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Concise Review: Pursuing Self-Renewal and Pluripotency with the Stem Cell Factor Nanog

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ABSTRACT

Pluripotent embryonic stem cells and induced pluripotent stem cells hold great promise for future use in tissue replacement therapies due to their ability to self-renew indefinitely and to differentiate into all adult cell types. Harnessing this therapeutic potential efficiently requires a much deeper understanding of the molecular processes at work within the pluripotency network. The transcription factors Nanog, Oct4, and Sox2 reside at the core of this network, where they interact and regulate their own expression as well as that of numerous other pluripotency factors. Of these core factors, Nanog is critical for blocking the differentiation of pluripotent cells, and more importantly, for establishing the pluripotent ground state during somatic cell reprogramming. Both mouse and human Nanog are able to form dimers in vivo, allowing them to preferentially interact with certain factors and perform unique functions. Recent studies have identified an evolutionary functional conservation among vertebrate Nanog orthologs from chick, zebrafish, and the axolotl salamander, adding an additional layer of complexity to Nanog function. Here, we present a detailed overview of published work focusing on Nanog structure, function, dimerization, and regulation at the genetic and post-translational levels with regard to the establishment and maintenance of pluripotency. The full spectrum of Nanog function in pluripotent stem cells and in cancer is only beginning to be revealed. We therefore use this evidence to advocate for more comprehensive analysis of Nanog in the context of disease, development, and regeneration. STEM CELLS *2013;31:1227–1236*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the preimplantation mammalian embryo and can be maintained indefinitely in culture [1, 2]. Along with their unlimited capacity for self-renewal in vitro, ESCs are also defined by their ability to give rise to all somatic and germ cell lineages of the developing embryo, with the exception of extra-embryonic tissues. Mouse ESCs harvested at embryonic day 3.5 (E3.5) from the naïve epiblast exhibit "ground state" pluripotency and require specific culture conditions for maintenance [3, 4]. The cytokines leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) have been shown to be sufficient for ESC self-renewal in the undifferentiated state in mouse embryonic fibroblast- and serum-free conditions, respectively [5–8]. LIF promotes self-renewal by activating the JAK/STAT3 and PI3K/AKT signaling pathways, and BMP4 upregulates transcription of inhibitor of differentiation (*Id*) genes through activation of SMAD (Small Body Size / Mothers Against Decapentaplegic) proteins 1, 5, and 8 (Fig. 1A). Maintenance of pluripotency in ESCs is governed by the expression of the core transcription factors Nanog, Oct4, and Sox2 as well as a variety of other factors. Nanog, Oct4, and Sox2 have been shown to repress the expression of developmental genes while modulating their own expression levels by binding to each other's promoter regions [1, 2, 9, 10]. ESCs can give rise to all three germ layers of the developing embryo [3, 4, 11, 12], including the primitive germ cells [5-8, 13, 14]. However, human ESCs exhibit gene expression profiles that are much more akin to mouse epiblast stem cells (mEpiSCs) derived at the postimplantation stage [5, 15, 16]. This "primed" state is a characteristic feature of human ESCs and is also what defines the epiblast at the post-implantation stage [3]. Unlike mouse (m)ESCs, human (h)ESCs (and mEpiSCs) do not require LIF or BMP4 for survival, but instead require basic fibroblast growth factor (bFGF) and insulin or insulin-like growth factor (IGF) signaling [17–19] (Fig. 1B). bFGF activates the mitogen-activated protein kinase (MAPK) as well as the Activin/Nodal signaling pathways, and IGF activates the Ras and PI3K pathways. In mouse ESCs (mESCs), LIF signaling upregulates *Klf4* and *Tbx3* via the JAK/STAT3 and PI3K/AKT pathways, which then go on to activate *Sox2* and *Nanog*, respectively [20]. In hESCs and mEpiSCs, on the other hand, SMADs 2 and 3 propagate Activin/Nodal signaling as well as directly bind and upregulate *NANOG* [21]. Taken together, these studies accentuate the elaborate and interconnected relationship between extrinsic survival signals and the transcriptional program in pluripotent stem cells. In this

