Developmental Biology 392 (2014) 182–192

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Nanog co-regulated by Nodal/Smad2 and Oct4 is required for pluripotency in developing mouse epiblast

Liang Tso Sun [a], Shinpei Yamaguchi [a], Kunio Hirano [a], Tomoko Ichisaka [b], Takao Kuroda [c], Takashi Tada [a,*]

[a] Department of Stem Cell Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan
[b] Department of Reprogramming Science, Center for iPS Cell Research and Application, Kyoto University, Kyoto, 606-8507, Japan
[c] Laboratory of Neural Differentiation, Graduate School of Brain Science, Doshisha University, Kyoto 619-0225, Japan

ARTICLE INFO

Article history:
Received 6 February 2014
Received in revised form
30 May 2014
Accepted 3 June 2014
Available online 12 June 2014

Keywords:
Nanog
Smad2
Nodal
Epiblast
Pluripotency
Oct4

ABSTRACT

Nanog, a core pluripotency factor, is required for stabilizing pluripotency of inner cell mass (ICM) and embryonic stem cells (ESCs), and survival of primordial germ cells in mice. Here, we have addressed function and regulation of *Nanog* in epiblasts of postimplantation mouse embryos by conditional knockdown (KD), chromatin immunoprecipitation (ChIP) using *in vivo* epiblasts, and protein interaction with the *Nanog* promoter *in vitro*. Differentiation of *Nanog*-KD epiblasts demonstrated requirement for *Nanog* in stabilization of pluripotency. *Nanog* expression in epiblast is directly regulated by Nodal/Smad2 pathway in a visceral endoderm-dependent manner. Notably, *Nanog* promoters switch from Oct4/Esrrb in ICM/ESCs to Oct4/Smad2 in epiblasts. Smad2 directly associates with Oct4 to form *Nanog* promoting protein complex. Collectively, these data demonstrate that *Nanog* plays a key role in stabilizing Epiblast pluripotency mediated by Nodal/Smad2 signaling, which is involved in *Nanog* promoter switching in early developing embryos.

Introduction

Nanog, a homeodomain-bearing transcription factor, plays a crucial role in early mouse embryonic development. *Nanog* expression has been detected in cells of the inner cell mass (ICM) of E3.5 blastocyst, the epiblast of E5.5–E7.5 embryos in the egg-cylinder into the primitive streak stages, and primordial germ cells (PGCs) of E8.5–E13.5 embryos in the headfold into the 52–55 somite stages (Chambers et al., 2003; Hatano et al., 2005; Mitsui et al., 2003; Yamaguchi et al., 2005). In pluripotent ICM cells and ICM-derived mouse embryonic stem cells (ESCs), *Nanog* has been identified as a central component of the regulatory circuitry in conjunction with Oct4 and Sox2. Disruption of *Nanog* halted development of ICM into epiblast at the peri-implantation stage (Mitsui et al., 2003), indicating requirement for *Nanog* in normal embryonic development. In ESCs, constitutive expression of *Nanog* entails cytokine-independent self-renewal in ESCs, and reduces the propensity to differentiate (Chambers et al., 2003), while cytokines such as leukemia inhibitory factor (LIF) and bone morphogenic factor-4 (BMP4) are required to maintain ESC identity *in vitro* (Qi et al., 2004; Smith et al., 1988; Williams et al., 1988). Further analysis has revealed that loss of *Nanog* severely compromises the robust pluripotency feedback networks, and leads to a concomitant cell fate decision to differentiate following deterioration of the self-perpetuating pluripotent state (MacArthur et al., 2012). In PGCs, *Nanog* is required for proper germ cell development. *Nanog*-knockout ESCs fail to contribute to germ cells of chimeric embryos (Chambers et al., 2007), and inducible *Nanog*-knockdown triggers apoptotic cell death of PGCs *via* disruption of a PGC-specific molecular network (Yamaguchi et al., 2009). Together, the data demonstrate that *Nanog* plays a crucial role in normal embryonic development through differential functioning between ICM cells and PGCs. However, it has not yet been investigated whether *Nanog* is required and how *Nanog* expression is regulated in the epiblast of E5.5–E7.5 embryos, between the egg-cylinder and primitive streak stages.

Mouse epiblast stem cells (EpiSCs), which are pluripotent cells derived from the epiblast, resemble human ESCs, but not mouse ESCs, in colony morphology and expression of pluripotency marker genes (Brons et al., 2007; Tesar et al., 2007). EpiSCs readily form teratoma, but not chimeric embryos following injection into blastocysts. Notably, core pluripotency regulators, *Oct4*, *Sox2*, and *Nanog* are expressed in both EpiSCs and ESCs, while expression of *Klf4*, *Stella*, and *Esrrb* are attenuated in EpiSCs, but not in ESCs. Furthermore, EpiSCs require bFGF and TGFb/ActivinA signaling, while ESCs

* Correspondence to: Department of Stem Cell Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogo-in, Sakyo-ku, Kyoto 606-8507 Japan. Fax: +81 75 751 4102.
E-mail address: ttada@frontier.kyoto-u.ac.jp (T. Tada).

http://dx.doi.org/10.1016/j.ydbio.2014.06.002
0012-1606/

respond to LIF/Stat3 and BMP4 signals, to support self-renewal under culture conditions. Collectively, EpiSCs are pluripotent stem cells maintained under a primed state, distinctive from ESCs under a naïve state (Nichols and Smith, 2009). As a model of the epiblast, EpiSCs have been used for investigating regulatory mechanisms of *Nanog* (Greber et al., 2010; Vallier et al., 2009). *Nanog* functions as a safeguard of pluripotency by blocking neuroectoderm and endoderm differentiation *in vitro* (Vallier et al., 2009). In EpiSCs, Nanog expression relies predominantly on ActivinA signaling through direct association of Smad2, but not Smad3, with the *Nanog* promoter region (Sakaki-Yumoto et al., 2013; Vallier et al., 2009). In contrast, in ESCs, Oct4, Sox2, and Esrrb, which are key factors of a master complex that also binds to the promoter region of *Nanog*, are responsible for upregulating *Nanog* expression (Kuroda et al., 2005; Rodda et al., 2005; van den Berg et al., 2008). Therefore, transcription regulation of *Nanog* in EpiSCs is different from that in ESCs, suggesting that switching of transcriptional regulation may occur during embryonic development from the ICM to the epiblast. However the underlying mechanism is unknown.

