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Review

Conserved and divergent paths that regulate self-renewal in mouse and human embryonic stem cells

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Abstract

The past few years have seen remarkable progress in our understanding of embryonic stem cell (ES cell) biology. The necessity of examining human ES cells in culture, coupled with the wealth of genomic data and the multiplicity of cell lines available, has enabled researchers to identify critical conserved pathways regulating self-renewal and identify markers that tightly correlate with the ES cell state. Comparison across species has suggested additional pathways likely to be important in long-term self-renewal of ES cells including heterochronic genes, microRNAs, genes involved in telomeric regulation, and polycomb repressors. In this review, we have discussed information on molecules known to be important in ES cell self-renewal or blastocyst development and highlighted known differences between mouse and human ES cells. We suggest that several additional pathways required for self-renewal remain to be discovered and these likely include genes involved in antisense regulation, microRNAs, as well as additional global repressive pathways and novel genes. We suggest that cross species comparisons using large-scale genomic analysis tools are likely to reveal conserved and divergent paths required for ES cell self-renewal and will allow us to derive ES lines from species and strains where this has been difficult.
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Keywords: Human embryonic stem cells; Self-renewal; Conserved and divergent paths

Early embryo development and ES cell derivation

A detailed description of early embryo development is beyond the scope of this review and readers are referred to excellent reviews by Dr. Gilbert (Developmental Biology, Sinauer Press) on species differences in early blastocyst development. A brief summary of blastocyst and inner cell mass (ICM) maturation is shown in Fig. 1. The primary trophoblast lineage segregates from the lineage of the embryo proper first followed by segregation of the hypoblast (extraembryonic endoderm) and mesoderm followed by amniotic endoderm. The epiblast or the embryo proper forms the embryonic ectoderm followed by the development of the primitive streak that leads to differentiation of mesoderm and endoderm that then differentiate into specific tissues and organs. It is important to note that primordial germ cells (PGC) develop early and are segregated extraembryonically (to the yolk sac) as the embryo develops further. The PGCs then migrate into the embryo proper and are localized to the gonadal ridges. PGCs retain Oct3/4 expression and acquire specific germ cell markers (reviewed in Johnson et al., 2003a; McLaren, 2003). ES cells are derived from the ICM of blastocyst before implantation and these cells retain many of the characteristics of ICM cells although unlike the ICM, which is a transient structure that rapidly differentiates, ES cells can be maintained relatively indefinitely in culture (Buehr and Smith, 2003). ES cells recapitulate the development

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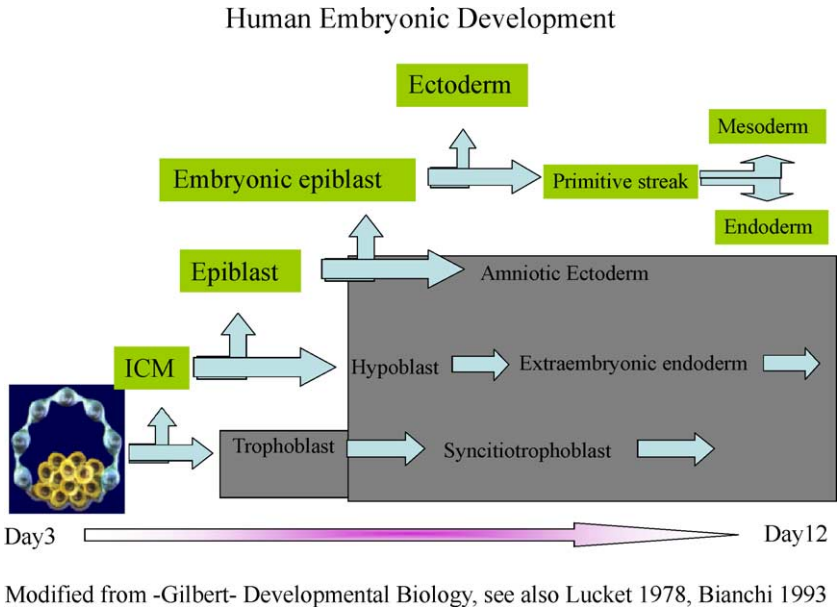

Fig. 1. Blastocyst development. A schematized sequence of development adapted from Gilbert's Developmental Biology is shown. Note the time period of development and the identified stages of differentiation that suggest that pluripotent cells of varying characteristics could be harvested depending on the method and time of isolation.

program of ICM cells and their differentiation is regulated by many of the same factors that regulate germ layer formation and cell type specification (Burdon et al., 2002; Loebel et al., 2003; Tiedemann et al., 2001). ES cells have been used successfully to identify regulatory pathways that direct differentiation (see for example Kikuchi et al., 2004) and confirming the role of a particular pathway in vivo using genetically manipulated mouse cells.

It is important to note, however, that pluripotent cells have been isolated from early blastocysts as well as from embryoid bodies and from germ cells (Papaioannou et al., 1984; Rossant and Papaioannou, 1984; Shamblott et al., 2001; Thomson and Odorico, 2000). These cells appear similar in their expression of Oct3/4 and their ability to contribute to multiple somatic tissues and germinal derivatives but likely differ in their imprinting status (Onyango, 2002), their ability to differentiate into extraembryonic tissue, and their relative frequency of contribution to chimeras after blastocyst injection. The relatively large time window from which cells can be isolated coupled with the dynamic changes that are taking place during this same time window perhaps explaining both the success in obtaining ES or ES-like lines has been reported from species as divergent as medaka and human as well as the difficulty in obtaining ES lines from some strains of mice, pigs, and rats. Further, species differences in development, differences in timing of isolation, and the variability in the types of cell that are potentially multipotent ensure that any pluripotent/totipotent cell lines obtained, while similar overall, will differ in subtle ways.

Most of our knowledge of ES cell biology is derived from studies performed in mouse embryos and ES cells (Carter et al., 2003; Tanaka et al., 2002; reviewed in Ko, 2004). In human embryos, information is limited to the detailed information on early blastocyst maturation, sequence of differentiation, and factors required for maturation and maintenance of blastocysts in culture that has been developed by IVF clinics. More recently, however, gene expression data developed by analyzing human ES cell lines (Abeyta et al., 2004; Bhattacharya et al., 2004; Brandenberger et al., 2004 a,b; Richards et al., 2004; Sperger et al., 2003) have provided additional insights.

Given the variation in early blastocyst maturation and the uncertainty in the stage at which the pluripotent cell population is isolated, it is possible that comparing across species may provide useful insights. Such comparisons may help distinguish between critical and redundant regulatory components required to maintain the pluripotent ES cell's state. In this review, we have summarized known pathways of ES cell self-renewal and identified potential additional pathways that are likely to be important and suggested how detailed inter- and intra-species comparisons may provide useful insight into generating and maintaining ES cells. The data on mouse ES cell self-renewal and known pathways are discussed first and comparisons are highlighted in subsequent sections.

Factors that maintain the ES cell state

A brief overview of mouse ES cell self-renewal is shown in Fig. 2 (see also Niwa, 2001) At this stage of development, most regulatory factors are either derived from the ES cells themselves or from cells that differentiate from them or from feeder cells that are required to maintain cells in culture. Current data suggest that more than one growth factor is

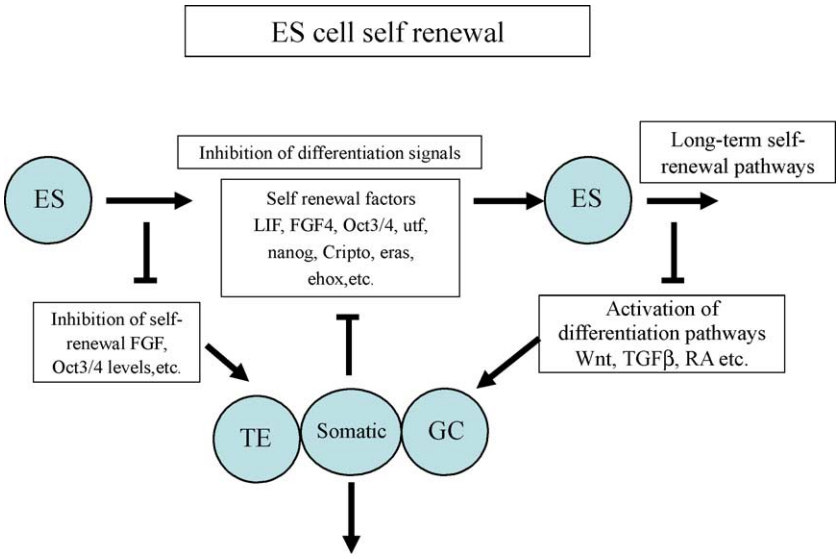

Fig. 2. ES cell self-renewal. A simple model of ES cell self-renewal and its potential regulation is shown. Positive and negative regulators act in concert to determine whether cells will self-renew or differentiate. Factors likely act over short term as well as over long term to maintain the ES cell phenotype.

