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EMBRYONIC STEM CELLS: CONCISE REVIEW

Lefty at the Crossroads of "Stemness" and Differentiative Events

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ABSTRACT

Stem cells are functionally defined by their ability to self-renew and generate a progeny capable of creation or reconstitution of various tissues. Microarray analysis has shown a member of the transforming growth factor (TGF)-β superfamily, *Lefty*, to be the single most abundant inhibitor in stem cells and in maternal decidua that supports embryo implantation. *Lefty* is regulated by pathways such as Smad (Sma and Mad [mothers against decapentaplegic]) and WNT (wingless-type) and by the transcriptional factor *Oct3/4* (octamer-binding transcription factor 3/4), which support "stemness." *Lefty* is also induced upon exit from the state of stemness, including forced in vitro differentiation, and leukemia inhibitory factor withdrawal. *Lefty* is a candidate in cell-fate decisions because of its unique ability to modulate the expression of TGF-β family proteins such as Nodal and by blanket inhibition of the activity of members of this family which require EGF-CFC (epidermal growth factor-Cripto, Frl-1, and Cryptic) as a coreceptor. STEM CELLS *2006;24:1998–2006*

INTRODUCTION

Self-renewal of unicellular organisms is achieved by cell division. Multicellular organisms are also derived from single cells that carry the potential of self-renewal, but at the same time these cells give rise to a progeny that differentiates into various cell lineages. Among cells in multicellular organisms, the ovum is totipotent and after fertilization is capable of giving rise to all tissues, including reproductive germ cells. This totipotency is maintained for one or two cell divisions and is narrowed in the pluripotent daughter cells that generate all three germ-layer derivatives. Although such cells still retain their self-renewal capacity, they become restricted in their differentiation potential and cannot make an entire embryo. With further development, these cells gradually differentiate further and make progenitor cells that give rise to differentiated somatic cells in tissues.

The ability of adult amphibians to regenerate an entire limb clearly shows that tissues in the adult organs can be fully repaired, and their function can be restored. It is believed that this restoration takes place by a group of unipotent or multipotent stem cells that, after development, remain within specific niches in the adult tissues. These cells replenish tissues that have outlived their natural lifespan and are subject to rapid cell turnover requiring repetitive cell renewal such as those in blood, skin, hair, colon, and endometrium. However, a similar repair can also take place during pathologic conditions such as after hepatectomy. For this reason, there is great hope that adult tissues can be repaired after injury or a disease that leads to the loss or aberrant function of somatic cells. Stem cells are, therefore, of great interest because of their potential use in tissue replacement therapy. Full insight into the molecular repertoire that stem cells use to maintain stemness or to differentiate into diverse tissues may ultimately allow reprogramming of adult somatic cells for use in regenerative medicine.

Our understanding of the transcriptional programming and the molecular mechanisms that underlie the stem cell self-renewal and differentiation has not reached the point of making the vision of "making tissues on demand" a reality. However, recent progress in identifying the molecular signature of embryonic stem cells (ESCs) has created the opportunity to delineate the pathways used by stem cells in maintenance of "stemness" and in cell-fate decisions.

Molecular Signature of Stem Cells Is Comprised of Transforming Growth Factor-β Proteins, Including *Lefty*

There is great interest in identifying differential molecular determinants and activities that allow distinction among totipotent, pluripotent, and terminally differentiated cells. Recent efforts

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have been invested in identifying the signature of stemness by the constellation of genes that stem cells express. DNA microarray has unveiled a plethora of genes that are thought to be the molecular signature of mouse ESCs (mESCs) and human ESCs (hESCs) [1]. The transcriptional profiling of stem cells and early embryos has identified signature genes such as those that participate in transcriptional control, RNA binding, signal transduction, and proteolysis [1].

Recently, the transcriptional profile of mESCs was compared with those expressed in hematopoietic and neuronal stem cells [2, 3]. These analyses show a cluster of genes that are common to both groups and are thought to be hallmark of stemness [2, 3]. However, comparison of the expression profiles reported for all these stem cells shows only one gene, integrin α-6, to be common to all three sets [4]. The inability to identify a common set of genes expressed in all stem cells might be related to several factors such as lack of representation of genes on the arrays used, use of a different set of genes by different cells to achieve the goal of self-renewal and pluripotency, transient expression of genes, or the method used for comparing the data. It is likely that, because of its importance, stemness is supported by multiple or redundant pathways that impart robustness to this vital cell characteristic.

Recently, global transcription profiles of undifferentiated hESCs and blastocyst have been reported by several groups [5–9]. Notably, among the highly enriched genes are octamer-binding transcription factor 4 (*Oct4*), receptors of the transforming growth factor (TGF)-β family, and the ligands of this family, including *Lefty A* and *B*, *Nodal*, and teratocarcinoma-derived growth factor-1 (*TDGF-1)/Cripto* [5, 8, 9]. Besides *Lefty*, other inhibitors of the TGF-β family such as *TMEFF* (tomoregulin-1), *Follistatin-like*, and *Ceberus*, although present, are not enriched [5]. *Lefty* is also highly expressed both in the inner cell mass and trophoectoderm [8].