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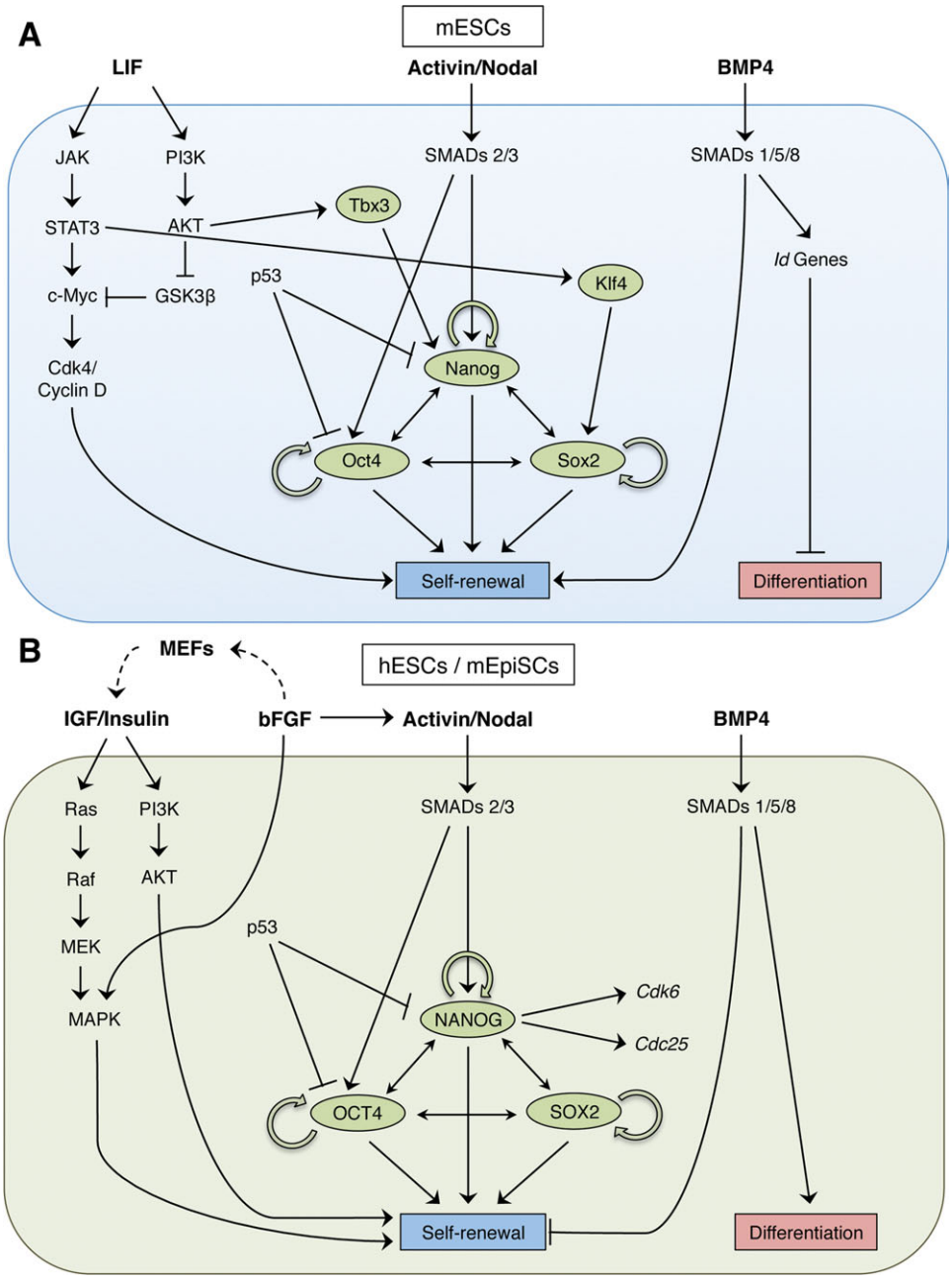

Figure 1. Mouse and human ESC survival pathways. **(A)**: mESCs require LIF and BMP4 for maintenance. **(B)**: Human ESCs and mouse EpiSCs require IGF/insulin and bFGF for maintenance. Human ESC-derived fibroblast-like cells and MEFs are also stimulated by bFGF in culture to secrete IGF (dashed arrows). In both cell types, Nanog, Oct4, and Sox2 form a positive autoregulatory loop. Abbreviations: bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; LIF, leukemia inhibitory factor; mESCs, mouse embryonic stem cells; MEF, mouse embryonic fibroblast; mEpiSCs, mouse epiblast stem cells.

review, we focus on the core transcription factor Nanog and present a broad range of evidence supporting its unique role in regulating pluripotency.

Genetic and Proteomic Features of *Nanog*

Nanog Pseudogenes and Isoforms

Upon analysis of the *NANOG* gene in the human genome, eleven pseudogenes were identified aside from the two *NANOG* alleles (Table 1). Among these, ten are retropseudogenes and one is an expressed tandem duplicate [22]. The ten pseudogenes were named *NANOGP2* to *NANOGP11*, and the duplication pseudogene *NANOGP1* (or *NANOG2*). The same group also uncovered two processed pseudogenes in the mouse genome, which they named *NanogPa* and *NanogPb*. Subsequently, Ian Chambers' group described two novel retrotransposed copies of murine Nanog, named *NanogPc* and *NanogPd* [24]. The differences between these two and the previously analyzed pseudogenes reside not only in their chromosomal locations but also in the fact that *NanogPc* and *NanogPd* open reading frames are 98% identical to *Nanog* and are potentially capable of expressing protein products

Table 1. Summary of Nanog pseudogenes and isoforms

Species	Name	Type	References
H. sapiens	*NANOGP1 (NANOG2)*	Tandem duplicate	[22]
H. sapiens	*NANOGP2*	Retropseudogene	[22]
H. sapiens	*NANOGP3*	Retropseudogene	[22]
H. sapiens	*NANOGP4*	Retropseudogene	[22]
H. sapiens	*NANOGP5*	Retropseudogene	[22]
H. sapiens	*NANOGP6*	Retropseudogene	[22]
H. sapiens	*NANOGP7*	Retropseudogene	[22]
H. sapiens	*NANOGP8*	Retropseudogene	[22, 23]
H. sapiens	*NANOGP9*	Retropseudogene	[22]
H. sapiens	*NANOGP10*	Retropseudogene	[22]
H. sapiens	*NANOGP11*	Retropseudogene	[22]
M. fascicularis	*NanogP*	Pseudogene	[22]
P. troglodytes	*NanogP4*	Pseudogene	[22]
M. musculus	*NanogPa*	Retropseudogene	[22]
M. musculus	*NanogPb*	Retropseudogene	[22]
M. musculus	*NanogPc*	Retropseudogene	[24]
M. musculus	*NanogPd*	Retropseudogene	[24]
M. musculus	*Nanog a*	Isoform	[25]
M. musculus	*Nanog b*	Isoform	[25]
M. musculus	*Nanog c*	Isoform	[25]

with roles in ESC maintenance [24]. Zhang et al. [23] demonstrated that the previously identified *NANOGP8* pseudogene is actually a retrogene that is expressed in different cancer cell lines, promoting proliferation.