required to maintain the ES cell state and differentiation is inhibited by the absence of differentiation genes as well as the presence of active repressors. It is likely that long-term self-renewal that is characteristic of ES cells requires additional specialized machinery to regulate genomic stability, epigenetic gene patterns, telomeric ends, and some aspect of a clock to maintain appropriate timing of lineage segregation as well as choosing between symmetric and asymmetric division (for a review, see Sommer and Rao, 2002). Knowledge of some of these pathways is surprisingly detailed while that of others is relatively sparse. Indeed, despite the widespread use of mouse ES cells and the successful isolation of ES cells from a variety of species, relatively little attention has been paid to identifying the regulators of self-renewal or determining why the efficiency of ES cell derivation is so species and strain dependent (Ishiwata et al., 2001; Meng et al., 2003). Nevertheless, much has been learned from examining well-characterized mouse ES cell lines and several factors involved in their self-renewal have been identified. Much of our knowledge has come from mouse ES cell lines (Burdon et al., 2002; Loebel et al., 2003) and more recently by comparison with human ES cell lines (Carpenter et al., 2003; Ginis and Rao, 2003; Richards et al., 2004), and these and other recent results are discussed below.

LIF–BMP–OCT–Sox pathways

LIF, serum, and BMP

The most critical pathways regulating self-renewal in mouse ES cells are those mediated by Oct3/4 and leukemia inhibitory factor (LIF), and a model of their interaction has been proposed by Niwa (2001). LIF, a member of the LIF–oncostatinM–Il-6 superfamily of cytokines, is critical for maintenance of feeder-free ES cell lines. LIF acts by binding to a LIFR–gp130 signaling complex that activates at least two downstream pathways: a Jak–STAT (Janus kinase, signal transducer and activator of transduction) pathway and an ERK–MEK–ras–raf pathway (Fig. 3). Experiments have shown that it is the activation of the Jak–STAT pathway that is critical for ES self-renewal (Niwa et al., 1998). Activation of STAT3 in the absence of LIF is sufficient for prolonged self-renewal (Matsuda et al., 1999) and activation of a modified receptor, which lacks the ability to activate the erk–MEK–ras–raf pathway, is as efficient (better) as the wild-type receptor in maintaining cells in an undifferentiated state (Burdon et al., 1999a; Burdon et al., 1999b). LIF signaling probably requires activation of c-abl and over-expression of bcr–abl allows for LIF-independent self-renewal (Table 1). The activation of the LIF pathway can be modulated by thrombopoietin acting via its receptor c-mpl probably at the level of STAT3 (Xie et al., 2002) and by insulin like growth factor-2 (IGF2) acting via the IGF receptor(s) (Takahashi et al., 1995; Viswanathan et al., 2003). CD9, a cell surface protein that is activated by LIF signaling, is likely important in LIF-mediated self-renewal as well (Oka et al., 2002).

LIF, however, does not act alone and an as yet unknown factor present in serum is required for efficient maintenance of ES cells. This serum factor is likely to be bone morphogenetic protein (BMP) acting via the bone morphogenetic protein receptor1 (BMPR1) and receptor activating SMADs (mothers against dpp related) as shown by recent experiments showing prolonged self-renewal in serum-free medium supplemented with BMP and LIF (Ying et al., 2003; Brandenberger et al., submitted). BMPs likely act via BMPR1a, which is expressed at high levels in ES, cell cultures, and is downregulated when ES cells differentiate

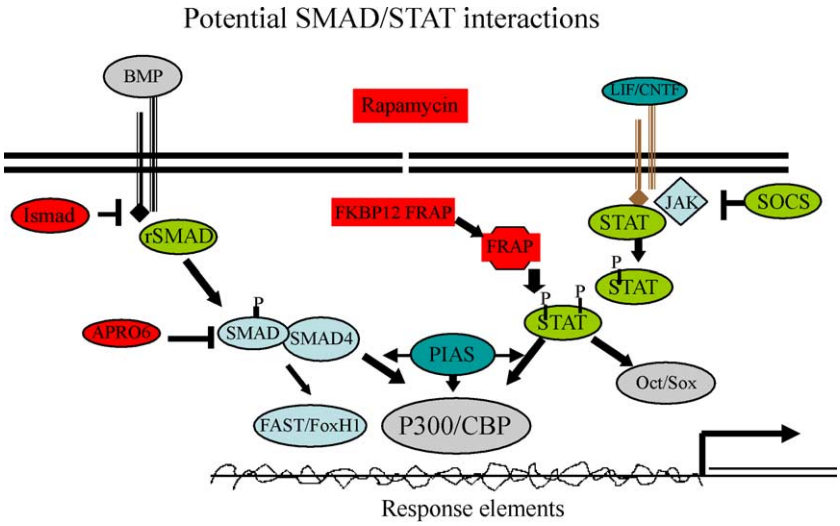

Fig. 3. LIF and BMP interactions. Potential interactions between LIF and BMP signaling pathways are shown. Note that these pathways can interact at multiple levels and suggest several candidate perturbation strategies. Highlighted in red are known modes of interaction that have not shown to be either expressed, active, or important in ES cells.

(Brandenberger et al., 2004b). The effect of BMP on self-renewal is dependent on the presence of LIF. In its absence, BMP is a strong inducer of mesodermal differentiation and an inhibitor of neuronal differentiation. Potential interactions between the LIF and BMP signaling pathways are summarized in Fig. 3. Interactions may occur at several different levels. FKBP12 may mediate interactions at the receptor level. Protein inhibitor of activated signaling (PIAS) may mediate interaction at the cytoplasmic messenger level while SMADS and STATS may interact with CPB ((CRE-binding protein)-binding protein)/p300 to regulate binding to appropriate signaling sites. Downstream modulating signals likely include suppressor of cytokine signaling (SOCS) and inhibitory SMADs (iSMADs), which in turn can regulate ligand signaling through appropriate receptors providing for a feedback regulatory loop. The importance of these interactions has been shown in other cell types including glial differentiation (Bright and Sriram, 1998; Nakashima et al., 1999; Rajan et al., 2003). It is unclear whether such interactions occur in ES cells. Nevertheless, components of each of these pathways as well as interacting molecules have been shown to be elevated in human and mouse cells (Rao, unpublished results).

Oct3/4 signaling

Equally important to maintaining the ES cell state is an octamer motif binding transcription factor Oct3/4. Most ES lines (with the possible exception of chicken cells) express high levels of Oct3/4 and precise levels of this gene are required to maintain the ES cell state and both overexpression and downregulation will alter ES cell fate (reviewed in Niwa, 2001 and references therein). While other members of the Oct family are widely expressed, the expression and binding of Oct3/4 are relatively specific to ES and germ cells, and its levels are regulated at the mRNA level (Donovan, 2001; Nishimoto et al., 2003). The promoter region of Oct3/4 has been well characterized, and proximal and distal enhancers, which are conserved across multiple species, have been identified. The proximal enhancer appears to be required for ES cell expression while the distal enhancer appears critical for germ cell expression (Pesce and Scholer, 2000, 2001). Binding sites for multiple transcription factors including the Sox family of transcriptional regulators have been identified Zhan et al., personal communication), and it has been shown that Oct3/4 is negatively regulated by retinoic acid (RA) signaling and possibly by TRIF (Toll/interleukin-1 receptor (TIR) domain-containing adapter protein) (Fuhrmann et al., 1999). Methylation likely regulates the spatial and temporal expression of this critical regulator of ES cell self-renewal (Hattori et al., 2004). Their results link regulation of the chromatin structure of the Oct-4 gene by DNA methylation status and by extension DNA-N-methyl transferase (DNMTs) (DNMT3β is another ES enriched gene) to regulating ES gene expression and highlight the importance of epigenetic mechanisms (discussed below). It is unclear as to what acts as a positive regulator to maintain appropriate levels of Oct3/4. No conserved Oct-Sox co-binding sites are present, and it is unlikely that Oct regulates its own expression directly. Likewise, no conserved STAT3 binding sites or FGF response element (FRE) or Nanog (Homeodomain) binding sites have been identified. How extracellular signals regulate Oct3/4 expression thus remains unknown. RA directly downregulates Oct3/4 expression and may be important in initiating differentiation.