In a recent study, Wei et al. compared the transcriptional profile of both mESC and hESC lines, and their results were validated by reverse transcription-polymerase chain reaction (RT-PCR) analysis [10]. The findings show that hESCs and murine ESCs differ significantly from each other on a global scale. The differences between murine and human transcript levels range from one- or twofold to more than 50-fold, suggesting that different cells might use different genes to achieve stemness. Even genes known to be important for ESC self-renewal, such as *Oct4*, vary severalfold. Such a difference has been validated for the leukemia inhibitory factor (LIF)/Stat3 (signal transducer and activator of transcription 3) pathway, which is sufficient to maintain stemness in mESCs but not hESCs [11, 12]. Wei et al. attribute the nonresponsiveness of hESCs to the lack of the LIF receptor and the lower level of Stat3 in hESCs. The difference between hESCs and mESCs is also noted in culture conditions required to maintain their stemness in vitro. Whereas basic fibroblast growth factor (FGF)-2 is used for propagation of hESCs in vitro, culture medium of mESCs is not supplemented with any of the known FGFs [11]. The components of signaling of the two pathways, wingless-type (WNT) and Smad (Sma and Mad [mothers against decapentaplegic]), also show differences in mouse and human stem cell sets. Although RT-PCR confirms the presence of most of the components of WNT and TGF-β in hESCs, they are found at a lower level in mESCs. Most members of the canonical WNT pathway and the transcription factors Smad1, -3, -5, and -8 are present in human and absent in mouse stem cells. However, members of the WNT pathway, including frizzled 2 (*FZD2*), *FZD8*, lipoprotein receptor-related protein 5 (*LRP5*), dsh homolog 1 (*DVL1*) and *DVL2*, glycogen synthase kinase-*3-*β (*GSK-3-*β), *Axin1*, and *CTNNB1* [catenin (cadherin-associated protein) β] and the Smad pathway, including *ACTVR2* [(activin receptor 2)], *Smad4*, and *Smad7* are present in both hESCs and mESCs. Such evidence suggests that human cells are better poised to respond to the WNT or Smad pathway than are the mouse cells.

Despite the differences in transcriptome complexity, the distribution of some signature sequences, based on their abundance, has been found to be strikingly similar in mESCs and hESCs. Genes such as *Oct4*, *Lefty*, *Nodal*, *Sox-2* (sex-determining region Y-box 2), *Utf-1* (undifferentiated embryonic cell transcription factor-1), tert (telomerase reverse transcriptase), and *TDGF-1/Cripto* all are highly enriched in both human and murine stem cell lines [10]. Based on the large number of differences and the small number of similarities, it can be reasoned that these genes represent components of the critical core pathways used by all stem cells.

Although *Lefty* expression is now believed to be a marker of stemness, this expression is not quenched upon differentiation. In mESCs, *LIF/Stat3* is important in the maintenance of stemness [13–15]. By binding to gp130 (signal transducing β-chain of the interleukin 6 receptor), LIF activates Jak (Janus tyrosine kinase)-mediated Stat3 phosphorylation and gene expression. When LIF is withdrawn, however, the expression of *Lefty* increases within 48 hours of cytokine withdrawal [16]. Similarly, the retinoic acid that induces differentiation leads to increased expression of *Lefty* in mouse embryonal carcinoma cells [17]. Differentiation of stem cells to embryoid bodies also leads to increased expression of *Lefty* in vitro [9]. Therefore, *Lefty* might be important both to the stemness and differentiation events that follow the exit from this state.

Two important issues are identifying factors that are supplied by feeder cells in vitro that keep the stem cells in an undifferentiated state and maintain a high level of expression of *Nodal*, *Lefty* A, and *Lefty B*. Likely candidates are those that induce either the WNT or Smad2/3 activation [12]. Consistent with this, it was recently shown that pluripotency can be maintained in hESCs in vitro in the absence of feeder layer or conditioned medium with a combination of Activin or Nodal supplemented with FGF-2 [18]. SB431542, which inhibits activin-linked kinase or activin receptor-like kinase (Alk) 4/5/7, leads to differentiation of stem cells. However, surprisingly, inhibition of Nodal by *Lefty* or Cerberus does not set up a similar differentiation process [18]. *Lefty* protein used at a high dose (200 ng/ml) also fails to induce differentiation of human embryonic cells grown over a feeder layer. *Lefty* is a known inhibitor of Nodal, so it is not clear why in this setting, Lefty cannot inhibit an observed effect of Nodal [19]. Possible explanations are that cells might be sensitive to the dose of Lefty, Lefty might be labile in vitro, or a special condition is required for responsiveness to Lefty.