Another way to potentially regulate Nanog function at the post-transcriptional level is through alternative splicing. Previous studies have reported that gene regulation by alternative splicing may affect about half of all genes in mammals [26]. More specifically, computational and experimental analyses have recently revealed that alternative splicing is fundamental for stem cell maintenance, pluripotency, and differentiation [26, 27]. Not surprisingly, a recent study has documented that the *Nanog* locus, via alternate promoter selection and alternative splicing, encodes two additional previously unknown protein variants, dubbed Nanog b and Nanog c, with reduced functions in mESC maintenance and pluripotency [25] (Table 1). For instance, although Nanog, Nanog b, and Nanog c can dimerize and interact with pluripotency factors such as Oct4 and Sall4, Nanog b cannot execute LIF-independent self-renewal. Both Nanog b and c are also slightly impaired in repressing transcription of primitive endoderm and trophectoderm markers such as *Gata6*, *Gata4*, *Sox17*, and *Hand1*.

Post-Translational Modification of Nanog

Post-translational modification (PTM) of proteins, particularly transcription factors, is a potent way to regulate functions such as transcriptional activity, DNA binding, cofactor association, subcellular localization, and protein stability. In many cellular contexts, important players such as p53 are heavily post-translationally modified by acetylation, phosphorylation, ubiquitination, methylation, and sumoylation, to extensively modulate their functions [28]. In ESCs, however, regulation of self-renewal and pluripotency factors has been broadly investigated at the transcriptional level, but lack of knowledge still exists about how their functions are modulated by PTMs. Nanog in particular has been known for quite some time to be a phosphoprotein in mESCs, since phosphatase treatment caused the disappearance of some slowly migrating forms of Nanog as detected by Western blot [29]. Since then, proteomic or site-directed mutagenesis analyses have revealed several Nanog phosphorylation sites in different cellular contexts [30]. Interestingly, only a few reports have investigated Nanog regulation by phosphorylation or by any other PTM in ESCs to date (Table 2). For instance, Moretto-Zita et al. [31] showed that Nanog is phosphorylated at several Ser/Thr-Pro motifs in mESCs. These modified sites are then recognized and bound by the prolyl isomerase Pin1, leading to Nanog protein stabilization by preventing proteasome-mediated degradation. Additionally, they demonstrated that those phosphorylated sites as well as Pin1 activity are important for ESC self-renewal and teratoma formation [31]. In a subsequent report, Ramakrishna et al. [34] reported that three out of the four Ser/Thr-Pro motifs mentioned above reside in a PEST domain that they previously identified, which regulates human NANOG stability by targeting it for proteasomal degradation. A recent report [35] has also shown that Nanog is ubiquitinated in mouse ESCs, which acts to maintain appropriate Nanog levels.

Two additional studies have described a role of Nanog phosphorylation in promoting tumorigenesis. In one, hNANOG was shown to be phosphorylated in vitro and in several cancer cell lines by focal adhesion kinase [32]. In another, Bourguignon et al. [33] illustrate that hNANOG, upon phosphorylation by protein kinase Cε, translocates to the nucleus and activates miR-21 production to promote tumor progression in breast cancer cells. All things considered, the current knowledge of Nanog regulation by PTMs may merely represent the tip of the iceberg. It would therefore not be surprising if Nanog modifications were as diverse and abundant as those found in p53, owing to the variety of functions that Nanog performs in stem cells and in cancer cells.

Nanog Dimerization Enhances ESC Self-Renewal and Pluripotency

Nanog is a homeodomain (HD) protein that was discovered in a screen for self-renewal factors that could sustain mESCs in the absence of LIF signaling [9, 36]. Nanog is critical for mammalian development and is required for specification of the ICM in the preimplantation embryo [37]. Similarly, the successful derivation of ESCs from the mouse blastocyst requires the expression of Nanog [9]. Because of the regulatory cooperation among Nanog, Oct4, and Sox2, it was believed that Nanog interacted with many other key factors in ESCs that govern pluripotency. The notion of ESC

Table 2. Summary of Nanog post-translational modifications

Nanog form	Modification	Modified residues	Function	References
Mouse	Phosphorylation	Ser 52	Interaction with Pin1, Nanog stabilization	[31]
Mouse	Phosphorylation	Ser 56/57	Interaction with Pin1, Nanog stabilization	[31]
Mouse	Phosphorylation	Ser 65	Interaction with Pin1, Nanog stabilization	[31]
Mouse	Phosphorylation	Ser 77/78	Interaction with Pin1, Nanog stabilization	[31]
Human	Phosphorylation	Tyr 35	Interaction with FAK, cancer cell motility and invasion	[32]
Human	Phosphorylation	Tyr 174	Interaction with FAK, cancer cell motility and invasion	[32]
Human	Phosphorylation	Serines (specific residues unknown)	Shuttling to nucleus, breast cancer cell survival and chemoresistance	[33]
Human	Ubiquitination	Lys 48	Targeting to 26S proteasome	[34]
Human	Ubiquitination	Lys 63	Targeting to 26S proteasome	[34]
Mouse	Ubiquitination	Lys 112	Targeting to 26S proteasome	[35]
Mouse	Ubiquitination	Lys 141	Targeting to 26S proteasome	[35]
Mouse	Ubiquitination	Lys 156	Targeting to 26S proteasome	[35]

Abbreviation: FAK, focal adhesion kinase.

maintenance as being a complex and multifaceted process was confirmed by the creation of the first pluripotency protein interaction network in mESCs [38]. This Nanog interactome connects with multiple corepressors such as the switch/sucrose non fermentable (SWI/SNF), NuRD, and Polycomb complexes, and outlines the proteins that physically interact with Nanog, and that are functionally important for ESC maintenance and early development [38].