Importantly, in the epiblast of postimplantation embryos, axial patterning occurs through extracellular signals provided by extraembryonic tissues (Beddington and Robertson, 1999; Tam and Loebel, 2007). One of the most important signaling molecules is Nodal (Brennan et al., 2001). In embryos at the egg-cylinder stage, embryonic visceral endoderm (VE) surrounds and supports growing pluripotent epiblast through Nodal signaling (Mesnard et al., 2006). Nodal processing by proprotein convertases Spc1 and Spc4 released from extraembryonic ectoderm (EXE) is essential for Nodal maturation and signaling in epiblast (Beck et al., 2002; Ben-Haim et al., 2006). The distal end of VE, which emerges in response to coordinated Nodal and BMP4 signaling in the E5.5 embryo (Brennan et al., 2001; Soares et al., 2005), migrates to the anterior region of the egg-cylinder embryo to form anterior VE (Kimura-Yoshida et al., 2005; Yamamoto et al., 2004). Anterior VE expresses Nodal antagonists Cer1 and Lefty1 that attenuate the effect of Nodal signaling and established the anterior–posterior patterning of the epiblast (Yamamoto et al., 2004). This anterior–posterior polarity is required for formation of the primitive streak. Ablation of Nodal expression promotes neural fate determination in the epiblast (Camus et al., 2006), suggesting that Nodal is associated with both maintenance of pluripotency and lineage specification.

Here, we have investigated the function of *Nanog* in postimplantation epiblast *ex vivo* using a conditional knockdown system. *Nanog* was required for maintenance of pluripotency in epiblast explants. In E6.5 embryos, the *Nanog* expression pattern overlapped with the *Nodal* expression pattern. *Nanog* expression in epiblasts was induced by Nodal in a VE-dependent manner. Notably, Smad2, a downstream effector of Nodal signaling, upregulated *Nanog* expression in association with Oct4 through binding to the *Nanog* promoter region that includes the Oct/Sox- and Smad2/Esrrb-binding elements. Switching of transcription regulation of *Nanog* occurred first in development of the epiblast in the early egg-cylinder embryos with silencing of *Esrrb*, followed by formation of anterior–posterior axis patterning in the epiblast of late egg-cylinder embryos with downregulation of Sox2 in the posterior region. Smad2 and Oct4 were core regulators of *Nanog* in the epiblast.

Results

Nanog is required for maintaining pluripotency in E6.5 epiblast

To explore function of Nanog in the epiblast, a 4-hydroxytamoxifen (TM)-inducible knockdown (KD) system (Yamaguchi et al., 2009) was applied to early postimplantation *in vivo* embryos, which were heterozygous for the *Nanog* shRNA transgene (NRi-Tg) and ER-Cre generated by mating of NRi-Tg (+/+) females with ER-Cre (+/−) males (Fig. 1A). Unfortunately, effects of Nanog knockdown was unable to be analyzed *in vivo* egg-cylinder stage embryos, because E6.5 double and single transgenic embryos died due to detrimental effect of TM peritoneal-injection into 5.0–5.5-day pregnant females. Nanog was downregulated within 24 h of TM induction as previously demonstrated (Yamaguchi et al., 2009). Hence, we examined functions of Nanog using *ex vivo* culture of E6.5 epiblasts isolated from surrounding extraembryonic (EXE) tissue and VE (Fig. 1A). Genotype of embryos was determined by PCR with EXE tissues. Epiblast was dissected into five pieces and plated onto MEF feeders with TM for 4 days (Fig. 1B). At day 1 after plating, all epiblast clumps formed flat-shaped colony morphology, which resembles primed-state pluripotent stem cell colony, and we call it EpiSC-like colonies here. Notably, colonies of the double transgenic ER-Cre/NRi-Tg (Nanog KD), but not single transgenic NRi-Tg (negative control), epiblast started dissociating into single cells at day 4 (Fig. 1B and C), indicating that TM-induced Nanog KD exerted deleterious effect on maintenance of EpiSC-like colonies. The Nanog KD-mediated effect was prominent at day 10 (Fig. S1A), where the survival rate of EpiSC-like colonies was dramatically reduced to less than 5% at day 10 from about 30% at day 4 in ER-Cre/NRi-Tg epiblasts (Fig. 1C). In contrast, more than 50% EpiSC-like colonies survived between day 4 and 10 in NRi-Tg epiblasts. These data demonstrated that Nanog was required for maintenance of stem cell properties in the epiblast of early postimplanation embryos.

Next, to investigate whether the Nanog KD-mediated disruption of EpiSC-like colony formation was caused by induction of cell differentiation, expression of pluripotency marker, *Oct4*, the mesoderm marker, *Flk1*, and the endoderm/trophectoderm marker, *Cdx2*, in three individual Nanog KD and negative control epiblast explants at day 10 were analyzed by qPCR. In Nanog KD epiblasts (ER-Cre/NRi-Tg), *Oct4* expression decreased, and lineage-specific markers, *Flk1* and *Cdx2* increased, compared to negative control epiblasts (NRi-Tg) (Fig. 1D). Furthermore, to address whether cell death played a role in loss of stem cell-like colonies, the pan-caspase inhibitor, Z-VAD-FMK, was supplemented in the media for *in vitro* epiblast culture. However, loss of colony forming ability was not rescued by the inhibition of apoptosis (Fig. S1B). Taken together, the data indicate that Nanog serves as a crucial factor in maintaining pluripotency in the epiblast of post-implantation embryos.

Nanog co-localizes with Oct4 but not Sox2 in E6.5 epiblast

Skewed expression of Nanog to the posterior region of the epiblast of E6.5 and E7.5 embryos has been previously demonstrated by immunostaining and mRNA *in situ* hybridization, in contrast with even expression in the epiblast of E5.5 embryos (Hart et al., 2004; Hatano et al., 2005; Osorno et al., 2012). To confirm skewed expression of Nanog in the E6.5 embryo, we visualized the reporter gene, GFP, in Nanog-GFP transgenic mice (Okita et al., 2007). Nanog expression was detected in the posterior region of the E6.5 epiblast, but not in the anterior region (Fig. 2A). Next, to compare the expression profile of other pluripotency core factors, Oct4 and Sox2, with that of Nanog, quantitative RT-PCR was carried out in the anterior and posterior regions of the E6.5 epiblast. Successful separation of the anterior and posterior regions was verified by high expression of the mesoderm-specific gene, Brachyury, in the posterior region (Rivera-Perez and Magnuson, 2005). Notably, in the posterior regions, *Nanog* and *Oct4* were highly expressed, while *Sox2* expression was extremely low (Fig. 2B), and the skewed expression of Nanog and Sox2 was confirmed by immunofluorescent staining of pluripotency markers on intact embryos in gastrulation (Hoffman et al., 2013). Inconsistent with this observation, both Oct4 and Sox2 have