Lefty Is a Member of the TGF-β Superfamily

The TGF-β superfamily of ligands is comprised of approximately 40 related proteins in humans [20]. Enrichment of TGF-β family members in stem cells suggests that these

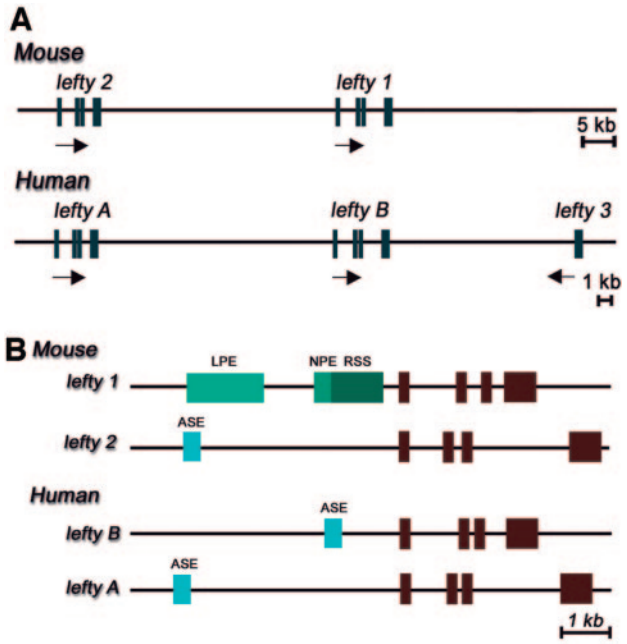

Figure 1. **(A):** *Lefty* loci in mouse and humans. Mouse *Lefty* locus encompasses two genes, *Lefty* 1 and *Lefty 2*. In humans, there are also two genes, *Lefty* A (ebaf) and *Lefty B*. A third gene, *Lefty* 3, is thought to be a pseudogene. The four *Lefty* exons are shown as black boxes. Arrows show the transcriptional direction. **(B):** Transcriptional regulatory elements of mouse and human *Lefty* genes as a basis for correlating the mouse and human genes. The four *Lefty* exons are shown as black boxes. Abbreviations: ASE, left side-specific enhancer; ebaf, endometrial bleeding associated factor; kb, kilobase; LPE, lateral plate-specific enhancer; NPE, neural plate-specific enhancer; RSS, right side-specific silencer ([21–24]).

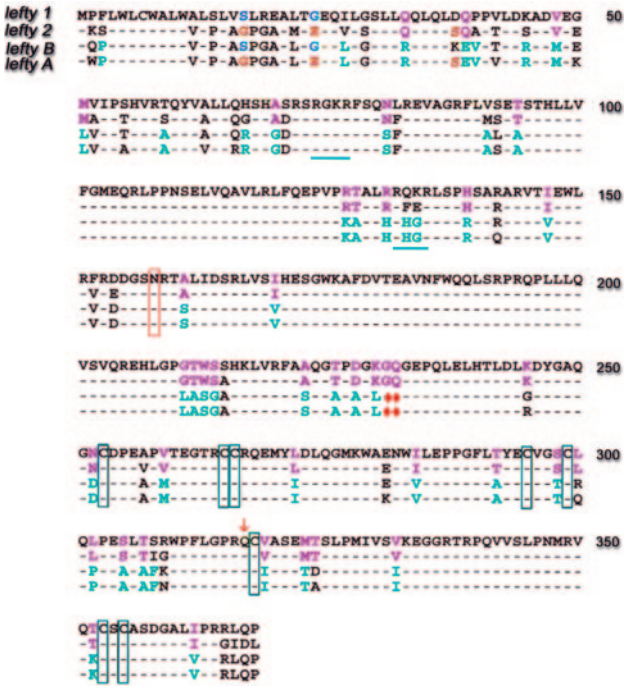

Figure 2. Predicted amino acid sequences of human and mouse Lefty proteins. Residues that are identical in all four proteins are replaced by dashes. Blue and pink show residues specific to human and mouse proteins, respectively. Residues specific to human and mouse Lefty 1 and Lefty 2 are shown in orange and blue, respectively. Two amino acid residues that are present in mouse Lefty proteins and absent in human proteins are shown by red diamonds. The *N*-glyocosylation site is marked by a red box. Proteolytic cleavage sites are underlined. Conserved cysteine residues, typical of members of the transforming growth factor-β superfamily, are boxed in blue. Lefty proteins lack the cysteine residue (aa^{317}) required for dimer formation (down arrow). Residues are numbered according to the sequence of Lefty 1 [24, 27–30].

proteins are part of critical pathways used by stem cells. Among these, *Lefty* is the only inhibitor that is highly enriched in stem cells.

In humans, mice, and zebrafish, the *Lefty* locus contains two genes with the same transcriptional orientation. Yashiro et al. refer to the genes at the 5′-side of the locus as *Lefty 2*, and based on the distribution of enhancer elements in these genes, human Lefty 1 is identical to Lefty B and Lefty 2 is identical to human Lefty A or ebaf (endometrial bleeding associated factor) [21–27] (Fig. 1). However, there is no tendency for Lefty B to be more related to Lefty 1 than to Lefty 2. Human Lefty proteins are more closely related to each other than to their mouse counterparts. For example, Lefty B has 96% sequence identity and shares 46 amino acids with Lefty A. These two proteins differ only in 16 amino acids (mostly in the pre- and pro-regions), whereas Lefty B has only 82% identity with Lefty 1 and has only two amino acids that are conserved in Lefty 1. There are only three amino acids that are conserved in Lefty A and Lefty 2 (Fig. 2, [24, 27–30]). Thus, based on these structural features, it has been suggested that Lefty proteins evolved independently in mouse and humans from duplication of a single *Lefty* gene [27].