We [39] and others [40] simultaneously demonstrated that mouse Nanog is able to form functional dimers through its tryptophan-rich (WR) domain. WR domain-mediated dimerization was further verified using an additional mutant form of Nanog containing 10 tryptophan to alanine substitutions (10WA) in the WR domain. This mutant form of Nanog was unable to dimerize with the wild-type form, confirming the importance of the WR domain for Nanog dimerization. In this study, we also found that the dimeric form of Nanog is essential for mESC self-renewal and pluripotency. We demonstrated by coimmunoprecipitation that factors within the Nanog interactome such as Sall4, Zfp198, Zfp281, Dax1, Nac1, and Oct4 preferentially interacted with wild-type Nanog versus the monomeric $Nanog^{10WA}$. Whether this differential interaction is due to a sequence-specific or monomer/dimer-specific effect needs to be further distinguished. We showed through colony formation assays and alkaline phosphatase (AP) staining that expression of a tethered Nanog dimer was sufficient to sustain the pluripotent phenotype of mESCs in the absence of LIF. Conversely, expression of a tethered Nanog monomer in the absence of LIF was insufficient to maintain mESCs in culture. Independently, Mullin et al. [40] found that wild-type Nanog only dimerized with Nanog mutants containing intact WR domains. Together these findings have demonstrated that Nanog dimers, but not monomers, are sufficient to support mESC self-renewal in the absence of LIF, and have delineated much of the physical and functional properties of Nanog in ESCs.

Nanog dimers interact with a subset of proteins identified in the Nanog interactome that promote pluripotency [38, 39]. One important Nanog binding partner is the Kruppel-like zinc finger transcription factor Zfp281, which was previously shown by coimmunoprecipitation to preferentially interact with Nanog dimers [39]. Our group recently demonstrated that Zfp281 functions as a transcriptional repressor of key pluripotency genes including *Nanog* [41] in mESCs. Zfp281 shares many target genes with Oct4, Sox2, and Nanog, and its promoter is also bound by Oct4, Sox2, and Nanog [42, 43]. Targeted deletion of *Zfp281* resulted in delayed mESC differentiation as measured by embryoid body formation, likely due to misregulation of Oct4 and Nanog at the transcript and protein levels, compared to wild-type mESCs. Zfp281 was also found to be required for Nanog binding to its own promoter by ChIP-PCR, suggesting that Zfp281 plays a critical role in regulating *Nanog* expression levels. These findings also suggest that Zfp281 helps to maintain the pluripotent state by fine-tuning Nanog expression in conjunction with other corepressors (see Nanog Autoregulation below) in ESCs.

Nanog Orthologs

The lower vertebrates chick and zebrafish both express Nanog and have been used extensively as developmental model systems. Chick and zebrafish Nanog exhibit low protein sequence similarity to mouse Nanog, due in part to the fact that neither chick Nanog nor zebrafish Nanog contains a WR domain (Fig. 2). Despite this caveat, it was recently shown that zebrafish Nanog is able to dimerize in vitro by a glutathione S-transferase (GST) pull-down assay and that zebrafish and mouse Nanog can functionally substitute for one another in vivo [44]. Unlike mouse Nanog, zebrafish Nanog requires the N-terminal domain and the HD for dimerization. It has not yet been determined, however, whether chick Nanog is able to form dimers or whether chick and mouse Nanog are functionally analogous.

Interestingly, it was recently discovered that chick and zebrafish Nanog can substitute for mouse Nanog during somatic cell reprogramming [45], supporting the idea of a functional conservation among vertebrate Nanog orthologs. Currently, it is believed that mouse Nanog dimers promote mESC self-renewal and maintenance of pluripotency [39, 40], and that Nanog monomers may be sufficient for the establishment of pluripotency [46]. It is not yet known, however, whether the same is true in hESCs, or if inherent differences exist between the inductive capabilities of the monomer and dimer forms in generating induced pluripotent stem cells (iPSCs). Human NANOG contains a WR domain that has 50% sequence identity with the mouse Nanog WR domain