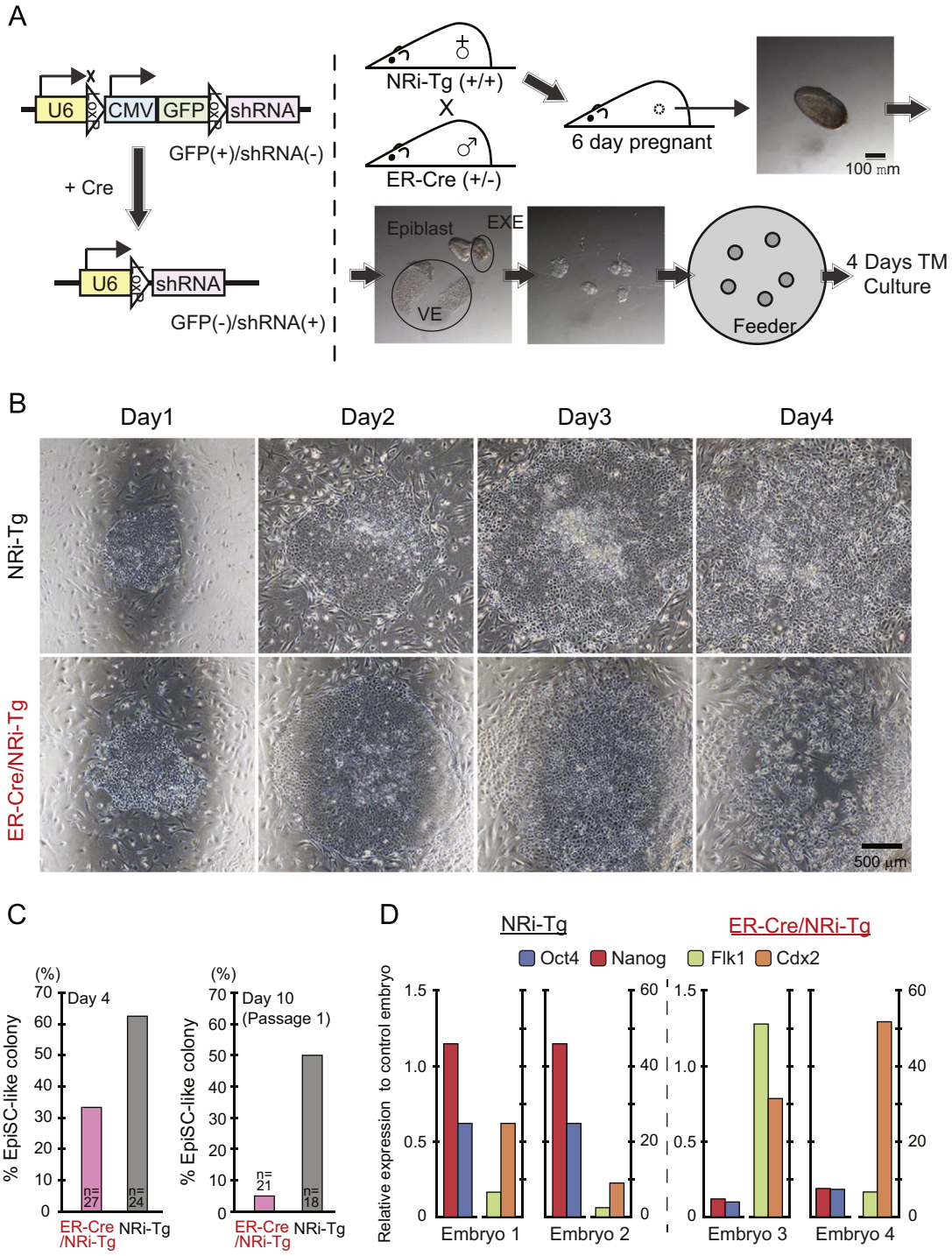

Fig. 1. Requirement of *Nanog* in E6.5 epiblast shown by conditional *Nanog* knockdown: (A) a Lentiviral vector for *Cre*-inducible *Nanog* RNA interference with shRNA (Left) and experimental scheme to collect epiblasts from E6.5 embryos for *in vitro* culture (Right). For obtaining E6.5 embryos, females homozygous for NRi-Tg were mated with males heterozygous for ER-Cre. GFP (green); green fluorescence protein, U6 (yellow) and CMV (blue); promoter sequences, shRNA (pink); short hairpin RNA inducing RNAi of *Nanog*, LoxP (white); LoxP sequences, between where *Cre* recombinase catalyzes DNA recombination event, TM; 4-hydroxytamoxifen, up-regulates *Cre* recombinase through binding to the ER promoter of the ER-Cre transgene, ER: estrogen receptor, VE; visceral endoderm, EXE; extraembryonic ectoderm. (B) Sequential changes of epiblast cell colonies *ex-vivo* cultured with *Nanog* knockdown inducer, tamoxifen (TM). Cell differentiation is observed in the double transgenic, ER-Cre/NRi-Tg, epiblast 4 days after TM treatment, while stem cell-like cells are propagated in the single transgenic, NRi-Tg, epiblast. (C) Generation efficiency of EpiSC-like colonies 4 and 10 days after TM treatment in culture. *Nanog* knockdown was induced in ER-Cre/NRi-Tg double transgenic (pink column), but not NRi-Tg single transgenic (gray column) epiblasts. The number of stem cell colonies at day 1 (indicated as *n*) was used as the reference to calculate the percentage of EpiSC-like colonies remaining after 4 and 10 days of TM treatment. (D) Comparative expression analysis of pluripotency and differentiation marker genes in epiblasts with or without *Nanog* knockdown (KD) by quantitative PCR. *Nanog* KD was induced in ER-Cre/NRi-Tg double transgenic epiblasts, while not in NRi-Tg single transgenic epiblasts. One of the NRi-Tg embryos was used as the reference. *Oct4*; pluripotency marker, *Flk1*; mesoderm marker, *Cdx2*; endoderm and trophectoderm marker.

been previously identified as core regulators of Nanog through binding to the Oct4/Sox2 motif upstream of the transcription-starting site of *Nanog* in ESCs (Kuroda et al., 2005; Rodda et al., 2005). We speculated that Nanog, which plays an important role in maintenance of pluripotency at the epiblast stage, was regulated by a mechanism distinct from ESCs.