Human *Lefty* locus contains a third gene, *Lefty 3*. *Lefty 3* has been proposed to be a pseudogene because of its reverse transcriptional orientation, presence of a single exon (exon 4), interruption by Alu repeats, and absence of human expressed sequence tag clones [27] (Fig. 1). *Lefty* genes have been localized to chromosome 1 (1q42.1) both in mice and humans [24, 28].

Multiple Pathways Regulate *Lefty* in Stem Cells

Lefty is regulated by transcriptional regulator *Oct3/4* and signaling is regulated by WNT and Smad2/3 pathways. *Oct3/4* is critical for the activity of LIF in the maintenance of self-renewal in mESCs [31]. *Oct3/4* regulates the cell-fate decisions in pluripotent and germline cells and is essential in the generation of the founder pluripotent cells in mammalian embryos [32–34]. Although gene expression is generally considered to be a binary on-off system for controlling cell function, Niwa et al. found that the varying levels of *Oct3/4* lead to three distinct fates in mouse embryonic cells [35]. A less than twofold increase in *Oct3/4* expression leads to differentiation of stem cells into primitive endoderm and mesoderm. This event is associated with increased expression of orthodenticle 1 (Otx1) and, to a lesser extent, *Lefty* A (Fig. 3) [36, 37]. On the other hand, repression of *Oct3/4*, which leads to loss of pluripotency and trophoblast differentiation, causes decreased expression of Otx1 and *Lefty* A but to increased expression of Hand1 and Cdx2 (caudal type homeo box 2) [38, 39].

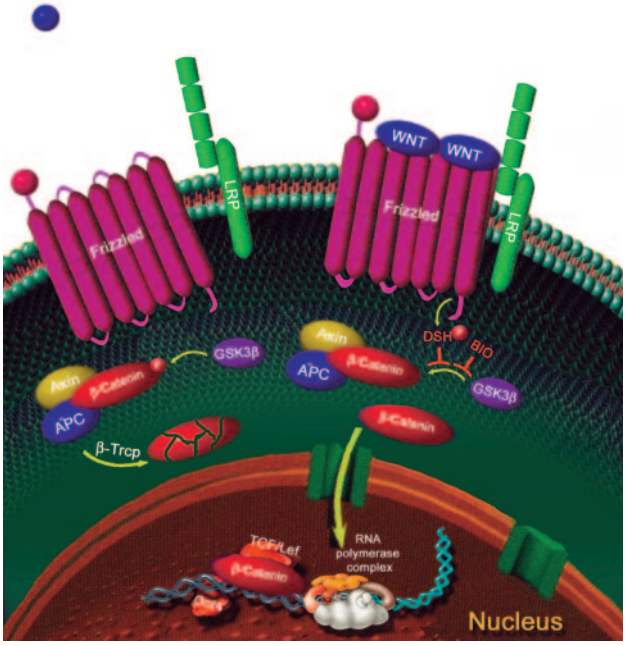

Figure 3. Induction of *Lefty* by WNT pathway and *Oct3/4*. *Lefty* is induced by *Oct3/4* and by WNT pathway. In the absence of an active WNT signaling, the cytoplasmic multifunctional protein, β-catenin, is anchored to the cell membrane where it participates in E-cadherin-mediated intercellular adhesion by adherens junctions. The cytoplasmic pool of β-catenin is controlled by a destruction complex comprised of two scaffolding proteins, APC and axin, which bind freshly translated β-catenin. After this binding, casein kinase-1 phosphorylates β-catenin at Ser^{45} at its N terminus. After this initial phosphorylation, GSK3β induces additional phosphorylations distal to Ser^{45} at conserved Ser and Thr residues. The phosphorylated β-catenin is ubiquitylated by the F-box-containing E3 ligase, β-TrCP, and is subsequently subjected to proteasomal degradation. In noncanonical WNT signaling, some WNT proteins bind to FZD receptors in the absence of LRP coreceptors and initiate planar cell polarity pathway. Canonical signaling is initiated by binding of WNT proteins to a complex comprised of a member of the FZD family of the seven-span transmembrane serpentine receptors and a single-span receptor of the low-density LRP family. Engagement of FZD/LRP by WNTs blocks the enzymatic activity of the destruction complex by an unknown mechanism. An adaptor protein, DSH, which is essential for signal transduction, is bound to the cytoplasmic tail of FZD. The function of the DSH is not quite clear, but it might stabilize axin binding to LRP/FZD, participate in the phosphorylation of LRP, or directly or indirectly downregulate activity of the destruction complex or of GSK3β activity. Regardless of the mechanism(s) involved, WNT signaling or inhibition of GSK3β activity by BIO leads to accumulation of unbound β-catenin in the cytoplasm which enters the nucleus. In the nucleus, β-catenin binds to the N terminus of members of the TCF/Lef family of transcription factors (TCF1, Lef-1, TCF3, or TCF4) and forms active transcriptional units due to the potent transactivational domain at the C terminus of β-catenin. By binding to their recognition elements in promoters and enhancers of WNT-responsive genes, these complexes initiate gene transcriptional activity [36]. Abbreviations: β-TrCP, β-transducin repeat containing protein; APC, adenomatous polyposis coli; BIO, 6-bromoindirubin-3′-oxime; DSH, Dishevelled; FZD, Frizzled; GSK3β, glycogen synthase kinase-3-β; Lef, late expression factor; LRP, lipoprotein receptor-related protein; *Oct3/4*, octamer-binding transcription factor 3/4; TCF, T-cell-specific factor; WNT, wingless-type.