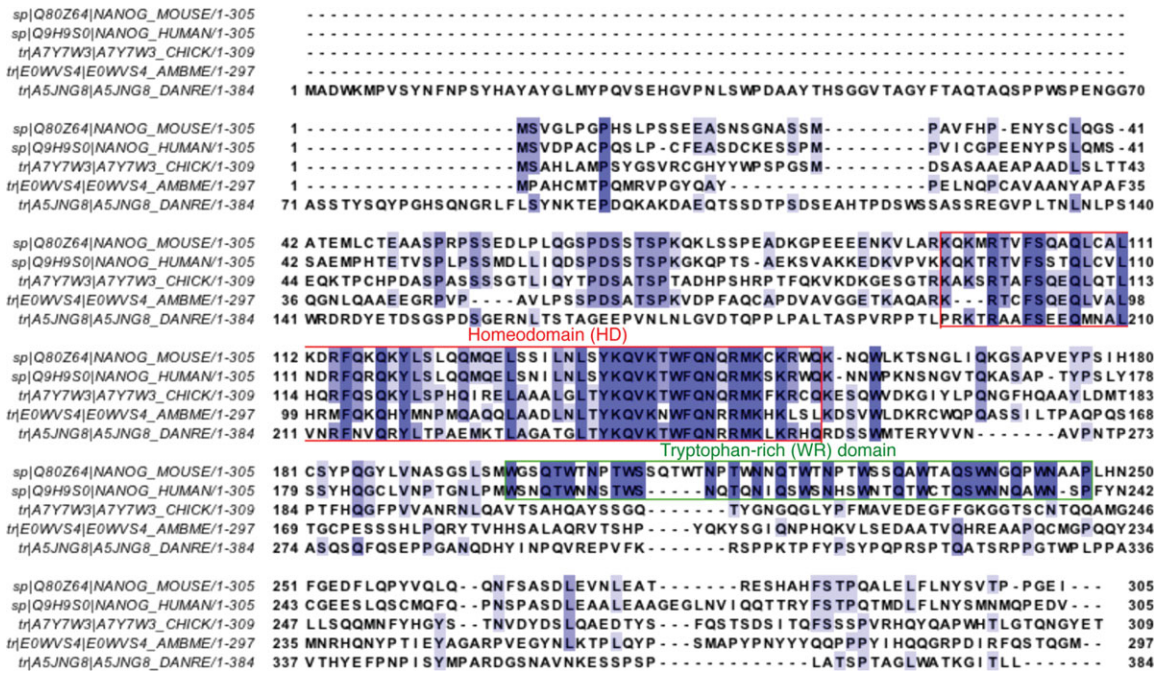

Figure 2. Sequence alignment of mouse, human, chick, axolotl salamander, and zebrafish Nanog (top to bottom). All five orthologs contain conserved residues, as indicated by shaded regions (darker = more conserved). All orthologs contain a homeodomain (boxed in red), but only mouse and human Nanog contain tryptophan-rich domains (boxed in green). Alignment created with ClustalW2 and analyzed in Jalview.

(Fig. 3). Despite this, it has been demonstrated that human NANOG can dimerize in vivo [47]. The highest percentage identity among mouse, human, chick, zebrafish, and axolotl Nanog sequences lies in the HD, which is also the only structural element in Nanog that has been solved to date by x-ray crystallography [48].

Dixon et al. [46] have confirmed that the WR domain of Nanog is required for maintaining pluripotency in mESCs. Sequence alignment with mouse Nanog revealed that axolotl Nanog does not contain a WR domain, but that it still contains a highly conserved HD, a domain important for DNA binding (Figs. 2, 3). Axolotl Nanog was also found not to form dimers, as measured by a protein complementation assay. This group also showed that induced axolotl Nanog dimerization is necessary and sufficient to support mouse ESC self-renewal in the absence of LIF.

Sequence identity relative to mouse Nanog (%)

	Full sequence	ND	HD	WR	Full CD
Human	57	49	87	50	50
Chick	29	25	67	-	26
Axolotl	27	26	55	-	20
Zebrafish	26	20	52	-	23

Figure 3. Vertebrate Nanog orthologs have conserved domains. Highest sequence identities relative to mouse Nanog reside in the homeodomain. Sequence identity percentages calculated in Jalview. Abbreviations: CD, C-terminal domain, Full CD = CD1 + WR + CD2; HD, homeodomain; ND, N-terminal domain; WR, tryptophan-rich domain.

Regulation of Nanog

Nanog Regulation by Transcription Factors

Due to the multifaceted functions of Nanog in ESC self-renewal and pluripotency, it does not come as a surprise that *Nanog* is extensively and promiscuously regulated in ESCs. Indeed, many transcription factors are recruited to the *Nanog* locus to activate and/or repress Nanog expression (Table 3). Moreover, Nanog expression is primarily monoallelic and fluctuates among mESCs in standard serum/LIF culture conditions, unless cultured in the presence of inhibitors of MAPK and glycogen synthase kinase 3 (GSK3), a condition known as "2i," with the addition of LIF (2i/LIF) [4, 77]. This suggests that signaling cascades also have important roles in regulating *Nanog* expression [77, 78]. Soon after Nanog was identified as an important factor for ESC self-renewal and pluripotency, much attention was focused on how the other core pluripotency factors regulate its gene expression. This led to the discovery that the proximal promoter region in the *Nanog* locus is responsible for most of the positive regulation of *Nanog* expression in mESCs [54, 55]. Not surprisingly, this region encompasses an Oct-Sox enhancer that is highly conserved among various mammalian species [54], demonstrating that Oct4 and Sox2 are major regulators of *Nanog* expression in mESCs.