Oct3/4 itself cooperates with other transcription factors to positively and negatively regulate downstream gene expression (summarized in Fig. 4). Oct3/4 binds to an Octamer motif in the promoter or enhancer regions of many ES cell-

Table 1
Candidate genes that may be important in maintaining ES cells

Gene	Reference	Phenotype
LIF related		
BCR-abl	(Coppo et al., 2003)	Overexpression activates STAT3 and allows LIF independent self-renewal
CD-9	(Oka et al., 2002)	Downstream of LIF activation
Thrombopoietin and c-MPL	(Xie et al., 2002)	Can activate Stat3 and may synergize with LIF
BMP	(Ying et al., 2003)	Cooperates with LIF to sustain self-renewal
Igf2	(Takahashi et al., 1995)	Cooperate with LIF to sustain self-renewal
FGF related		
FGFR1	(Deng et al., 1994; Esner et al., 2002)	Blocks maturation of visceral endoderm and cavitation
FGF	(Chen et al., 2000)	Blockade inhibits embryoid body differentiation
FGFR2	(Arman et al., 1998)	Preimplantation abnormalities
O-linked carbohydrates	(Jirmanova et al., 1999)	Absence reduces cell proliferation
Other pathways		
Nanog	(Mitsui et al., 2003)	ES cell self-renewal independent of Oct and LIF
E-Hox	(Jackson et al., 2002)	Essential for differentiation
PARP-1	(Hemberger et al., 2003)	Biased to trophoblast giant cells
Eras	(Takahashi et al., 2003)	Essential for ES cell proliferation
Dicer	(Bernstein et al., 2003)	Embryonic lethal and failure to generate ES lines
PEM	(Fan et al., 1999)	Overexpression inhibits differentiation
E-cadherin	(Oloumi et al., 2004)	Interacts with Wnt and integrin pathways
Rad51–XRCC2	(Tsuzuki et al., 1996)	Appears critical for ES cell growth
Interferon gamma	(Zou et al., 2000)	Induces cell death
makorin	(Du et al., 2001; Hirotsune et al., 2003)	Downstream of Oct3/4 signaling, expressed pseudogene regulates gene expression
CD98	(Tsumura et al., 2003)	Embryonic lethal when knockouts made
SRp20	(Jumaa et al., 1999)	Mice fail to form blastocysts
β-1 integrin	(Stephens et al., 1995)	Impairs post implantation development
Evx-1	(Spyropoulos and Capecchi, 1994)	Early embryonic lethality
NrOB1–DAX	(Clipsham et al., 2004)	Lethal in ES cells involved in germ cell development
EZH2–eed complex	(O'Carroll et al., 2001)	Failure to implant or derive ES cell lines
GlcNAC-1 phosphotransferase	(Marek et al., 1999)	Preimplantation embryonic lethality
MLL	(Ayton et al., 2001)	Preimplantation lethality in homozygous nulls
Moesin	(Doi et al., 1998)	No phenotype but synctiaum formation abnormal
ESP	(Lee et al., 1996)	ES cell specific phosphatase
MicroRNAs	(Houbaviy et al., 2003)	ES specific MicroRNAs noted
Fbx15	(Tokuzawa et al., 2003)	Knockout has no phenotype
asrij	(Mukhopadhyay et al., 2003)	No phenotype reported
Jumonji	(Toyoda et al., 2000)	High in ES cells and a negative regulator for growth
Hsp90alpha &β	(Voss et al., 2000)	Hsp90β null fail to develop placental labyrinth
Raly	(Michaud et al., 1993)	Preimplantation embryonic lethality
Glut1 and Glut 3	(Saijoh et al., 1996)	Downstream of Oct-3

Molecules that are either highly expressed, required, or affect ES cell or blastocyst development are listed. Note not all known genes are listed, rather a representative subset was selected to illustrate the multiple pathways that are likely critical in regulating ES cell proliferation.

specific genes and regulates their expression, and expression of Oct3/4 is critical to maintaining a self-renewing ES cell. Experiments modulating Oct3/4 levels have shown that precise levels of Oct3/4 expression are required to maintain ES cells in an undifferentiated state. Oct3/4 probably interacts with other transcription factors to regulate downstream genes, and some interactions have been delineated. A cooperative binding mechanism has been proposed where Sox2 and Oct3/4 bind to adjacent domains and regulate expression. Indeed, Sox-Oct sites have been found adjacent to each other in regulatory regions of Sox2, Utf1 (undifferentiated cell transcription factor 1), Rex1–zfp42 (zinc finger protein-42), FGF4 (fibroblast growth factor-4), and so forth (Nishimoto et al., 1999; Tomioka et al., 2002; Yuan et al., 1995; Zeng et al., 2004), and several additional genes that have been shown to be important in ES cell self-renewal or differentiation. In addition to Oct–Sox cooperative binding, interaction between FoxD3 (forkhead family member D3) and Oct has also been reported (Guo et al., 2002; Shivdasani, 2002). How critical is the expression of FoxD3, Rex1, Utf1, and other ES cell-enriched transcription factors in maintaining ES cell self-renewal and whether their role is conserved in human ES cells is unclear. FoxD3, for example, appears to be nonessential, and differing expression has been reported in various human ES cell lines (Ginis et al., 2004; Richards et al., 2004). Likewise, Rex1 is present at high levels in most human ES cell lines but was shown to be absent in HES4 (human ES cell line designated

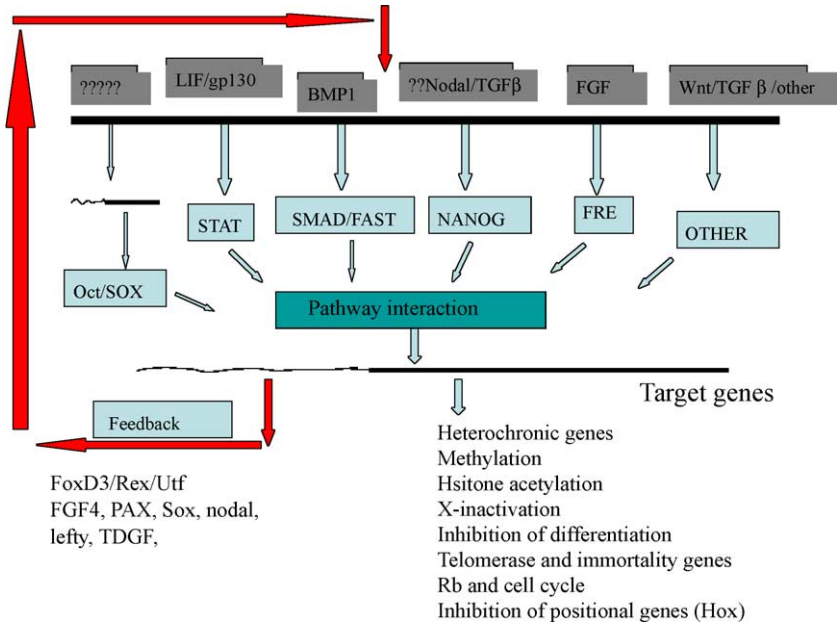

Fig. 4. Summary of pathways. Oct3/4 activation of downstream genes and potential interactions with other pathways regulating ES cell-specific gene expression are shown.