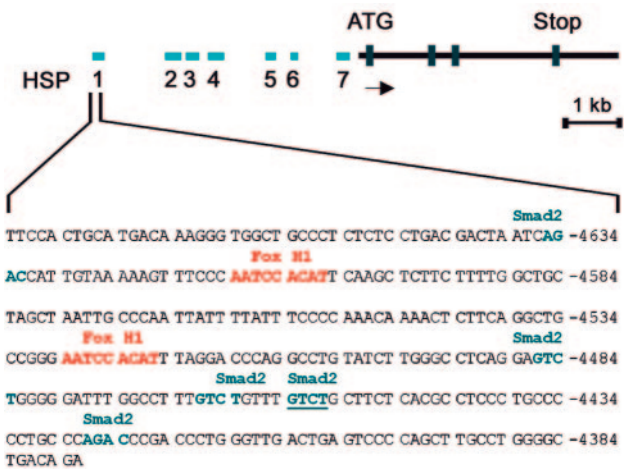

Figure 4. Organization of the Lefty A locus and localization of Fox H1 and potential Smad (Sma and Mad [mothers against decapentaplegic]) binding sequences in *Lefty* promoter. There are seven homology sequence pairs (HSPs) (shown in blue) upstream from transcription start site (arrow) in the *Lefty* A locus. *Lefty* A is translated from four exons (shown in black) from the translation start site (ATG). The sequence of HSP1 (−4684 to −4377) upstream from transcription start site includes two Fox H1 (Fast1) binding sites (shown in red). Potential Smad (2/3) binding sites are shown in blue. The underlined Smad binding site is shared in mouse and human [21, 22, 27, 42].

Little is known of upstream signals that regulate *Oct3/4*. Recently, it was shown that canonical WNT signaling can positively regulate expression of this key molecule both in human and mouse cells [12]. The activation of WNT pathway is initiated by binding of the WNT protein to the Frizzled receptor at the cell surface. This leads to inactivation of GSK-3-β that normally keeps β-catenin in a phosphorylated state anchored to the cell membrane (Fig. 3). The released β-catenin accumulates in the nucleus, where, in collaboration with TCF/Lef (T-cell-specific factor/late expression factor), it activates the transcription of WNT target genes [40, 41]. WNT signaling can also be activated by direct, intracellular inhibition of GSK-3-β function by specific inhibitors such as 6-bromoindirubin-3′-oxime BIO [37]. By using this inhibitor, it was demonstrated that pluripotency can be maintained both in mESCs and hESCs [12]. In addition to maintenance of pluripotentcy, BIO also leads to the high expression of Nodal, *Lefty A*, and *Lefty B* in hESCs, suggesting that *Lefty* is inducible by WNT signaling [42]. Consistent with this, analysis of genes that are downstream of nuclear localization of β-catenin in early embryos included *Lefty* as well as protocadherin, *cadherin II*, *Eph* (ephrin), *CCND1* (Cyclin D1), *netrin*, and *frizzled3/6* [43]. All these genes are expressed in vegetal blastomeres between the 16- and 110-cell stages. Thus, these genes might be important in the first step of embryonic cell specification and axis formation after nuclear localization of β-catenin. Induction of expression of *Lefty* by BIO has been shown to require ALKs 4/5/7, which are receptors that convey the messages of the TGF-β family members through Smads [42] (Fig. 4). BIO as well as lithium chloride, another inhibitor of WNT pathway, activate both WNT and Smad signaling [43].

The promoter regions of human *Lefty A* and mouse *Lefty 2* contain conserved regulatory sequences comprised of seven homology sequence pairs (HSPs). In the most upstream of first homology sequence, two FoxH1 (Fast1) binding sites and five potential binding sites for the Smad2/4 heterodimer exist [23,

42] (Fig. 4). These binding sites, which are recognized by their respective transcription factors, are required for in vivo expression of *Lefty A* in the embryonic node [23]. Consistent with these findings, treatment of differentiating hESCs with Activin A, which causes activation of Smad2/3, leads to the expression of Nodal, *Lefty A*, and *Lefty B*. Inhibition of ALK4/5/7 by the kinase inhibitor, SB431542, which blocks activation of Smad2/3, downregulates the expression of *Lefty A* and *B* in the undifferentiated stem cells [42]. The activity of *Lefty A* is downregulated in differentiated ESCs as compared with undifferentiated cells and is found to be responsive to activation of Smad2/3 and to BIO [42]. Together, these findings show that, in undifferentiated stem cells, expression of *Lefty* is conveyed by ALK4/5/7- and Smad2/3-mediated pathway. In these cells, bone morphogenetic factor (BMP) signaling, which leads to Smad1/5/8 activation via ALK2/3/6 receptors, is blocked and gets activated when cells start to differentiate [42].