Fine-tuning the expression of *Nanog* is also achieved via modulation of the recruitment and activity of additional transcription factors in response to specific cues. In fact, the Wnt signaling-responsive transcriptional regulator Tcf3 binds to an upstream regulatory region in the *Nanog* locus to downregulate *Nanog* levels and to ensure proper differentiation [79]. Recently described as a major downstream target of Nanog in regulating many functions in mESCs and miPSCs [63, 64], Esrrb also directly binds to the *Nanog* locus and activates its transcription in collaboration with Oct4 [56]. To further dissect the mechanism of Esrrb regulation of *Nanog*

Table 3. Summary of Nanog regulators

Factor	Mode of regulation	Effect on Nanog	References
Nanog	Transcriptional and epigenetic	Activator and repressor	[41, 49–52]
Zfp281	Transcriptional and epigenetic	Repressor	[41, 51]
Zfp143	Transcriptional	Activator	[53]
Oct4	Transcriptional and epigenetic	Activator	[49, 50, 53–58]
Sox2	Transcriptional and epigenetic	Activator	[49, 54, 55, 57, 59]
Klf4	Transcriptional	Activator	[20, 49, 60, 61]
Tcf3	Transcriptional	Activator and repressor	[57, 62]
Esrrb	Transcriptional	Activator	[56, 57, 63–66]
Ncoa3	Transcriptional	Activator	[65, 66]
Zic3	Transcriptional	Activator	[67]
Cdx2	Transcriptional	Repressor	[68]
Gcnf	Transcriptional	Repressor	[69]
Sp1	Transcriptional	Activator	[70]
Sp3	Transcriptional	Activator	[70]
Timp2	Transcriptional (Nanog promoter-driven luciferase assay)	Activator	[71]
Hig2	Transcriptional (Nanog promoter-driven luciferase assay)	Activator	[71]
Mki67ip	Transcriptional (Nanog promoter-driven luciferase assay)	Activator	[71]
Esrrg	Transcriptional (Nanog promoter-driven luciferase assay)	Activator	[71]
Dusp7	Transcriptional (Nanog promoter-driven luciferase assay)	Activator	[71]
Spi1	Transcriptional (Nanog promoter-driven luciferase assay)	Repressor	[71]
Prkaca	Transcriptional (Nanog promoter-driven luciferase assay)	Repressor	[71]
Jun	Transcriptional (Nanog promoter-driven luciferase assay)	Repressor	[71]
Tbx3	Transcriptional	Activator	[20]
Stat3	Transcriptional	Activator	[57, 72]
Brachyury	Transcriptional	Activator	[72]
PBAF complex	Transcriptional and epigenetic	Repressor	[57]
p53	Transcriptional	Repressor	[73]
Sin3a/HDAC complex	Transcriptional and epigenetic	Activator and repressor	[59, 73]
Mof	Epigenetic	Activator	[74]
Wdr5	Transcriptional and epigenetic	Activator	[58]
Ezh2	Epigenetic	Repressor	[75]
Satb1	Transcriptional and epigenetic	Repressor	[76]
Satb2	Transcriptional and epigenetic	Activator	[76]
NuRD/NODE complexes	Transcriptional and epigenetic	Repressor	[50, 51]

Abbreviations: HDAC, histone deacetylase; PBAF, polybromo BRG1 (Brahma-Related Gene 1) associated factor.

transcription, two groups independently demonstrated that the nuclear receptor coactivator 3, Ncoa3, binds directly to Esrrb, is recruited to the *Nanog* promoter, functions as a coactivator of Esrrb, and couples Esrrb to the basal transcription machinery by binding to RNA polymerase II [65, 66]. While Esrrb requires Oct4 for binding to the *Nanog* locus, the zinc finger protein Zfp143 stimulates *Nanog* transcription by modulating Oct4 binding [53]. Klf4 also binds to the distal and proximal promoter regions of *Nanog* to activate transcription in mESCs [60], and to the proximal promoter in hESCs to upregulate *NANOG* levels [61].

Common among the core pluripotency factors is their reciprocal feedback loop regulation [49, 62]. For instance, the two Nanog target genes *Sox2* and *Oct4* can regulate *Nanog* expression. However, Nanog shares this feature with another transcription factor, Cdx2, which is not a pluripotency factor, but is instead a lineage specific marker. Indeed, Daley's group demonstrated that Cdx2 can bind to the *Nanog* promoter in mESCs and repress its transcription [68]. Another way in which *Nanog* is downregulated to trigger differentiation is via the transcriptional repressor Gcnf. Indeed, Gu et al. [69] showed that upon retinoic acid (RA)-induced differentiation, Gcnf binds to its DR0 consensus sequences located 2.5 kb upstream of the transcription start site, and in the 3′ untranslated region in the *Nanog* locus to directly reduce *Nanog* expression. To additionally confirm that not only ESC-specific factors but also ubiquitously expressed transcription factors can regulate *Nanog* expression in pluripotent cells, Yao's group [70] demonstrated that Sp1 and Sp3 bind to the *Nanog* proximal promoter and activate its transcription. To complement studies done on the endogenous *Nanog* locus, Abujarour et al. [71] used a luciferase reporter assay driven by the *Nanog* promoter coupled with a cDNA library screening. They identified several factors, such as Timp2, Hig2, Mki67ip, Esrrg, and Dusp7 that activated the reporter, and others including Spi1, Prkaca, and Jun that repressed it.

However, they did not investigate whether these proteins could bind to the endogenous *Nanog* locus.

Finally, to properly regulate *Nanog* expression, several signaling transduction cascades come into play. For instance, *Nanog* is activated in response to LIF via two parallel pathways: the JAK/STAT3 pathway via Klf4 and the PI3K/AKT pathway via Tbx3 [20]. Interestingly, STAT3 can also directly activate *Nanog* transcription by binding to an enhancer region upstream of the *Nanog* promoter, together with Brachyury [72]. Likewise, other signaling cascades, including FGF/MEK [80], GSK3β [81], and TGFβ [82, 83], as well as local changes in chromatin structures by chromatin remodeling complexes such as polybromo BRG1 (Brahma-Related Gene 1) associated factor (PBAF) [57], are also important for maintaining *Nanog* levels in undifferentiated cells and for downregulating *Nanog* in order to execute differentiation programs.