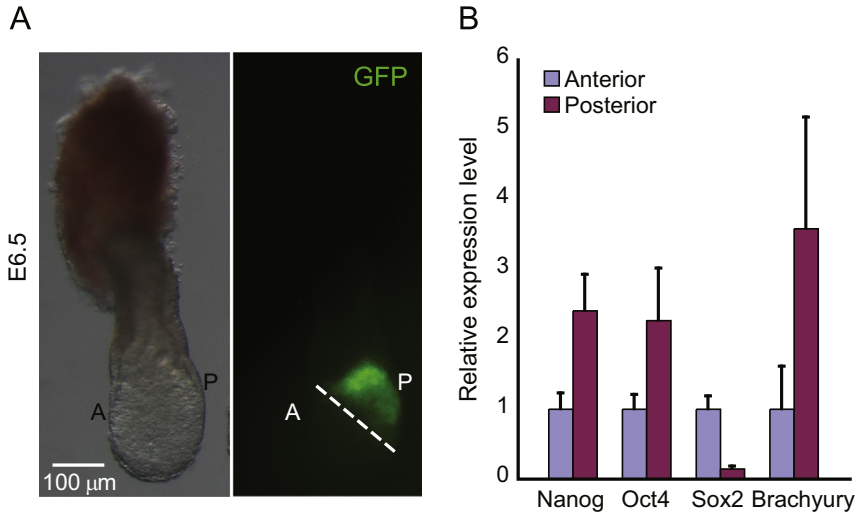

Fig. 2. Skewed Expression of *Nanog* in E6.5 Epiblast: (A) skewed expression of Nanog to the posterior region of E6.5 epiblast in *Nanog-GFP* transgenic embryo. Dashed line represents the border of GFP-positive posterior region in epiblast. A, anterior; P, posterior. (B) Comparative analysis of expression levels of pluripotency marker genes between the anterior and posterior regions of E6.5 epiblast by quantitative PCR. *Nanog* and *Oct4* are pluripotency marker genes, while *Sox2* and *Brachyury* are ectoderm and mesoderm differentiation marker genes respectively, in E6.5 embryos. *Sox2* and *Brachyury* are expressed in the anterior and posterior regions of E6.5 embryos respectively. Data are shown as mean ± SD ($n=3$).

Nanog expression is regulated by Nodal signaling in a visceral endoderm-dependent manner

To explore transcriptional regulation of Nanog in E5.5 Nanog-GFP embryos, epiblast was either carefully removed from the encapsulating VE or kept with VE intact (Fig. 3A). GFP expression was detected in 7 out of 8 intact epiblasts, while only 1 out of 7 VE-free epiblast was GFP positive (Fig. 3B). Expression of Nodal, a protein related to transforming growth factor-beta (TGF-beta), patterns VE and activates phosphorylation of Smad2, which recruits transcriptional factors to promote gene expression in developing embryos (Brennan et al., 2001; Kumar et al., 2001; Schier, 2003). Recombinant Nodal has previously been shown to rescue *Cripto* expression in VE-free epiblasts (Beck et al., 2002). Hence, epiblasts without VE were treated with or without Nodal. Remarkably, and GFP expression was detected with (7 out of 8) but not without (1 out of 7) Nodal in VE-free epiblasts. Consistent with our findings, the *Nodal* expression pattern agreed with that of *Nanog* in E6.5 mouse embryos (Conlon et al., 1994). Our results with the previous reports (Brennan et al., 2001; Kumar et al., 2001; Schier, 2003) indicated that expression of *Nanog* in the epiblast is regulated through Nodal/Smad2 signaling pathway in a VE-dependent manner (Fig. 3C).

For further analysis of *Nanog* expression in the epiblast of early implantation embryos, one of the core regulators of *Nanog*, Sox2, which plays crucial role in regulating *Nanog* transcription through binding to the Oct4/Sox2 element in ESCs, was examined by immunohistochemistry. Interestingly, Nanog was repressed in E5.5 VE-free epiblasts, in which Sox2 was expressed (Fig. 3C), suggesting that Oct4 and Sox2 alone may not maintain *Nanog* expression and transcriptional regulation of *Nanog* has been switched either partially or fully from Oct4/Sox2 motif-dependent to Nodal/Smad2-dependent mechanism around E5.5.

Binding of Oct4 and Smad2 to Nanog promoter in E6.5 epiblast

To examine which transcription factors are binding to the promoter region of Nanog in the E6.5 epiblast, chromatin immunoprecipitation (ChIP) assays were performed. Recently, it has been shown that Esrrb plays an important role in maintaining pluripotency and regulating *Nanog* expression through binding to a region located upstream of the Oct4/Sox2 element in the *Nanog* proximal promoter region in ESCs (van den Berg et al., 2008). The Esrrb binding site overlaps with Smad2 binding site previously reported in EpiSCs (Fig. 4B) (Greber et al., 2010; Vallier et al., 2009). Therefore, Esrrb was included in ChIP assays to reveal the relationship among Nanog regulators in developing embryos. ESCs and EpiSCs were used as references in ChIP. In all ChIP experiments, there were no significant difference between the control regions A and C (Fig. 4C).

In ESCs, *Nanog*, *Oct4*, *Sox2*, *Esrrb*, and *Smad2* were expressed (Fig. 4A), and Oct4 and Esrrb predominantly bound to the *Nanog* promoter (Fig. 4B and C), as previously reported (van den Berg et al., 2008). In EpiSCs, *Nanog*, *Oct4*, *Sox2*, and *Smad2*, but not *Esrrb* were expressed (Fig. 4A), and Oct4 and Smad2 bound to the *Nanog* promoter (Fig. 4B and C). Notably, in the E6.5 epiblast, *Nanog*, *Oct4*, and *Smad2*, but not *Sox2* and *Esrrb* were expressed in the posterior region, and Oct4 and Smad2 bound to the *Nanog* promoter (Fig. 4B and C). The expression profile of pluripotency genes in ESCs and EpiSCs corresponded to that of inner cell mass cells (ICM) of the blastocyst and the E5.5 epiblast, respectively (Fig. 4A), with respect to *Nanog* transcriptional regulation. Therefore, our data demonstrated that occupancy of the *Nanog* promoter switched from Esrrb/Oct4 in the ESC/ICM to Smad2/Oct4 in the EpiSC/E5.5 epiblast. Smad2/Oct4 continued to occupy the *Nanog* promoter in the posterior region of E6.5 epiblast for driving *Nanog* expression despite down-regulation of *Sox2*. Due to technical difficulties, we could not obtain quality Sox2 ChIP results with limited epiblast samples, and hence whether Sox2 participates in regulating Nanog expression in E6.5 epiblast remains unclear. However, our observations in Sox2 patterning (Fig. 2B) and immunostaining of E5.5 epiblast (Fig. 3C) indicated that Sox2 is dispensable for Nanog expression in developing epiblast.

ChIP analysis of Esrrb was not performed since *Esrrb* was repressed in the EpiSC (Fig. 2A) and the epiblast (Osorno et al., 2012).