HES4, Richards et al., 2004). F-box containing protein 15 (Fbx15), a gene known to be highly expressed in mouse ES cells and regulated by Oct3/4, appears to be dispensable in knockout mice and is absent or expressed at undetectable levels in human ES cells (Bhattacharya et al., 2004; Tokuzawa et al., 2003). Sox binding sites are present in the Oct3/4 promoter and suggest a potential regulatory interaction as well (Zhan et al., personal communications).

How LIF extracellular regulatory pathways interact with the Oct–Sox pathway is unknown. Many of the genes that are regulated by Oct–Sox signaling also contain STAT binding sites, suggesting that these two pathways could act cooperatively in regulating the expression of ES cell-specific genes at the transcriptional level (Fig. 4). LIF itself does not appear to regulate Oct3/4 and Oct3/4 does not appear to regulate Jak–STAT signaling, suggesting that the Oct3/4 pathway is a parallel pathway for maintaining self-renewal. A possible additional mechanism by which LIF–STAT signaling may interact with Oct3/4 is suggested by a recent report describing Oct1–Stat5 interactions. Magne et al. (2003) have recently shown that Stat5 contains a motif in its carboxyl terminal that is similar to the POU interacting motif on two well known partners of Oct1 (obf-1–Bob and Snap90), suggesting that a physical interaction between these factors stabilizes the binding of Stat5 to its consensus binding motif. A similar motif is present in Stat3, and thrombopoietin, which activates Stat5 (Kirito et al., 2002) and modulates ES cell self-renewal (Xie et al., 2002), can modulate the effect of LIF on ES cell self-renewal.

A potential candidate ribonuclear protein has been identified, originally called Dppa5 (Astigiano et al., 1991; Bierbaum et al., 1994) that was then independently cloned by Tanaka et al. (2002) and named embryo specific gene 1 (ESG1). We have cloned the human homologues of ESG1 and shown that it is highly expressed in human ES cells and is downregulated as ES cells differentiate as much as in mouse ES cells (Ginis et al., 2004; unpublished data). ESG1 appears to be downstream of both LIF and Oct3/4 pathways (Tanaka et al., 2002), and its downregulation as cells differentiate is more rapid than that of Oct3/4 or Nanog, indicating that this gene may be a useful marker of the undifferentiated cell state. How ESG1 integrates Oct3/4 and LIF signaling remains to be determined.

In summary, the data suggest that two parallel pathways exist that are required to maintain mouse ES cells. The LIF–Stat3 pathway is modulated by the BMP pathway likely at multiple levels and interacts with the Oct3/4 pathway to maintain ES cell self-renewal. Oct3/4 levels are precisely maintained and how this is achieved remains unclear. Much is known about downstream interactors of Oct3/4 signaling; however, little is known about upstream regulators and it is unlikely that Oct3/4 regulates its own expression directly. The Oct3/4 pathway appears conserved in most species (with the exception of chicken ES cells), while components of the LIF pathway appear to be redundant in some human ES cells. LIF and Oct pathways appear to converge on common downstream targets that include many ES cell-specific genes and suggest potential autoregulatory circuits.

FGF, Nanog, and other pathways

FGF signaling

Although, LIF is critical to maintaining mouse ES cells in culture, experiments generating LIF receptor-null, gp130-

null embryos have shown that such embryos can develop and ES cell lines can be established. These data provide compelling evidence that factors other than LIF are important in the ICM and that pathways independent of LIF–gp130 signaling may be sufficient to maintain undifferentiated ES cells (Berger and Sturm, 1997; Gendall et al., 1997). Candidate factors that may be important remain elusive although a series of experiments suggest that FGF acting via fibroblast growth factor receptors (FGFRs) may be important. Modulation of FGF signaling as in FGFR1- and FGFR2-null mice or in FGF 2 mutants (Chen et al., 2000; Deng et al., 1994; Esner et al., 2002) alters blastocyst development, cavitation, or differentiation. FGF4 is required for appropriate differentiation, its expression is regulated by Oct3/4, and in the absence of FGF4 endoderm, differentiation is altered (Wilder et al., 1997). Receptor binding of FGFs is modulated by extracellular matrix (ECM) molecules and O-linked carbohydrates on ECM molecules appear to be important at this early stage of differentiation (Jirmanova et al., 1999). More recently, it has been shown that human cells can be maintained with FGF in the absence of LIF (reviewed in Carpenter et al., 2003). This finding is consistent with the possibility that this may be a candidate alternate pathway. Whether FGFs could substitute for LIF in maintaining mouse ES cells or whether some other pathway exists is unclear. FGFRs are present on mouse ES cells and can be activated by external FGF application (see above, and data not shown), and it appears that this question can be rapidly addressed. Known downstream mediators of FGF signaling are summarized in Fig. 5. Two aspects of FGF signaling may be of particular relevance to ES cell self-renewal. Recently, Dr. Ghosh et al. showed that FGF2 facilitates access of the STAT–CBP complex to the GFAP promoter by inducing Lys4 methylation and suppressing Lys9 methylation of histone H3 at the STAT binding site (Song and Ghosh, 2004). This FGF-mediated regulation of chromatin remodeling provides a possible mechanism by which FGF can regulate multiple ES cell-specific genes and permit the self-renewal of ES cells in the absence of LIF. In addition, Haremaki et al. (2003) showed that Xcad3, a gene downstream to FGF activation, may contain an FRE. The authors showed that an FRE was comprised of juxtaposed Ets and TCF–LEF binding sites that served to integrate FGF-mediated signaling with that of other growth factors. In a detailed functional and physical analysis, the authors showed that FGF, BMP, and Wnt signals are integrated on these FREs through positively acting Ets and Sox family transcription factors and negatively acting TCF–LEF family transcription factor(s). Whether these two pathways can be extended to ES cell cultures remains unknown. It is interesting, however, that Ets family transcription factors are expressed at high levels in ES cells (unpublished data) and, recently, Wnt and TGF have been suggested as important in regulating human and mouse ES cell self-renewal.

Nanog and other homeobox proteins

Recently, a factor that may act in parallel to maintain ES cell self-renewal has been identified (Chambers et al., 2003; Mitsui et al., 2003). This homeobox domain-containing protein was initially identified as a gene highly expressed in ES cells (Wang et al., 2003). Two groups subsequently showed that it is required for mouse cell self-renewal called Nanog (after Tir a nanog or land of the ever young). Nanog was shown to be essential for self-renewal of ES cells and overexpression was sufficient to maintain Oct3/4 levels. Nanog acts in concert with LIF but does not modulate the LIF signaling pathway and does not seem to be involved in the BMP regulatory pathway either (Chambers et al., 2003; Mitsui et al., 2003). How Nanog and Oct3/4 signals converge downstream remains to be determined. How Nanog expression is regulated remains unknown, and it is unlikely that LIF or Oct3/4 directly regulates Nanog

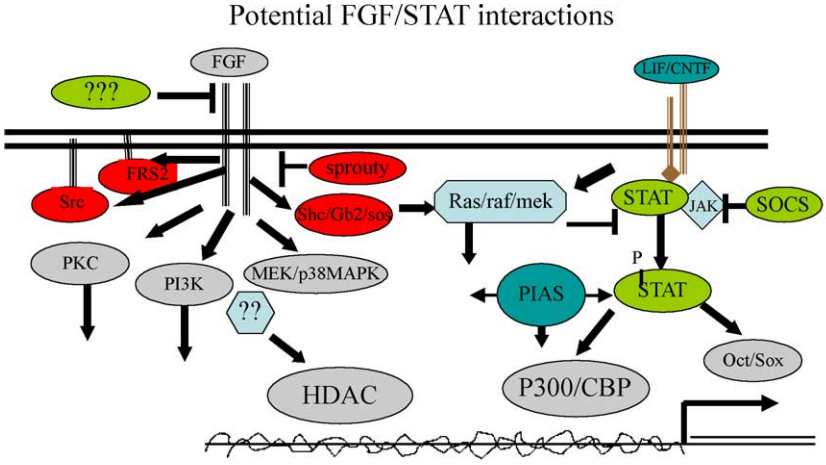

Fig. 5. FGF signaling. Potential downstream signaling pathways are shown. Note potential interactions with STATs, histones, Wnts, and IGF signaling. FGF has been shown to regulate TERT expression as well though this is not shown in this figure. Highlighted in red are genes known to modulate the FGF pathway that have not been shown to our knowledge to be important in ES cells.