Biologically Active Forms of *Lefty* Require Cleavage by Convertases

There is evidence that biologically active forms of *Lefty* proteins require processing of their precursors by members of the convertase family of enzymes. Most TGF-β proteins are produced in precursor form that are cleaved to release the C terminus monomeric active proteins. This cleavage occurs at RXXR motifs by subtilisin-like proprotein convertase (SPC) [44]. The proform of TGF-β is cleaved intracellularly by the endopeptidase furin, of the convertase family of molecules, at a single RXXR site. This enzymatic digestion releases a 12.5-kD mature C terminus protein and a 75-kD N terminus prosegment (latency-associated peptide [LAP]). Lefty is also cleaved by convertases. The mutation of both of the potential consensus sequences for protein convertase (PC) cleavage in *Lefty* protein allowed these sites to be recognized as authentic cleavage sites (Fig. 2). The mutation of the sequence RGKR to GGKG (aa 74–77) and of RHGR to GHGR (aa 132–135) prevents the proteolytic processing of *Lefty* precursor to two processed forms of 34 kD and 28 kD, respectively [29]. Thus, in contrast to TGF-β, *Lefty* has two RXXR cleavage sites, which lead to the secretion of two cleaved products of 28- and 34-kD proteins [27, 29].

Lefty 1 precursor protein is cleaved by SPC1, SPC4, SPC6A, and SPC6B at both sites, whereas Lefty 2 precursor is cleaved to its long cleaved product by SPC1 and SPC6A but is not cleaved by any of the SPCs tested [45]. As compared with other PCs tested, SPC6A(PC5A) has been found to be most efficient in cleavage of Lefty A at the RGKR cleavage site [29, 46]. It is speculated that cleavage at RFER site of the Lefty 2 precursor may require an unknown SPC that is expressed only in certain cell types or that this site may not be cleaved at all in vivo [45].

There might be a distinct difference(s) between the large and small forms of *Lefty* with respect to their biological activity, their diffusion potential, stability, or proteolytic cleavage. However, at least in one bioassay, both the small and large processed forms of *Lefty* proteins inhibit Nodal signaling to the same extent [45].

Lefty Mechanism of Action

By acting as a dimer, TGF-β proteins bring together two transmembrane serine/threonine kinases, the type I and II receptors. The assembly and oligomerization of these receptors lead to phosphorylation of receptor-regulated Smads (R-Smad) and release them from their docking site on the receptor and allow them to heterodimerize with a common Smad, Smad4. These complexes accumulate in the nucleus, where they interact with other transcription factors, bind to DNA, and activate transcription of TGF-β-responsive genes [20].

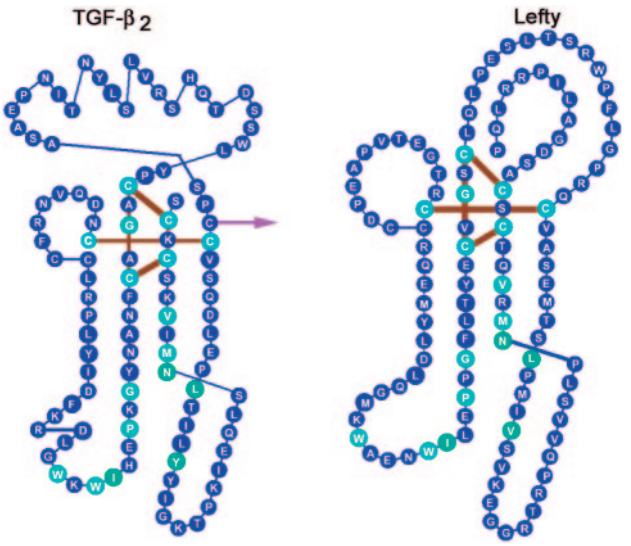

Figure 5. Comparison of secondary structure of transforming growth factor (TGF)-β 2 and Lefty. Shared amino acid residues are shown in light blue, and conserved substitutions are green. Red lines mark the intrapeptide bonds. The cysteine residue used in TGF-β for dimerization is shown by arrow (adapted and reproduced with permission from [47]).

The TGF superfamily of ligands signal through two main pathways. The BMP branch activates activin-linked kinases (ALKs) (TGF type I receptors 2/3/6) and leads to the phosphorylation and activation of the transcription factors Smad1/5/8. The second branch, Activin/Nodal, involves the activation of the type I receptors, ALK4/5/7, and subsequent phosphorylation and activation of R-Smad2/3 [23]. Nodal uses ALK4 and either ActRIIA or ActRIIB as type I and type II receptors, respectively [45].

TGF-β family members have a characteristic signature motif that is comprised of a series of seven cysteine residues at their C-termini (Fig. 5). All of these residues are used for the formation of intrapeptide bonds with the exception of the third cysteine residue from the C terminus of mature protein. Active dimerized TGF-β ligands are formed by the formation of disulfide-linked bonds between the fourth cysteine residues at the C terminus of the protein [47–49] (Fig. 5). Lefty, its *Xenopus* homolog, *antivin*, GDF-3/Vgr2 (growth and differentiation factor), and GDF-9, all lack the cysteine residue necessary for the formation of intermolecular disulfide bond [24, 28, 47–53] (Figs. 2 and 5). Therefore, Lefty appears to belong to a subgroup of the TGF-β superfamily with an unpaired cysteine residue that does not exist as a dimer and for this reason is poised to be an inhibitor.