Smad2/Esrrb and Oct4 binding sites are required for Nanog expression

Due to the limited options for assays that could be applied to early embryonic tissues, we assessed the possibility of using

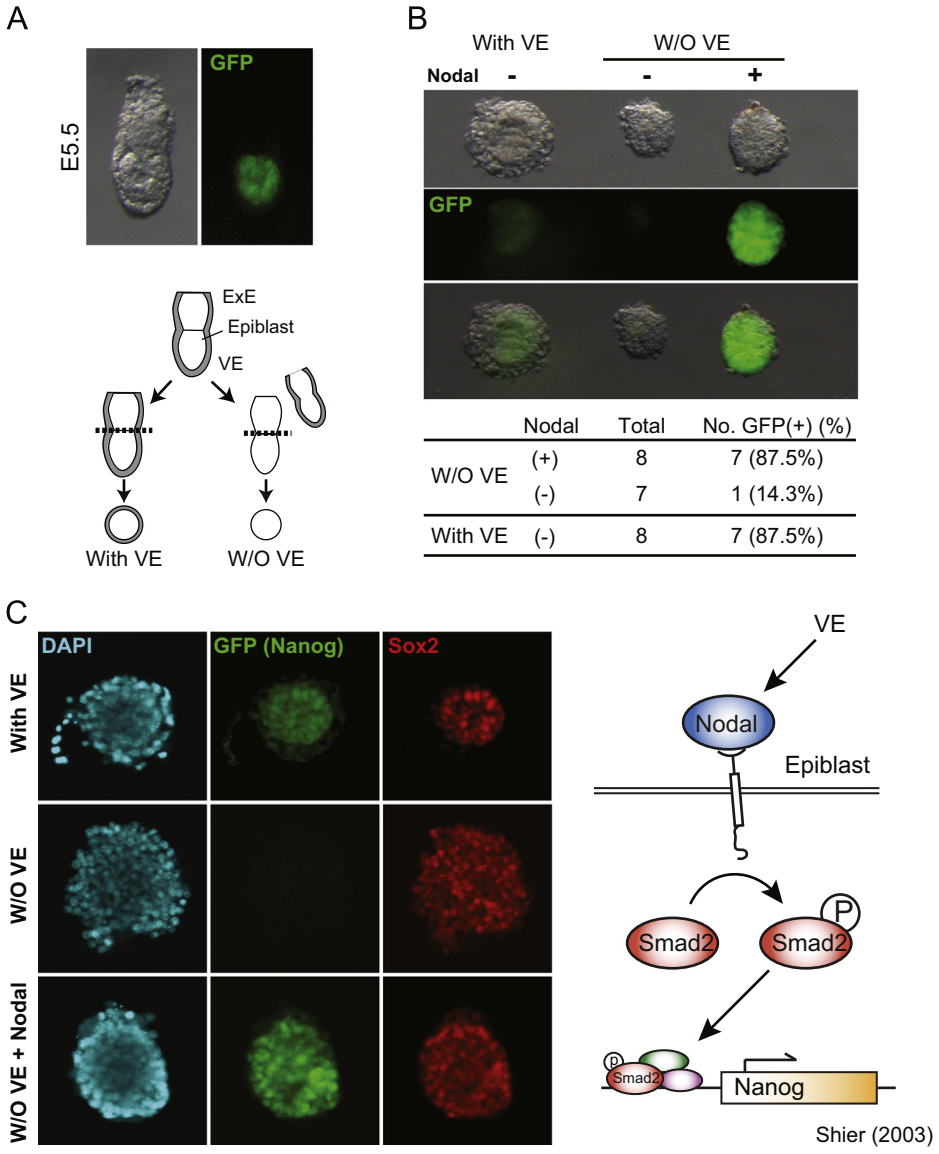

Fig. 3. Nodal-dependent Expression of Nanog in E5.5 Epiblast: (A) collection of green fluorescence (GFP)-positive E5.5 epiblasts with or without visceral endoderm (VE) by micro-dissection of *Nanog-GFP* transgenic embryos. GFP expression is detected evenly in E5.5 epiblast (top). EXE; extra-embryonic ectoderm, W/O; without. (B) Nanog-*GFP* expression in E5.5 epiblasts with or without visceral endoderm (VE) or with Nodal for 12 h. The number of embryos examined is summarized in the bottom. W/O; without. (C) Nodal-, but not Sox2-dependent Nanog-GFP expression in E5.5 epiblasts. Nanog-GFP expression is dependent on visceral endoderm (VE) or Nodal, but not Sox2. W/O; without. The Nodal-smad2 signaling pathway is linked to Nanog expression.

EpiSCs as a reliable model for investigating Epiblast biology. With multiple lines of evidence shared between epiblast and EpiSCs, including downregulation of *Esrrb*, Nodal/ActivinA/Smad2 dependency of Nanog expression, and occupancy of both Smad2 and Oct4 on the *Nanog* promoter (Fig. 4C), we concluded that EpiSCs was a suitable model for specifically analyzing the *Nanog* promoter landscape in the epiblast.

To examine whether the Oct4/Sox2 and Smad2/Esrrb binding sites are required in promoting *Nanog* expression, triple point mutations were introduced by replacement of DNA residues in each of Oct4, Sox2, Smad2, and Esrrb binding site (Fig. 5A) into previously cloned Nanog promoter-activated luciferase constructs (Kuroda et al., 2005). Wild type and mutated constructs were transfected into ESCs, EpiSCs, and NIH3T3 fibroblasts, which do not express *Nanog*. In ESCs and EpiSCs, all constructs with the Oct4 mutation showed a dramatic reduction in luciferase activity (Figs. 5B and S1D), indicating that Oct4 binding to the *Nanog* promoter is essential for upregulation of *Nanog*. Mutation of Sox element or Esrrb binding site with retention of the wild type Oct4 binding site induced considerable reduction of luciferase activity in both ESCs and EpiSCs (Fig. 5B). Conversely, mutations of the Smad2 binding site alone reduced luciferase activity only slightly in ESCs, but the effect of this mutation was significant in EpiSCs. Since Esrrb binding site overlapped with a significant portion of Smad2 binding site, a slight reduction in luciferase activity in ESCs transfected with Smad2-mutated construct was expected. Yet, it was remarkable that mutation in Smad2 and Esrrb binding sites demonstrate a similar level of luciferase activity in EpiSCs. This observation indicated that Esrrb was not a dominant promoter of Nanog in EpiSCs, and Esrrb and Smad2 binding sites might respond to a common promoter, while they were functionally separable and distinctively different in ESCs, suggesting a promoter-changing event might occur between ESCs and EpiSCs. Since *Esrrb* expression was nearly silenced in EpiSCs (Fig. 4A), Smad2 might substitute for Esrrb to bind the *Nanog* promoter.