expression. Nanog likely acts by transcriptional activation via binding to a homeobox domain in downstream target genes (Pan and Pei, 2003; Zhang et al., 2004). Potential downstream targets include Rex and GATE6 (Chambers et al., 2003). Nanog expression is high in human ES cells as well (Bhattacharya et al., 2004) and is downregulated as the cells differentiate. Zhang et al. (2004) have shown that potential Nanog binding sites exist in several genes that are expressed in ES cells and that TGF β signaling family may be regulated by Nanog. It is interesting to note that another homeobox domain containing protein E-Hox (ES specific homeodomain protein) is important in ES cell differentiation (Jackson et al., 2002). This protein when overexpressed inhibits differentiation and its expression levels appear high in mouse ES cells. No human ortholog of E-Hox exists and the most closely related paralogs are not expressed in human ES cells (Zhang et al., 2004). E-Hox appears, however, to be a member of a large family that includes several genes that are important in early development, and it is possible that the function of E-Hox is performed by another family member. Overall, the data suggest that Nanog represents a relatively independent pathway that is required in both mouse and human cells. Nanog is also expressed by rat ES-like cells, and the gene appears relatively well conserved. Other homeobox proteins are likely important as well, but it is likely that the particular family member expressed will be dependent on the species.

Wnt and TGFβ1 signaling

Recently, Sato et al. showed pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling (see Fig. 6) by a pharmacological GSK-3-specific inhibitor (Glycogen-synthetase Kinase, Sato et al., 2004). The authors found that activation of the Wnt pathway by 6-bromoindirubin-3′-oxime (BIO), a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK-3), maintains the undifferentiated phenotype in both types of ES cells and sustains expression of the pluripotent state-specific transcription factors Oct3/4, Rex1, and Nanog. The authors found that Wnt signaling is endogenously activated in undifferentiated murine ES cells and is downregulated upon differentiation, suggesting that GSK-3 inhibition is maintained by Wnt signaling. These results are supported by earlier results by Shibamoto et al. (2004), which showed that a blockade of Wnt signaling was activated when F9 teratomacarcinoma cells were induced to differentiate. Expressed sequence tags (EST) scan analysis of human ES cells and massively parallel signature sequencing (MPSS) analysis of mouse and human cells suggest that the major components of the Wnt pathway are represented in detectable levels in undifferentiated cell cultures (unpublished results, Bhattacharya et al., 2004; Brandenberger et al., 2004a). A potential additional interaction between Wnt and ECM signaling is suggested by the known effect of E-Cadherin on preventing β-catenin nuclear localization and β-catenin–LEF-1-mediated transactivation (Orsulic et al., 1999). Expression of E-cadherin is seen in the epiblast and appears important in regulating differentiation in several tissue types and is important in regulating the localization and levels of β-catenin (summarized in Fig. 6 and reviewed in Oloumi et al., 2004). Absence of E-cadherin in E-cadherin−/− embryonic stem (ES) cells leads to an accumulation of free β-catenin and its association with LEF-1 (T cell factor–lymphocyte expressed factor), thereby mimicking Wnt signaling.

The Nodal–TDGF1–Cer-1 signaling pathway is involved in ES cell differentiation (Fig. 7), impacting left–right axis formation, neural patterning, and mesoderm development

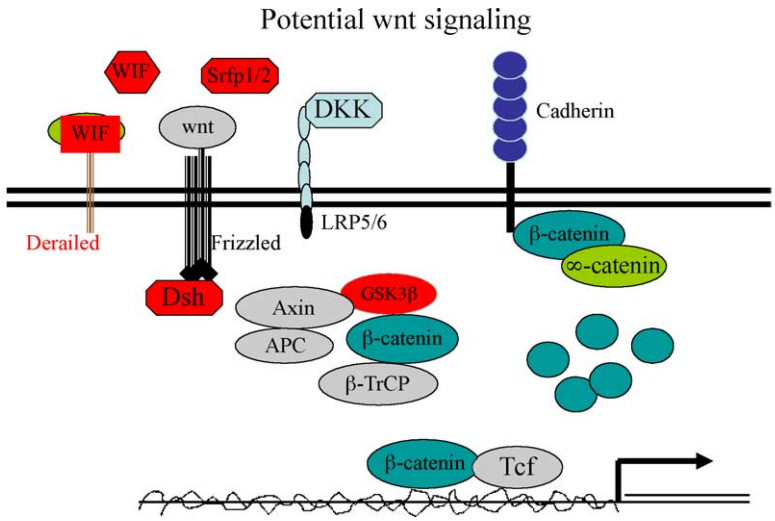

Fig. 6. Wnt signaling. The canonical pathway and its interaction with cadherins are shown. Note that β-catenin performs differing functions at the membrane, cytoplasm, or nucleus, and multiple pathways act to regulate its levels and location. Highlighted in red are genes known to modulate the Wnt pathway that have not been shown to our knowledge to be important in ES cells. In blastocysts, cadherins and their interaction with β-catenin have been shown to be important (see text).

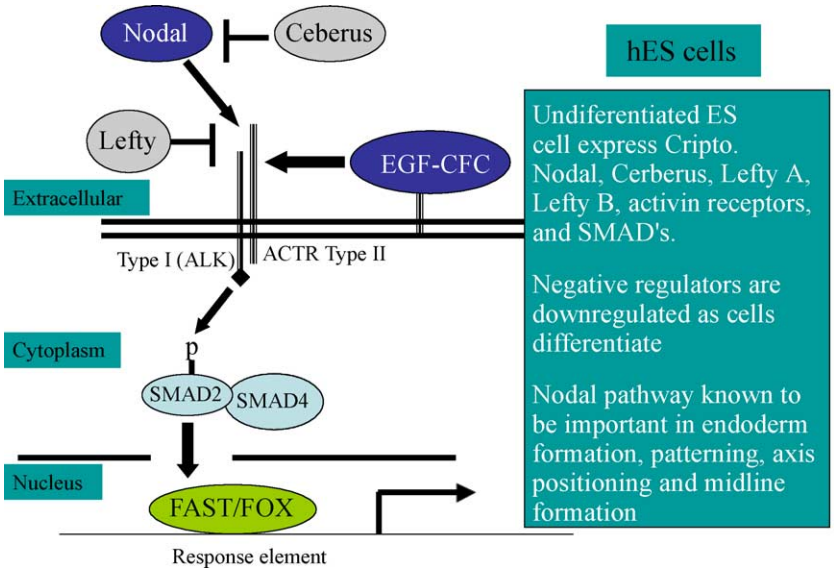

Fig. 7. TGFβ signaling. Potential TGFβ signaling pathways are highlighted. Note the multiple steps at which positive and negative regulators can modulate receptor–SMAD–fox signaling. Members of this extensive family shown in the figure have been shown to be present, active, or important in ES cell or blastocyst development.

(Parisi et al., 2003; Shiner et al., 2003). Nodal, TDGF1 (teratoma derived growth factor 1), and Cer-1 (Cerberus 1) are all expressed in ES cells. TDGF1, an Epidermal growth factor–cryptic family (EGF–CFC) family member, is an obligate coreceptor or a coligand for Nodal (Yan et al., 2002). Amit et al. (2004) in a separate set of experiments used a defined medium, feeder-free, and serum-free condition to assess the role of other factors. Their results suggest that in a defined medium when cells are grown on fibronectin with 15% serum replacement medium, addition of LIF, TGFβ1, and FGF is sufficient to maintain human ES cells over multiple passages in an undifferentiated state. Cells retain the expression of ES cell markers and the ability to form teratomas after implantation in immune compromised mice. These results are consistent with the known expression of molecules in this signaling pathway during early development and in ES cell cultures. Moreover, several modulators of signaling are expressed early as well (see Fig. 7), including Nodal, lefty and TDGF1, Cer-1, and so forth. TDGF1 can alter proliferation of teratomacarcinoma cells (Baldassarre et al., 1997), consistent with the idea that TGF1 signaling may regulate cell proliferation. In addition, genes downstream of TGF superfamily signaling are upregulated in cultured ES cells (Zeng et al., 2004). However, TGFβ1, activin, and other members of the family are thought to promote differentiation and inhibit proliferation as well (Shiner et al., 2003), raising the possibility that the results may be specific to the particular combination of culture conditions used.