Nanog Regulation by Epigenetic Factors

Chromatin modifiers can also modulate *Nanog* transcription in ESCs (Table 3). For example, the tumor suppressor p53 binds to the *Nanog* promoter, and upon RA-induced differentiation, recruits the Sin3a/histone deacetylase (HDAC) complex to reduce histone H3 acetylation and to directly repress *Nanog* expression in mESCs [73]. Interestingly, another group [59] demonstrated that the Sin3a/HDAC complex can activate *Nanog* when associated with Sox2. The H4 histone acetyltransferase Mof and the H3K4 methyltransferase mixed-lineage leukemia (MLL) complex subunit Wdr5 have also been recently implicated in regulating *Nanog* activity [58, 74]. Unlike other histone acetyltransferases (HATs), Mof directly binds to and actively upregulates transcription of *Nanog* [74]. Similarly, Wdr5 is recruited to the *Nanog* promoter in an Oct4-dependent manner to stimulate H3K4 trimethylation as well as to activate *Nanog* transcription [58]. Another epigenetic regulator, Ezh2, is involved in fine-tuning *Nanog* expression. In fact, even though the *Nanog* locus is not bivalent in mESCs, Ezh2 and its catalyzed trimethylation of histone H3 at K27 are both detectable by ChIP assays in the *Nanog* promoter in mouse ESCs and iPSCs, which inversely correlates with *Nanog* expression levels [75]. Modulation of higher order chromatin structure is also essential for stemness. In particular, the special adenine/thymine (AT)-rich sequence-binding protein Satb1 binds to and negatively regulates the expression of *Nanog*. Conversely, the related factor Satb2 is involved in the positive regulation of *Nanog* expression [76].

Nanog Autoregulation

Even though Oct4 and Sox2 protein levels are relatively stable in undifferentiated ESCs, Nanog protein levels fluctuate extensively [78]. Nanog itself can bind to its own promoter and regulate its own transcription either positively, by cooperating with Sox2 and Oct4 for instance, or negatively, by interacting with the transcriptional regulator Zfp281, bound to the NuRD repressor complex [41, 50, 51] (Table 3). Contrary to the common assumption that *Nanog* upregulation requires Oct4 and Sox2, Lim et al. [67] found that the transcription factor Zic3 binds to the *Nanog* promoter in vitro and in vivo, and that it activates *Nanog* expression even in the absence of Oct4/Sox2 binding regions. Similarly, Navarro et al. [52] recently found that *Nanog* autorepression, an endogenous negative feedback loop that prevents overexpression of *Nanog*, occurs independently of Oct4 and Sox2. This finding confirms our original report of *Nanog* autorepression [51] and further emphasizes the dual role of Nanog in transcriptional regulation.

Nanog Function in Stem Cell Pluripotency

The Smith group [84] has coined the term ground state, which refers to the pluripotent state of undifferentiated mESCs isolated from the naïve epiblast. Oct4 and Sox2 upregulate *Fgf4* levels, which in turn activate the MAPK pathway and poises ESCs for differentiation [85]. The combination of LIF and BMP4 is sufficient to maintain mESCs in vitro, but these factors are insufficient to block autoinductive MAPK signaling [4]. In trying to recapitulate the ground state, they hypothesized that the blocking of lineage commitment by LIF and BMP4 was downstream of FGF4-mediated MAPK signaling. To test this, Ying et al. [4] cultured mESCs in 2i/LIF and found that they could be maintained indefinitely in serum- and feeder-free conditions. Nanog is crucial for ICM development, and therefore *Nanog*$^{-/-}$ embryos are unable to form viable epiblasts [9]. It was later found, however, that conditional deletion of *Nanog* in cultured mESCs rendered them more prone to differentiation, but that it did not compromise their cellular integrity or pluripotent status [78].

Nanog has been shown to be heterogeneously expressed in mESCs in culture [49, 78, 86]. A recent report [77] indicates that this may be explained by variable allelic expression of *Nanog*, corresponding to its expression pattern during early embryonic development. *Nanog* exhibited monoallelic expression from the two-cell blastomere stage to the early blastocyst stage. By the late blastocyst stage, however, *Nanog* expression transiently became biallelic, coinciding with establishment of the pluripotent ground state in the ICM. A subset of cells also underwent allelic switching of expression, which could explain the heterogeneic expression pattern of Nanog observed in mESCs. Culturing mESCs in 2i/LIF further confirmed that biallelic *Nanog* expression promotes the transition to ground state pluripotency, as this condition significantly increased the level of biallelic *Nanog* expression compared to the standard serum plus LIF condition. 2i/LIF treatment also enriched the *Nanog* locus for trimethylated lysine 4 on histone 3 (H3K4Me3), an active chromatin mark as well as for RNA polymerase II. *Nanog* expression is thus controlled by chromatin modifications at each allele, which occurs during preimplantation embryonic development.

Nanog Function in Somatic Cell Reprogramming

The initial report of iPSC generation by Takahashi and Yamanaka did not include Nanog as one of the four canonical reprogramming factors [87]. However, addition of Nanog to the Oct4, Sox2, Klf4, and c-Myc cocktail can enhance reprogramming kinetics in a predominantly cell division rate-independent manner [88]. In addition, initial reprogramming of human fibroblasts by Thomson's group included NANOG along with OCT4, SOX2, and LIN28 [89]. Nanog can also enhance fusion-based reprogramming [90] as well as mEpiSC reprogramming [91].