It was evident that binding of Oct4 to the Oct4/Sox2 motif was crucial for *Nanog* upregulation (Fig. 5B). To address whether the Oct4 transcription complex contained Smad2 as a cofactor,

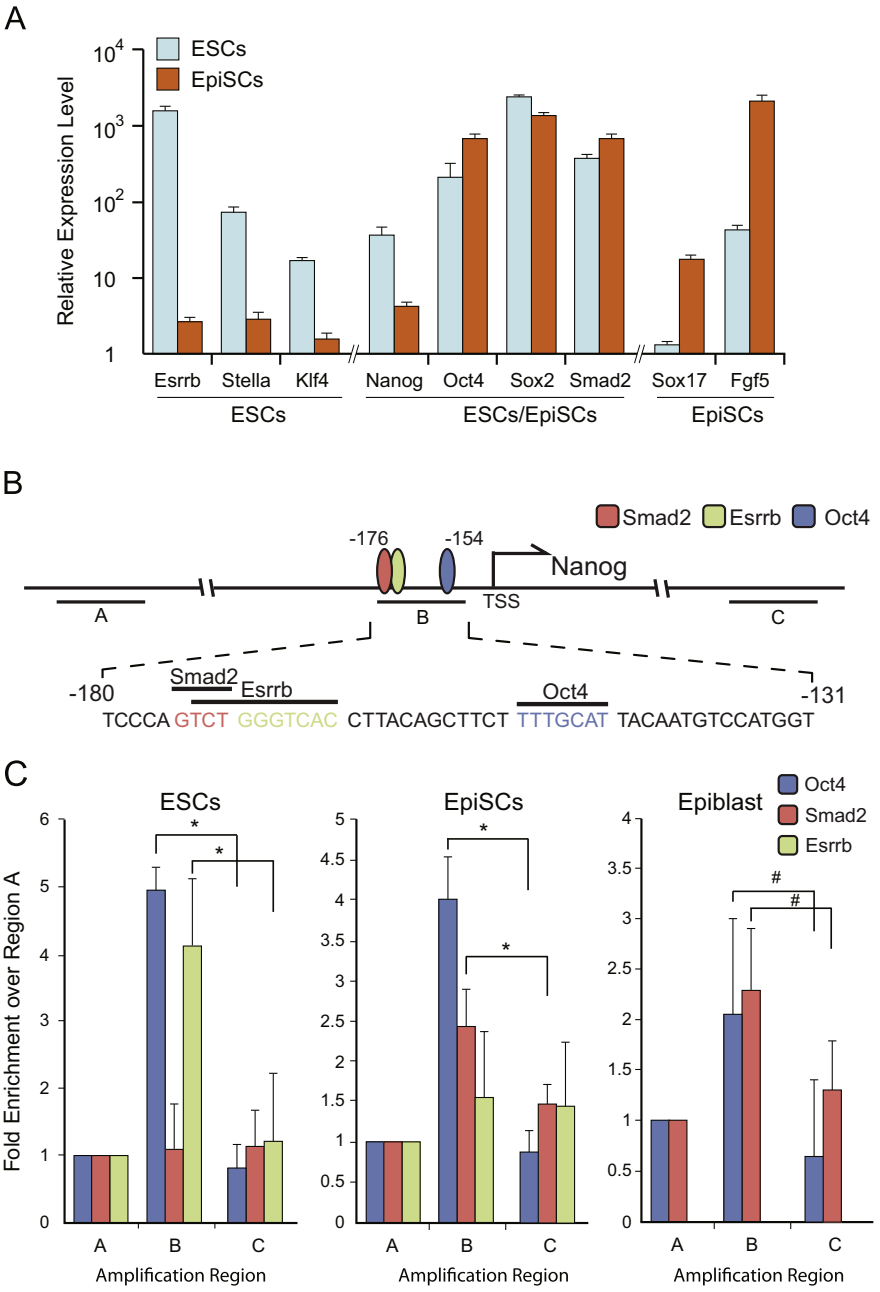

Fig. 4. Binding of Smad2 and Oct4 to *Nanog* promoter in E6.5 epiblast: (A) expression of pluripotency marker genes relative to *Gapdh* in ESCs and EpiSCs. Expression level of *Gapdh* is set at 10^5. *Esrrb* is expressed in ESCs, but not EpiSCs, while *Smad2* is in both ESCs and EpiSCs. *Oct4*, *Sox2*, and *Nanog* are expressed in both ESCs and EpiSCs. Data are represented as mean ± SEM. (B) Schematic representation of Smad2, Esrrb, and Oct4 binding sites upstream of *Nanog* transcription start site (TSS). The Smad2/Esrrb element is 12-base pairs away from the Oct/Sox element. A, B, C are PCR-amplified regions in chromatin immunoprecipitation (ChIP) assay. (C) Binding of Oct4, Smad2, and Esrrb to *Nanog* promoter in ESCs, EpiSCs, and E6.5 epiblasts. Quantitative PCR-amplified DNAs obtained by ChIP with anti-Oct4, Smad2, and Esrrb antibodies were normalized to control region A. Results are expressed as mean ± SD ($n=4$) ($^*p<0.05$, $\#p<0.1$).

immunoprecipitation assays were performed with anti-Oct4, anti-Nanog, and anti-Smad2 antibodies. In ESCs, Smad2 was free from the Oct4/Nanog complex (Fig. 5C), consistent with the observation that Esrrb occupied the Smad2/Esrrb binding site, and made a complex with Oct4 (van den Berg et al., 2008). In EpiSCs, Smad2 bound Oct4, but not Nanog (Fig. 5C), suggesting that Smad2 could induce *Nanog* upregulation as a co-factor of the Oct4 complex through direct binding with Oct4, but not Nanog. Low expression of Sox2 at the posterior region of E6.5 epiblast suggested that binding of a complex containing Oct4 and Smad2 to the Nanog promoter might be sufficient for promoting Nanog expression in the posterior region of the E6.5 epiblast.

In conclusion, *Nanog* is required for maintenance of pluripotency in E5.5–E6.5 early implantation embryos. *Nanog* transcription in ~E6.5 epiblast is regulated by Nodal/Smad2 signaling pathways in a VE-dependent manner. In embryonic development, a complex binding to the *Nanog* promoter changes from Esrrb/Oct4/Sox2 in ES /ICM cells to Smad2/Oct4/Sox2 in EpiSCs/E5.5 epiblast. Subsequently, Nanog expression skews toward posterior E6.5 epiblast and coincides with Oct4 and Nodal expression pattern, which is opposite of Sox2 patterning, suggesting a potentially reduced role of Sox2 in Nanog regulation (Fig. 6).