Lefty-induced inhibition can be rescued by excess ActRIIA or ActRIIB, suggesting that Lefty also antagonizes Nodal signaling through competitive binding to the common receptor, ActRIIA or ActRIIB [45]. However, unlike Activin, Lefty does not directly interact with the extracellular domain of ActRII. Such interaction has been proposed to require a cofactor that

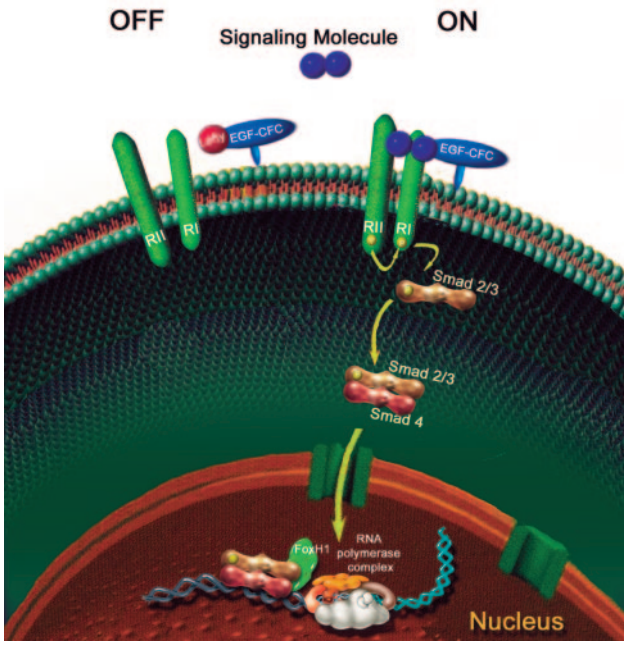

Figure 6. Lefty inhibits Smad signaling by TGF-β family members requiring EGF-CFC as a coreceptor. By virtue of binding to EGF-CFC, Lefty inhibits gene transcriptional activity induced by Smad pathway, including expression of Nodal and possibly Lefty itself. In the absence of Lefty, dimerized signaling molecules such as Nodal, Vg1/GDF1, and GDF3 bind type II receptor (RII) and a type I receptor (RI [ALK 4/5/7]) in conjunction with the coreceptor, EGF-CFC protein. This leads to cross-phosphorylation of RI by RII and subsequently to phosphorylation of a receptor-regulated SMAD (Smad2/3), allowing this protein to associate with Smad4. This complex moves into nucleus, associates with a DNA-binding partner (Fox H1), binds to specific enhancers in targets genes, and activates their transcription [45, 50–57]. Abbreviations: EGF-CFC, epidermal growth factor-Cripto, Frl-1, and Cryptic; Smad, Sma and Mad (mothers against decapentaplegic); TGF, transforming growth factor.

does not appear to be endoglin or betaglycan [45]. This cofactor has been recently recognized to be epidermal growth factor-Cripto, Frl-1, and Cryptic (EGF-CFC) proteins. These proteins are extracellular GPI (glycosylphosphatidyl-inositol)-linked factors that share a conserved cysteine-rich domain (CFC motif). These include one-eyed pinhead (*Oep*) in zebrafish and mammalian *Cripto*, *Frl-1*, and *Cryptic* [52, 53]. Lefty inhibits the signaling of proteins such as Nodal, Vg1/GDF1, and GDF3, which require EGF-CFC proteins for efficient signaling [45, 54] (Fig. 6). It has been suggested that Cripto mediates its effect by virtue of activating mitogen-activated protein kinase (MAPK) pathways independently of the TGF-β signals and Activin receptors [54–56].

Role of *Lefty* in Embryogenesis and Pluripotency

The importance of TGF signaling in the earliest cell-fate decisions during embryogenesis is now well recognized. Nodal induces mesoderm and endoderm, patterns the nervous system, and specifies left-right (L-R) asymmetry in vertebrates [50]. Lefty 1 and Lefty 2 both block Nodal signaling by binding Nodal and its EGF-CFC coreceptors such as TDGF-1/Cripto. By virtue of these bindings, lefties prevent the assembly of an active Nodal/Activin receptor complex [45, 50, 52, 57] (Fig. 6). These findings show that lefties serve as regulatory molecules for Nodal. Consistent with these findings, loss of function of Nodal prevents mesoderm formation [58], whereas excessive mesoderm is formed and the primitive streak is expanded in *Lefty 2*-deficient mice [19]. Consistent with inhibition of Nodal, in *Lefty* 1-deficient mice, there are abnormalities in the L-R axis formation [59]. Moreover, the *Lefty 2* mutant phenotype is partially suppressed by heterozygosity for *Nodal* or by overexpression of the *Nodal*-related genes, *Cyclops* and *Squint* or the extracellular domain of ActRIIB [19]. Nodal and both lefties are under the control of Activin/Nodal and Smad2/3 signaling in the embryonic node [60]. It is possible that Nodal and lefties establish a signaling network via Smad2/3 activation, driving correct induction of mesoderm, endoderm, and nervous system. Within such a network, expression of Nodal via the activation of Smad2/3 induces its own expression, whereas both lefties inhibit the Nodal and possibly lefties.