Overall, these results suggest that FGF, Wnt, and TGFβ are likely important candidates in regulating ES-cell self-renewal. Wnt likely acts via the canonical pathway and its effect can be modulated by integrins and cadherins, and TCEF–LEF may interact downstream with the FRE providing an additional mechanism of interaction. TGFβ likely signals thru SMADs and Forkhead-related family members. It is important to note that BMP, Wnts, and TGFs have been shown to regulate multiple aspects of ES cell differentiation as well, and it is likely that levels of the cytokines, their interaction with other molecules, and the overall state of the ES cells will determine if cells respond with self-renewal or initiation of differentiation. It will be important as with BMP (see above) to assess the effect in culture with appropriate cofactors that modulate or alter their activity, and these results highlight the importance of developing defined culture conditions to compare results across laboratories.

Other pathways that regulate preimplantation development or ES cell self-renewal

In addition to LIF–FGF–Oct3/4–BMP and Nanog, several other molecules that modulate preimplantation development or prevent derivation of ES cells lines or whose loss causes embryonic lethality have been identified. A list of candidate molecules is shown in Table 1, and some of the pathways that these may reflect are discussed below. These include signaling molecules such as Eras, homeobox domain containing proteins such as evx (even skipped homeobox), and E-Hox, molecules that modulate LIF and FGF pathways as well as metabolic regulators such as cell cycle regulators and so forth. While it is clear that these molecules are important in blastocyst development or ES cell self-renewal, it is unclear how these factors are regulated or interact with known signaling pathways and significant work will be required to delineate interactions between these molecules.

Epigenetic remodeling, HDACs (histone deacetylase), X inactivation

Over the past few years, the importance of heritable epigenetic remodeling has been highlighted in regulating stem cell proliferation, cell fate determination, and carcinogenesis (Beaujean et al., 2004; Huntriss et al., 2004; Meehan, 2003; Ohgane et al., 2004; Vignon et al., 2002). These pathways summarized briefly in Fig. 8 are likely important in early embryonic development as well. HDACs and methyl-CpG-binding protein (MECPs) are expressed in ES cells and their levels are dynamically regulated as cells differentiate (Christodoulou and Weaving, 2003; Young and Zoghbi, 2004; and data not shown). Further, as discussed above, FGF that is critical for maintaining human ES cells may regulate gene expression by histone acetylation. In addition, Hattori et al. (2004) have shown that methylation of CpG islands is important in the expression of Oct3/4. These investigators showed that Oct3/4 activity was undetectable and severely repressed in trophoblastic lineage, including stem cells, and that treatment with 5-aza-2′-deoxycytidine (5-aza-dC) or trichostatin A (HDAC regulators) caused activation of the Oct-4 gene. The authors further showed that in the placenta of Dnmt1 null mutant mice, most of the CpGs in the enhancer–promoter region were unmethylated and Oct-4 gene expression was aberrantly detected and that Oct-4 enhancer–promoter region was hyperacetylated in ES cells compared to TS (trophoectoderm) cells. Finally, in vitro methylation suppressed the Oct-4 enhancer–promoter activity in a reporter assay. This demonstrates that DNA methylation status is closely linked to chromatin structure of the Oct-4 gene. Further evidence of methylation regulating gene expression in ES cells is provided by Liu et al., who showed that the alpha subunit of human chorionic gonadotrophin was silenced by methylation that was directed by direct binding of the Oct3/4 protein to the site responsible for silencing (Liu et al., 1997). Other investigators (Hori et al., 2002) have identified a dyad Oct-binding sequence that functions as a maintenance sequence for the unmethylated state within the H19–Igf2-imprinted control region, providing further evidence of importance of methylation. In ES cells, specific demethylases DNMT3β and DNMT3L appear to be expressed at high levels (Bhattacharya et al., 2004) and are rapidly downregulated upon differentiation, and expression of these genes is critical for nuclear reprogramming (Huntriss et al., 2004). Interestingly, DNMT3L appears to be expressed during oogenesis in mice, while it begins to be expressed only after fertilization in humans. This suggests differential regulation of methylation in these species (Huntriss et al., 2004).

The importance of methylation of CpG islands as well as histones in regulating gene expression during development has been recognized (Chow and Brown, 2003; Sims et al., 2003). Its role in regulating X inactivation and in imprinting (reviewed in Hemberger, 2002; Monk, 2002) and in appropriate development after somatic nuclear transfer (Bortvin et al., 2003; Rideout et al., 2001; Tucker et al., 1996; Wutz and Jaenisch, 2000) has been described. An additional role for Polycomb repressors such as enhancer of zeste homolog and early embryonic ectoderm (eed) that bind to chromatin and regulate gene expression has also been identified (Chadwick and Willard, 2003; Okamoto et al., 2004; Silva et al., 2003). Eed-Enx1 Polycomb group

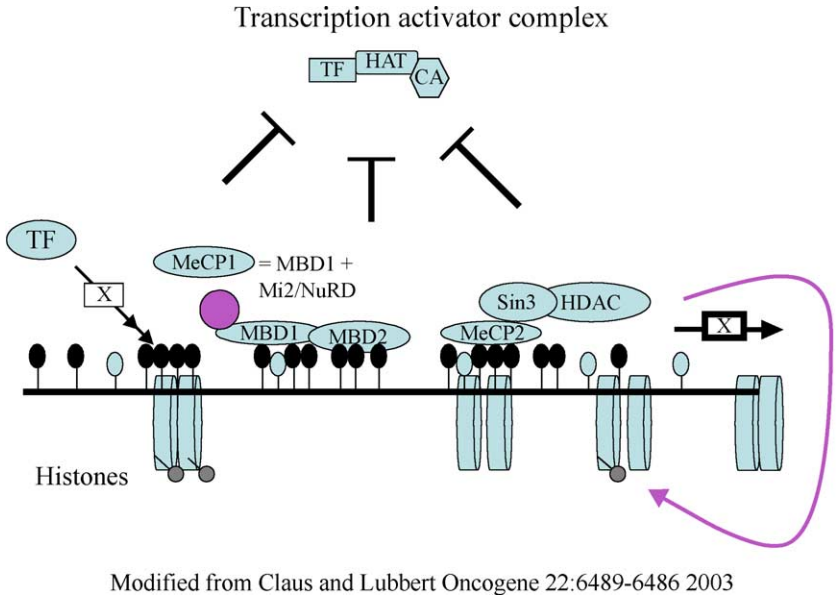

Fig. 8. Epigenetic remodeling. Possible epigenetic pathways to regulate gene expression are schematized. Note that histones can be acetylated, phosphorylated, or methylated. Methylation of genomic DNA at CpG island and modulation of histone interactions with other transcriptional regulators can all serve to regulate cell type-specific gene expression. Genes shown in the figure have been shown to be present, active, or important in ES cell or blastocyst development.

complex is required not only in the maintenance of imprinted X inactivation in the trophectoderm lineage in mouse but also for recruitment of Eed-Enx1 to the inactive X chromosome (Xi) in random X inactivation in the embryo proper. Localization of Eed-Enx1 complexes to Xi occurs very early, at the onset of Xist expression, but then disappears as differentiation and development progress (Silva et al., 2003). Overall, the data suggest that epigenetic remodeling may regulate multiple aspects of stem cell self-renewal, thus providing a mechanism for integrating and coordinating multiple signals directing self-renewal versus differentiation. Comparison between mouse and human suggests that many of these pathways are similar, and genes, which cause embryonic lethality in mouse embryos, are expressed in human ES cells and are downregulated as ES cells differentiate.