Discussion

Here, we demonstrate that (1) Nanog plays a crucial role in stabilizing pluripotency of cells in the epiblast of post-

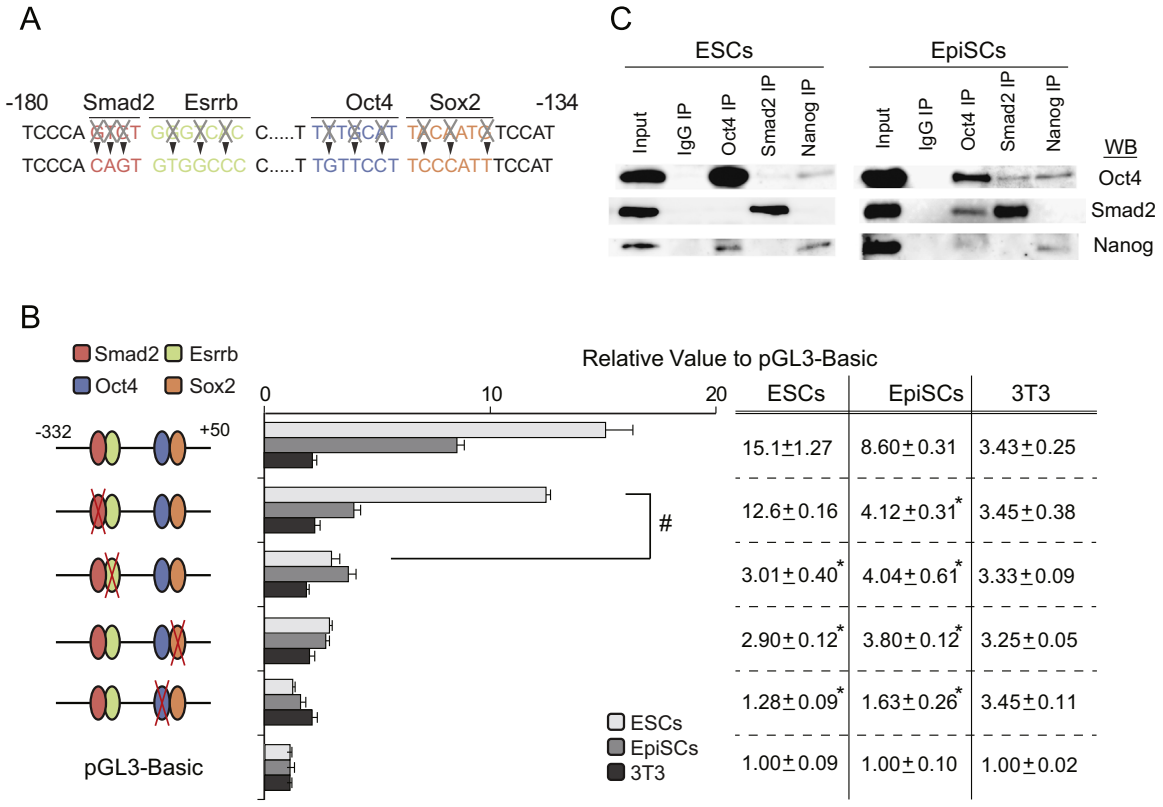

Fig. 5. Protein complex binding to Smad2 and Oct4 motifs in *Nanog* promoter: (A) mutation integration sites in Smad2, Esrrb, Sox2 and Oct4 binding sequences in *Nanog* promoter. Crosses on the wild-type sequence (top) indicate mutation integration residues. Mutated DNA sequence is shown below. (B) Requirement of binding of Smad2, Esrrb, Sox2 and Oct4 for up-regulating *Nanog* expression by luciferase assay in ESCs and EpiSCs. NIH3T3 cell was used as negative control, in which no expression of *Nanog* was detected. Luciferase activities are shown relative to those of pGL3-Basic. Data are represented as means ± SD ($n=4$). ($^*p < 0.005$ against WT, and $\#p < 0.001$). (C) Protein complex containing Smad2 in ESCs and EpiSCs by immunoprecipitation (IP) assays. Smad2 binds with Oct4, but not Nanog, in EpiSCs, while no Oct4 complex containing Smad2 is detected in ESCs. Input nuclear extracts and IP samples were hybridized with the indicated antibodies.

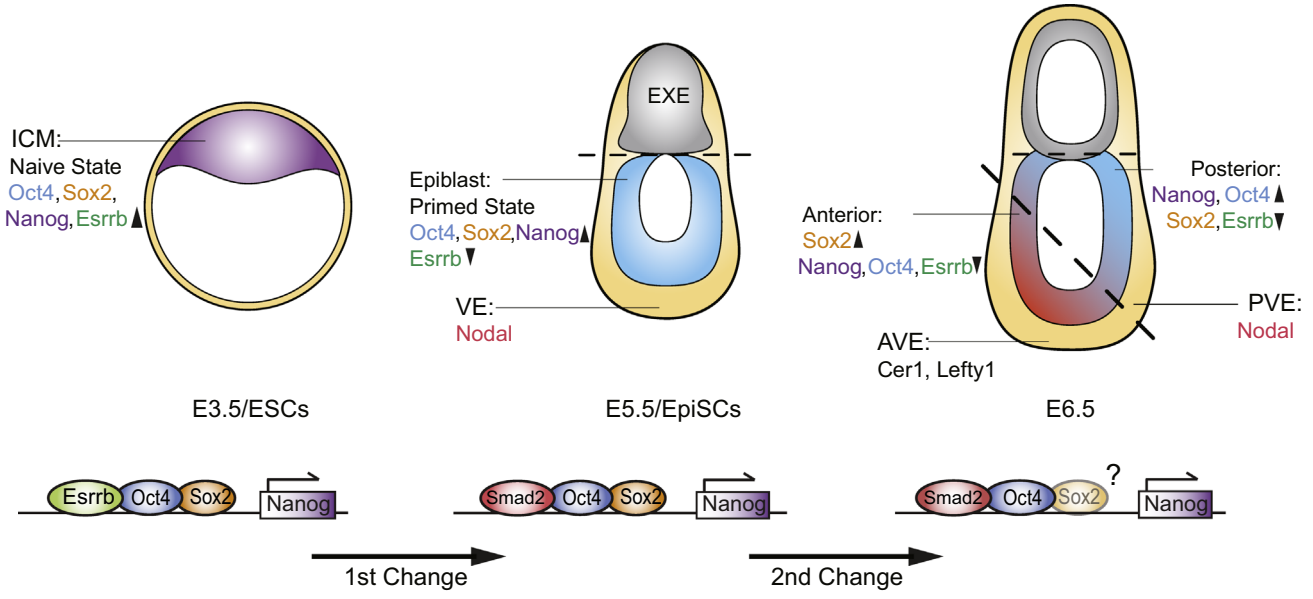

Fig. 6. Dynamic change of function and regulation of *Nanog* in early developing embryos. Function and expression pattern of *Nanog* dramatically changes between inner cell mass (ICM) of the blastocyst stage at E3.5 and epiblast of the primitive streak stage at E6.5 (Top). Dynamic changes of Nanog activator complexes bind to the Nanog promoter between naïve (E3.5) and primed (E5.5) state, and between egg-cylinder (E5.5) and primitive streak (E6.5) stages (Bottom). VE: visceral endoderm; AVE: anterior visceral endoderm; PVE: posterior visceral endoderm; and EXE: extra-embryonic ectoderm.