Although obvious defects in the inner cell mass are not observed in mice deficient for *Lefty 1*, *Lefty 2*, or Nodal [58], recent findings support the viewpoint that TGF-β signaling is required for the maintenance of pluripotency [18, 61]. Lack of obvious defects in the inner cell mass in *Lefty*-deficient mice is likely due to significant homology in Lefty proteins, establishing a redundancy in their function.

Lefty-Induced Differentiation Versus Re-Rerouting of Differentiation

Currently, only limited information supports the role of Lefty in differentiation events. Increased expression of Lefty 1 or 2 in animal cap explants of *Xenopus laevis* leads to neuralization as evidenced by expression of neural markers, including *NCAM* (neural cell adhesion molecule) [30]. Whereas the animal caps of the uninjected embryos remain as a mass of atypical ectodermal cells, the animal caps of the injected caps develop visible cement glands. Elongation, a hallmark of mesoderm formation, is absent, but there is evidence of neuralization in the animal caps. Using human *Lefty* A RNA, we have obtained results that are in line with these published reports (S. Tabibzadeh and H. Brivanlou, unpublished data).

Lefty More Than a Nodal Inhibitor

Inhibition of *Lefty* by morpholino oligonucleotides during gastrulation has produced results in *Xenopus*, which are unexpected from mere inhibition of *Nodal* by *Lefty* [62]. Increased *Nodal* leads to severe hyperdorsalization or twining. Thus, it is expected that inhibition of *Lefty* should lead to a phenotype similar to that induced by increased Nodal signaling. However, inhibition of *Lefty* leads to exogastrulation. In this case, the ectodermal and endodermal tissues, which normally reside within the embryo, lie externally. In addition, in these embryos, both *Nodal* (Xenopus nodal-related 2 [*Xnr2*], *Gsc* [Goosecoid], *Cer*, *Xbra* [*Xenopus* brachyury]) and WNT responsive organizer (*Gsc*, *Xnr3*) gene expression are altered and appear to be extended from their primary site of expression. *Lefty* overexpression, on the other hand, inhibits the expression of *Nodal* and WNT-responsive genes in mesoderm. Together, these findings show that *Lefty* is more than a simple Nodal inhibitor and that it is required for normal patterning by the organizer and in normal gastrulation.

Development of L-R Asymmetric Territories by *Lefty*

Embryogenesis in vertebrates includes development of numerous L-R asymmetries that involve major organs such as cardiovascular system, spleen, liver, and gastrointestinal tract. L-R polarity is determined early during development, at approximately the presomite stage in mammals [63–65]. Creation of such an asymmetry might include asymmetric distribution of cytoskeletal components [66], extracellular matrix [67], and morphogens. Leftys and Nodal are among the morphogenic factors that are asymmetrically expressed [28, 68]. In the mouse, *Lefty 1* is expressed predominantly in the prospective floor plate, whereas *Lefty 2* is expressed in the lateral plate mesoderm. Lack of *Lefty 1* results in bilateral expression of Nodal, *Lefty 2*, and *Pitx2*, a homeobox gene that is normally expressed in the left side [59]. These observations suggest that the role of *Lefty 1* is to restrict the expression of *Lefty 2* and Nodal to the left side and that *Lefty 2* or Nodal are signals for "leftness". The behaviors of green fluorescent protein (GFP)-Nodal and GFP-*Lefty 2* proteins have been investigated in chick embryos. Both proteins can diffuse over a long distance, but Nodal diffuses faster than *Lefty* [45]. Such findings suggest that differentiation by Nodal takes place outside the territory set by *Lefty* expression.

Because *Lefty* is induced by WNT pathway and by β-catenin, an intriguing possibility is that *Lefty* is involved in the development of polarization during early embryogenesis. Microarray data validated by Northern blot analysis have shown *Lefty* to be highly enriched in decidua that supports embryo implantation [69, 70]. During implantation, *Lefty* is increased and peaks on day 5 of pregnancy in mouse uterine horn [71, 72]. PC5, which cleaves Lefty to its biologically active form, is also coordinately increased in decidua during implantation and in cells decidualized in vitro [72–75]. Although ovum also expresses PC5, this expression is lost at the two-cell stage [76]. This suggests that Lefty is not processed in the developing embryo during early embryogenesis. However, during implantation, the embryo is exposed asymmetrically to the decidual *Lefty*, which is processed by PC5. For this reason, it is tempting to speculate that decidual *Lefty* is involved in the embryogenesis and in the asymmetric development of placenta.

CONCLUSION

WNT and Smad pathways have recently been shown as being able to support stemness. In addition, the critical role of *Oct3/4*, which participates in pluripotency and in cell-fate decisions by stem cells, is gradually becoming clear. *Lefty* has emerged as a common denominator of these apparently diverging pathways and transcriptional requirement of stemness. Moreover, transcriptional profiling has shown *Lefty* to be the most abundant inhibitor both in mESCs and hESCs. *Lefty* is induced upon exit from the state of stemness, including forced in vitro differentiation. Together, these findings support the view that *Lefty* resides at the crossroads of stemness and "differentiative events." This is likely because of the unique ability of *Lefty* to reroute the cell fate by making cells nonresponsive to diverse differentiation factors such as Nodal or other signals that require EGF-CFC as a coreceptor.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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