Cell cycle Rb and myc, and pathways regulating telomere ends

ES cells possess unique properties in that they appear spontaneously immortal, can be maintained as a karyotypically stable cell in undifferentiated culture conditions for prolonged periods, do not show contact inhibition, express high levels of telomerase, and maintain telomere length, and when transplanted in immune-compromised animals generate teratomas. These properties, which are relatively unique to ES cells, suggest that the pathways regulating long-term self-renewal and transition through the cell cycle may be unique to or modulated differently in ES cells. Several investigators have examined cell cycle protein expression in ES cells (see review by Burdon et al., 2002). ES cells appear to have a short G1-S transition, and Rb (retinoblastoma gene), which is critical in regulating this transition in most dividing cells, appears to be inactive (hyperphosphorylated or present at low levels). Other Rb-related proteins (p107 and p130) appear to be low or absent as well (unpublished results). Further, mouse ES cells appear refractory to growth inhibition by $P16^{ink4a}$, triple KO ES cells where all three Rb family genes are knocked out show normal growth rates, and DNA damage does not lead to arrest at G1 as is typical of all other dividing cells. In addition, it appears unlikely that the ras–raf–erk pathway that transduces cytokine proliferation signals to cell cycle machinery plays an important role in regulating ES cell division (Jirmanova et al., 2002). Potential regulatory pathways that have been suggested to regulate ES cell proliferation include a novel ras family member Eras whose overexpression is sufficient to maintain cell proliferation and whose expression is limited to ES cells (Takahashi et al., 2003), a PI3K (phosphoinositide-3 kinase)-mediated regulation of cyclins (Pacold et al., 2000; Sun et al., 1999), and myc activation (Davis and Bradley, 1993; MacLean-Hunter et al., 1994) that appears to regulate proliferation. Myc activation rather than the ras–raf–erk pathway may be critical in linking cytokine signaling to the cell cycle as myc overexpression can render cells cytokine independent (Shirogane et al., 1999; S. Dalton, personal communication), and myc expression and activation can be detected in human ES cells. Myc-null ES cell lines can, however, be generated (Davis and Bradley, 1993; MacLean-Hunter et al., 1994), suggesting that compensatory pathways exist. Data on human ES cells are still incomplete, but preliminary expression data by MPSS and EST scan analysis (Brandenberger et al., 2004a,b; Ginis et al., 2004) suggest that as in mouse cells the Rb pathway is relatively unimportant while the PI3K and myc pathways are active. No perturbation experiments, however, have been performed to our knowledge.

Given the ability of ES cell to self-renew for prolonged periods, one would predict that telomerase activity will be present and tightly regulated. Mouse ES cells, however, can be obtained from telomerase reverse transcriptase (TERT) KO mice, and these cells can be maintained in culture. Blastocysts from TERT KO mice appear to implant and develop normally for several generations (reviewed in Cheong et al., 2003). This ability may be unique to mice, which have unusually long telomeres and may not be generalized to other species. Indeed, human ES cells express all the major components of the TERT pathway and levels of some of these components are dynamically regulated as cells differentiate. TERT assays have shown that TERT levels are downregulated as cells differentiate but maintained if cells are kept in non-differentiating conditions over at least 2 years (Carpenter et al., 2004; Rosler et al., 2004). Perturbation experiments knocking down TERT activity in human cells remain to be performed.

In summary, we would suggest that the cell cycle is regulated differently in ES cells, and this differential regulation appears to include many critical aspects of cell cycle control including Rb, p53, ras, myc, and the DNA repair machinery. Many of these unique aspects are shared between mouse and human ES cells; however, some differences exist (Ginis et al., 2004). These differences include the pathway by which Rb is regulated, how telomerase activity is regulated, and how the cell cycle pathway is coupled to growth factor signaling (see above).

MicroRNAs and antisense regulation

MicroRNAs are small noncoding RNA genes found in most eukaryotic genomes and are involved in the post-transcriptional regulation of gene expression. The microRNAs are transcribed in the cell nucleus where they are processed into pre-microRNAs. Further processing occurs in the cytoplasm, where the pre-microRNAs are cleaved into their final approximately 22-nucleotide-long form summarized in Fig. 9. MicroRNAs appear to be processed by Dicer, and double-stranded RNAs appear to regulate gene expression via transcriptional, translational, or protein degradation regulation (Bartel, 2004; Szymanski and Barciszewski, 2003). Recent reports have identified global

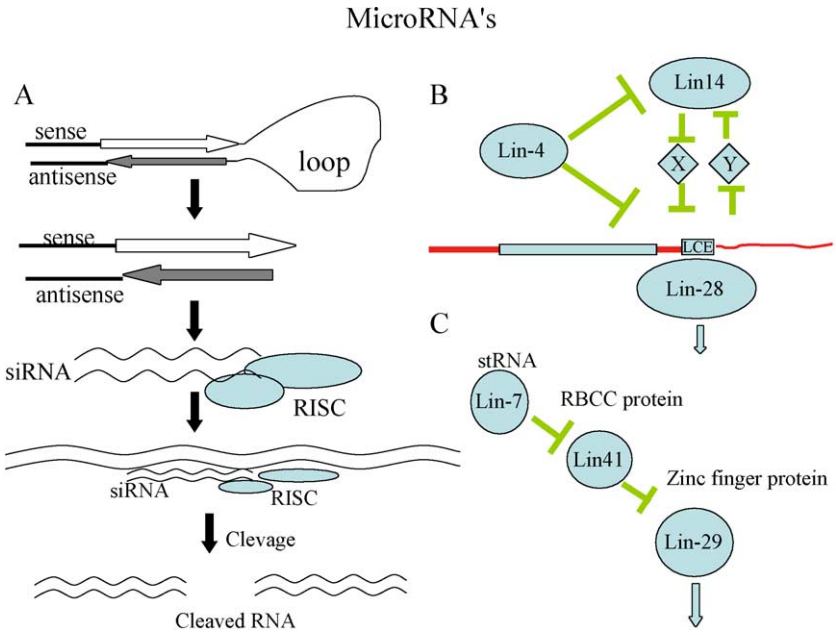

Fig. 9. MicroRNAs. Processing of microRNA is summarized in Panel A. Components shown are present in mouse and human ES cells. Panels B and C summarize possible pathways of heterochronic gene regulation as identified in *C. elegans*. Lin 41, Lin 28, and binding domains for Let-7 and Lin-4 have been identified in mouse and human ES cells.

strategies for identifying microRNAs and over 250 such untranslated RNAs have been identified (see for example Houbaviy et al., 2003; Lewis et al., 2003; Rajewsky and Socci, 2004) and in particular showed that several microRNAs were ES cell specific and were downregulated as cells differentiate or absent in differentiated tissue. Other expression data have suggested that these microRNAs are dynamically expressed at specific stages and may play an important role in cell fate and differentiation in multiple species (Chen et al., 2004). Knockouts of Dicer show embryonic lethality at early stages of development, and it has not been possible to derive ES cell lines from Dicer-null embryos (Bernstein et al., 2003), further confirming the importance of this pathway. Functionally, at least two microRNAs play a role in regulating timing of development in *C. elegans*, lin-4 and let-7, both of which have been identified as being expressed in ES cells (Zhan et al., personal communication). In *C. elegans*, these microRNAs bind to conserved sequences in the untranslated region (UTR) of genes involved in regulating the appropriate timing of differentiation (Banerjee and Slack, 2002). This heterochronic pathway includes Lin-28 and Lin-41 (Johnson et al., 2003b; Moss and Tang, 2003), and recent microarray and serial analysis of gene expression (SAGE) analysis has suggested that the human homologues of these genes are highly expressed in undifferentiated ES cells (Bhattacharya et al., 2004; Brandenberger et al., 2004b; Richards et al., 2004). Examination of their three prime UTR shows that Lin-4 and Let-7 sites are evolutionarily conserved as well (Zhan et al., personal communication), suggesting that this pathway may be important in regulating ES cell self-renewal and differentiation.