Upon discovery that mESCs could be maintained in the absence of extrinsic factors, the Smith group set out to determine whether the 2i condition could enhance iPSC generation. They found that the initial products of somatic cell reprogramming existed in a "pre-iPSC" state, resting on the threshold of pluripotency [17]. Pre-iPSCs exhibit qualities quite different from ESCs. For example, they incompletely express

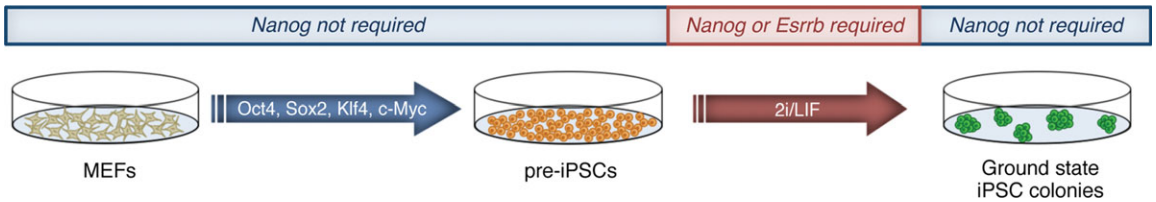

Figure 4. Nanog or its direct target *Esrrb* is required in the final stages of somatic cell reprogramming. MEFs transduced with the Yamanaka factors yield pre-iPSCs. Nanog is required in the pre-iPSC to ground state iPSC transition, as shown in red. Once the pluripotent ground state is established, Nanog is no longer required. Abbreviations: iPSCs, induced pluripotent stem cells; MEFs, mouse embryonic fibroblasts.

pluripotency markers, retain silencing of an X chromosome in female cells, are unresponsive to LIF, and are unable to contribute to chimeras. Remarkably, Silva et al. [17] found that serum- and feeder-free medium supplemented with 2i/LIF was able to drive pre-iPSCs toward ground state pluripotency to become bona fide iPSCs. Shortly afterward, this group [91] determined that Nanog is not required for the early stages of iPSC generation, but that it is required for the final transition from the pre-iPSC state to the fully induced ground state (Fig. 4). Using *Nanog*$^{-/-}$ neural stem cells and three (Oct4, Klf4, and c-Myc) of the four Yamanaka factors, they observed that 2i/LIF medium was insufficient for establishing ground state iPSCs. Contrastingly, upon addition of a floxed *Nanog* transgene as a reprogramming factor in the same conditions, pre-iPSCs were then able to fully transition to the ground state. Cre recombinase-mediated excision of the *Nanog* transgene had no effect on these iPSCs once pluripotency was established, as they were then able to contribute to chimeras. These results are also consistent with previous findings in *Nanog*$^{flox/flox}$ ESCs, wherein *Nanog* excision did not affect pluripotency [78]. Interestingly, it was recently shown that *Esrrb*, a direct downstream target of Nanog, can drive pre-iPSCs to the pluripotent ground state [63], further supporting the critical role of Nanog in establishing pluripotency. We also recently demonstrated that Nanog colocalizes with the methylcytosine hydroxylases Tet1 and Tet2 to a subset of pluripotency genes in mESCs, and that Nanog synergizes with these key epigenetic regulators during somatic cell reprogramming [92]. How Nanog precisely orchestrates the genetic and epigenetic events during the pre-iPSC to iPSC transition is only beginning to be defined.

Conclusions

Nanog dimers have been shown to be critical for maintenance of mESCs; however, the specific functions of Nanog monomers and dimers in ESCs and during somatic cell reprogramming are not yet clear. Although it has been shown that human NANOG can form dimers in vivo, it is not yet known whether the dimeric form is sufficient for self-renewal of hESCs. Zfp281 preferentially interacts with Nanog dimers in mESCs and is required for Nanog autorepression. Interestingly, vertebrate Nanog orthologs can bind to and activate transcription of mouse Nanog target genes [45]. This and the fact that these orthologs can replace mouse Nanog in reprogramming *Nanog*$^{-/-}$ pre-iPSCs demonstrate a functional conservation among Nanog orthologs. Additional functional studies of Nanog orthologs in ESCs and iPSCs could create a novel platform for interrogating Nanog function. The lack of functional data regarding Nanog PTMs emphasizes the importance of future studies designed to assess the implications of these modifications in regulating self-renewal and pluripotency. A number of Nanog phosphorylation sites have already been identified, yet it is unknown how phosphorylation or other modifications at these sites plays a role in Nanog protein-protein interactions or transcriptional activity.

iPSCs exhibit striking similarities to ESCs, that is, the capacity for unlimited self-renewal and multilineage differentiation. Although Nanog is crucial for the establishment of ground state pluripotency, it appears that it is not required for maintaining this state once it is established. Because of the positive and negative transcriptional activity that Nanog exerts, it is likely that Nanog cooperates with epigenetic activators or repressors to enhance the establishment of pluripotency. Further investigation into the interactions between Nanog and epigenetic regulators aside from Tet1 and Tet2 may point to additional synergistic effects on somatic cell reprogramming upon coexpression of Nanog and these regulators. Future work aimed at delineating the behavior of Nanog in ESCs and iPSCs will provide much needed mechanistic insights into the establishment and maintenance of pluripotency, and importantly, will enhance our understanding of the highly dynamic process of somatic cell reprogramming.

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Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

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