripotency in vitro.[9,11,138] FGF-mediated PI3K/AKT signalling may be subject to regulation by BMP2, which is thought to inhibit the degradation of the tumour suppressor protein, PTEN,[146,147] a known negative effector of PI3K.[148] The PI3K/AKT pathway is also thought to inhibit GSK3β,[160,162] thereby contributing to β-catenin nuclear accumulation in co-ordination with Wnt.

tumours.[182] Additionally, in prostate cancer cells, nuclear accumulation of β-catenin through PI3K/AKT-mediated inhibition of GSK3β promotes androgen receptor activity,[162] which is known to contribute to the maintenance and survival of prostatic tumours.[183]

A specific requirement for all self-renewing populations is the capacity to maintain telomere length during prolonged proliferation in order to avoid critical telomere shortening and subsequent senescence or apoptosis. Approximately 90% or more of all human tumours maintain telomere length by transcriptional up-regulation of hTERT and subsequent reactivation of telomerase activity.[184–187] Inhibition of telomerase leads to a cessation of tumour growth and/or programmed cell death,[188–190] highlighting the importance of telomerase activity in tumour progression and thereby exposing hTERT as a logical target for immunotherapy and gene therapy in the treatment of cancers.[191,192] However, a minority of tumours (approximately 10%[73]) do not display telomerase activity and maintain telomere length using ALT-mediated mechanisms.[70] It should also be noted that telomerase activity and ALT mechanisms are not necessarily mutually exclusive and it is possible for both to co-exist within individual cells,[193] thereby highlighting the necessity for suppression of both telomerase and ALT activities in anticancer therapies.[72]

The capacity of ESCs for self-renewal renders them ideal targets for tumourogenesis. It is therefore essential for cells such as these to have regulatory mechanisms to ensure protection against transformation. One such candidate is the tumour suppressor p53, which becomes activated in response to stress signals such as DNA damage and promotes cell-cycle arrest or apoptosis.[194] However, in comparison to somatic cells, certain mechanisms of DNA damage, such as ribonucleotide depletion and DNA double-strand breaks, fail to elicit an efficient p53 response in undifferentiated ESC populations, which is in part due to compromised nuclear localisation of p53.[195,196] Fortunately, this limitation can be overcome by the binding of p53 to the promoter region of *Nanog*, which subsequently suppresses *Nanog* gene expression[196,197] and induces ESC differentiation into cell types which are vulnerable to p53-induced apoptosis/cell-cycle arrest.[195,196] These interactions are reversible as ChIP analysis reveals a reduction in p53/*Nanog* binding following the onset of differentiation.[196]

6. Summary

Self-renewal in ESCs is accomplished by a fine balance between proliferative potential, inhibition of differentiation and prevention of senescence/apoptosis. Each of these responses are themselves governed by intrinsic and extrinsic

factors, many of which are intimately linked through cross-talk between signalling pathways. Several of the signalling molecules and pathways involved in ESC self-renewal have a paradoxical role in that they promote both self-renewal and differentiation in a context-dependent manner. Some signalling ligands may elicit different responses in different cell lineages, and some even instigate varying responses within the same cell. The overall resulting cellular response appears to depend on signal strength, the degree of interplay between synergistic and antagonistic signalling cascades and may also be modulated by cell type, cell density and cell maturity. Disparity between mESCs and hESCs must also be taken into account. Differences in both cell surface marker expression and in the dependence upon specific self-renewal pathways (e.g. LIF) illustrate the dangers in assuming these ESC populations are interchangeable when discussing mechanisms of self-renewal. As the ICM is a transient population in vivo, care should be taken in assuming equivalence with ESCs, which exist only in vitro. For this reason, it is important not to assume that in vitro observations in ESC self-renewal signalling necessarily recapitulate what is occurring in the embryo. In addition, the inherent genetic differences between individual embryos from which the hESC lines are derived cannot be ignored and may contribute to subtle response differences between different hESC lines. In vitro culture conditions may also vary between research groups; small changes in concentration of signalling molecules and in cell density could potentially have large impacts on the differentiation status of ESCs in vitro. Such variation may explain the contradictory nature of some reports. In summary, a deeper understanding of ESC self-renewal mechanisms, and identification of the molecules and factors involved, may lead to higher efficiency ESC expansion in animal-free in vitro culture systems. Furthermore, insights into the maintenance and regulation of proliferative potential may aid in the discovery of novel treatments for deregulated proliferative cells such as cancer.

Conflict of interest statement

None declared.

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