Some recent results suggest that antisense RNA may also regulate gene expression and ES cell self-renewal. Perhaps the clearest example comes from the expression of TSIX, which is antisense to XIST, and makorin, which is an Oct3/4-regulated gene that is antisense to RAF1 (serine–threonine protein kinase). Both TSIX and makorin 2 are expressed at early stages of development and at relatively high levels in ES cells. Alteration of XIST and makorin alters development (Chow and Brown, 2003; Gray et al., 2001; Hirotsune et al., 2003). In addition to makorin2 being antisense to RAF1, a phylogenetically conserved genomic orientation (Gray et al., 2001), a processed pseudogene of makorin has also been identified (Hirotsune et al., 2003). Knockdown of either makorin or its pseudogene affects development and dedifferentiation, and the phenotype can be rescued by either gene, suggesting that RNA stability is being regulated by makorin. Given that makorin and its antisense partner RAF1 are both expressed in human and mouse ES cells and that makorin was earlier identified as a downstream target of Oct3/4 (Du et al., 2001), it suggests that antisense regulation of gene expression may be an important aspect of maintenance of the stem cell state and that other such examples may exist. While it is clear that many components of these pathways are shared between mouse and human ES cells, relatively little is known and further comparisons await additional data.

Conserved and divergent pathways and additional unknown pathways

The ability to compare ES cells from human and mice has allowed one to assess conserved pathways likely to be

important in ES cell self-renewal. A list of potential conserved pathways is listed in Table 2. Nanog, Oct3/4, Sox, Rex, Utf, TERT, connexin, Glut-1, Dicer, DNMT, and so forth all appear to be conserved and are likely to be important in ES cell self-renewal. Interestingly, the heterochronic gene regulatory pathway appears to be highly conserved as well. Assessing BMPR, Wnt, TGFβ, and other pathways suggests that the effect of BMPR1 postulated in mES cells is likely true in human ES cell cultures as well. The pathway appears conserved, as does downstream signaling via SMADS. In contrast, the Wnt and TGFβ signaling pathways do not appear to be conserved in the same manner. Wnts appear to affect differentiation in mouse cell, as does TGFβ, while these molecules appear to be important in maintaining primate ES cell self-renewal (see above). Nevertheless, the conservation of key pathways suggests a core ES cell identity that is conserved across species. This core identity would include Oct, Sox, Rex, Utf, TERT, and so forth and will be clearly distinct from genes expressed in other "adult–non-ES" stem cell populations. We suggest that additional conserved pathways probably remain to be identified. Note that several development and pluripotency associated genes (Dppa) were identified based on early embryonic expression, and recent work has confirmed the importance of some of these genes including Dppa3/stella and Dppa5/ESG1. Nanog was identified based on a digital differential display strategy that identified 15 other genes that are enriched or specific to ES cells. Many of these are also expressed in human ES cells (Bhattacharya et al., 2004), confirming that additional pathways exist and remain to be characterized. A detailed cross species comparison is likely to provide important insights.

Many but not all of these pathways are likely conserved between species. Differences in gene expression and regulation have been reported though the number and extent of these differences remain unknown as no comprehensive cross species comparisons have been undertaken. Some of these comparisons are technically difficult currently as neither markers, microarrays, nor genomic data sets are available at a resolution that would allow such a comparison. However, several differences reported have been in

Table 2
Conserved pathways of self-renewal in mice and humans

Conserved signaling pathways	
Stat3 signaling	Evolutionarily conserved gene, STAT binding sites present in multiple ES cell specific genes
Nanog	Shown to be critical in both mouse and human, rat gene not yet cloned
Oct-Sox	Conserved genes and cobinding sites conserved, Oct3/4 not present in chick
BMPR1a	Cooperates with LIF to sustain self-renewal, function conserved in human and mouse
TGFβ signaling	Cripto, nodal, lefty appear to show similar patterns of expression and function though some differences exist
Igf2–H19	Highly expressed in both mice and human ES cells
FGF signaling	Critical for blastocyst development in mice and required for human ES cell. Differences in which FGF used likely
MicroRNAs	Dicer required for blastocyst development, expressed in human ES cells, and heterochronic gene expression (Lin-28) conserved
Methylation–X Inactivation	Polycomb genes (EZH2–eed complex, Xist, TSIX, DNMT3β and DNMT3-like show conserved patterns of expression
Cell cycle	While differences exist both human and mouse ES cells show distinct Rb regulation when compared to other cell types
Others	DNA repair machinery, telomerase biology, some aspects of cell death, and several novel gene pathways (Dppa2, 4 and so forth) are likely conserved.

A list of genes or pathways known to be important in mouse and human ES cells is summarized. Data on the importance of these pathways in human ES cells and is largely based on high levels of expression in multiple lines and downregulation as cells differentiate.

Table 3
Some known differences between mice and human ES cells

Divergent signaling pathways	
LIFR–gp130	LIFR gene regulatory domains not well conserved, expression divergent in mouse and human
Eras and EHox	Conserved in mouse and rat no functional ortholog in humans
Fox-D3	Required in mice but expression variable in human cell lines
Rex1	Variable expression in human ES cell lines
FGF signaling	FGF2 appears high in human ES cells while FGF4 is high in mouse ES cells. SNP in FGF4 alters Sox-Oct binding in three prime UTR of hFGF4.
Lefty A	No orthologue in mice or rats identified
SSEA antigen	Differential expression seen
Tert signaling and aging associated genes	Several components of this pathway are differentially expressed
Fbx15	Does not appear to be expressed in human ES cells
Cell cycle and cell death	Ubiquitination seems important in regulating human Rb levels while mdm2 appears more important in mouse. Likewise pattern of caspases and other cell death genes expressed are quite distinct
Expression of trophoblast markers	Mouse ES cells do not differentiate into trophoblast while human cells appear to do so and express early trophoblast markers in maintenance conditions
Claudin 6	Shows a reverse pattern of expression. High in ES and low in EB in human
Decorin	Differentially expressed in mouse and human ES cells
NROB1	steroid (NROB1–DAX) axis appears divergent
Others	MPSS analysis show very low concordance in gene expression suggesting multiple additional differences exist

Pathways known to be important in mouse ES cells that are either not critical or appear to not be evolutionarily conserved are listed.

genes important for self-renewal and survival of mouse ES cells. For example, Oct3/4 homologues likely do not exist in chicken embryos (Soodeen-Karamath and Gibbins, 2001), while LIF signaling that is critical for ES cell self-renewal in mice does not appear to be critical or even required for human ES cells (see for example Ginis et al., 2004). No paralogs of E-Hox have been identified in humans, and Eras appears to be a pseudogene in humans (Bhattacharya et al., 2004). A list of known differences is summarized in Table 3. As can be seen, this include genes that have become redundant during evolution, factors that have been recruited to different functions, and gene expression patterns that have been flipped. It is useful to remember that this was not entirely unexpected given known differences in hematopoietic and neural stem cell populations across species (Ginis and Rao, 2003). However, the number of differences reported from limited comparisons was quite surprising. It suggests to us that additional mechanisms driving change, perhaps evolutionary pressure for speciation, may underlie the larger than expected difference observed. In any case, however, it is clear that the self-renewal state of ES cells can be achieved by multiple independent pathways and different species likely use overlapping but distinct strategies for self-renewal. Inter-species comparisons therefore will continue to be illuminating. The low overall concordance rate between human and mouse ES cells (in one comparison was around 40%) relative to that seen in human-to-human cell comparisons (90% between human ES cell samples) provides additional support for this hypothesis (Wei et al., personal communication). Given that approximately 25% of all genes identified as being expressed in human cells were genes of unknown function (Brandenberger et al., 2004b), it is likely that an additional large number of differentially expressed genes exist of which a significant number will be functionally important but divergent.

Summary

Progress in our understanding of ES cell biology has provided crucial insights into the mechanisms of self-renewal and differentiation. Perhaps the most important insights have been the determination that species-specific pathways to ES cell self-renewal exist and that additional pathways remain to be discovered. We expect that large-scale genomic comparisons between species and across stages of differentiation will provide a better understanding of the interaction between known and unknown pathways and the role of heterochronic genes, microRNAs, antisense, polycomb repressors, genes involved in telomere length regulation, and other poorly characterized modulators of self-renewal. Further detailed analysis of conserved and divergent pathways may suggest strategies to isolate ES cell lines from difficult to isolate species and provide information on how to monitor cells in culture. These observations highlight both the importance as well as the caveats of extrapolating across species and emphasize the remarkable divergence in the expression of genes that are critical to maintaining the ES cell state.

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