implantation embryos from the egg-cylinder to primitive streak stages, (2) *Nanog* expression is regulated by Nodal/Smad2 signaling in a VE-dependent manner, (3) the transcription regulator binding to the Smad2/Esrrb element in the *Nanog* promoter region switches from Esrrb in ICM/ESCs to Smad2 in epiblast/EpiSCs, and (4) skewed *Nanog* expression to the posterior region of the E6.5

epiblast is regulated by Smad2 and Oct4. In conclusion, expression of *Nanog*, which is a key player in safeguarding pluripotency of the epiblast, is regulated by Smad2/Oct4, which has switched from Esrrb/Oct4 in ICM/ESCs, binding to the Smad2/Esrrb and Oct/Sox elements in the *Nanog* promoter region.

Functional diversity of Nanog in the developing embryo

Nanog is a core factor for maintaining molecular pluripotency circuitry (Boyer et al., 2005). In mouse, disruption of *Nanog* function by gene targeting or knockdown induces differentiation of ICM and ESCs (Hough et al., 2006; Mitsui et al., 2003), indicating that *Nanog* plays a crucial role in maintaining pluripotency through inhibition of cell differentiation. Moreover, constitutive *Nanog* expression supports cytokine-independent self-renewal of ESCs (Chambers et al., 2003), suggesting that maintenance of pluripotency is associated with robust cell proliferation. Interestingly, *Nanog* exhibits distinctive functions in mouse PGCs. Conditional *Nanog* knockdown *in vivo* induces apoptotic cell death through disruption of a specific molecular network in migrating PGCs (Yamaguchi et al., 2009). Therefore, *Nanog* plays an important role in safeguarding against cell differentiation in ICM, and apoptotic cell death in PGCs, indicating that *Nanog* is a stage-specific multi-functional gene in developing embryos. Notably, in the epiblast of embryos from the egg-cylinder until primitive streak stage, *Nanog* plays a role in maintaining pluripotency similar to *Nanog* in the ICM of E3.5 blastocysts. In addition to being a pluripotency factor, *Nanog* is involved in axis patterning as shown by skewed expression to the posterior region relative to the anterior region, where specification to ectoderm occurs (Tam and Loebel, 2007) in the epiblast of E6.5 embryos. Collectively, diverse functions of *Nanog* are required for proper development of the ICM, epiblast, and migrating PGCs.

Nanog function in the posterior epiblast

Skewed expression of *Nanog* to the posterior region of the epiblast of the E6.5 embryo is associated with other posterior-specific genes including *Oct4*. The anterior region is specified for ectoderm fate characterized by *Sox2* expression (Avilion et al., 2003), while the posterior region maintains pluripotency and develops into mesendoderm lineage. A possible explanation for the unique spatial orientation of pluripotency-associated gene expression is that maintenance of pluripotency is required for subsequent developmental events, including primitive streak formation (Conlon et al., 1994) and germ cell specification (Saitou et al., 2012). One of the primary requirements for proper development of primitive streak in the posterior-proximal epiblast is the anterior–posterior axis dictated by Nodal/Nodal-antagonists (Conlon et al., 1994; Perea-Gomez et al., 2002). Here, we report that Nodal maintains pluripotency through Nanog expression in the posterior epiblast, suggesting that Nodal plays a crucial role in spatial and temporal regulation of pluripotency to specify germ-layer formation. Furthermore, Germ cells, marked by *fragilis*, an interferon-inducible transmembrane protein (Saitou et al., 2002) are induced by bone morphogenetic protein (BMP) signaling from the extraembryonic ectoderm at ~E6.5 (Lawson et al., 1999). BMP signaling induces expression of the transcriptional regulators Blimp1 and Prdm14 in the proximal epiblast of E6.5 embryos (Ohinata et al., 2005; Saitou et al., 2012). Blimp1- and Prdm14-positive cells form a cluster of alkaline phosphatase (AP)-positive PGCs at the proximal region of allantois at ~E7.25 (Ginsburg et al., 1990). Thus, it is likely that Nanog inhibits differentiation of the posterior epiblast cells at E6.5 until PGCs emerge. It is speculated that the extraembryonic BMP-responsible Smad signaling (Xu et al., 2008) may be related to *Nanog* upregulation in the posterior region of E6.5 epiblasts.

Requirement of Nanog in development

Nanog expression fluctuates among mouse ESCs at the single-cell level (Chambers et al., 2007; Hatano et al., 2005), suggesting that transient downregulation of *Nanog* predisposes the cells toward differentiation but does not mark commitment. Surprisingly, *Nanog*-null ESCs can self-renew indefinitely, although the cells are prone to differentiation (Chambers et al., 2007). *Nanog*-null ESCs can contribute to embryonic germ layers including PGCs, but not mature germ cells, in fetal and adult chimeras (Chambers et al., 2007). Consistent with this, *Nanog* function in anti-apoptotic cell death in migrating and colonized PGCs into genital ridges is indispensable (Yamaguchi et al., 2009). Importantly, *Nanog* function in stabilizing pluripotency could be substituted by other factors or pathways, as shown by *Nanog*-null ESC self-renewal in specific culture conditions, and their ability to contribute to embryonic development in chimeras (Chambers et al., 2007; Theunissen and Silva, 2011). *Esrrb* has been identified as a potent pluripotency factor that can functionally replace *Nanog in vitro* (Festuccia et al., 2012), suggesting that *Esrrb* may substitute *Nanog* function of pluripotency stabilization in *Nanog*-null ESCs. However, no expression of *Esrrb* is detected in the epiblast, and hence, it is unclear whether *Nanog* function can be replaced by other factors in the epiblast. In EpiSCs, pluripotency is maintained with gene expression profile characterized by a low level of *Nanog* (Silva et al., 2009). Moreover, it was reported that Nanog-null EpiSCs could be derived from both *in vitro* and *ex vivo* schemes, suggesting the existence of an as yet unidentified pathway that could sustain primed pluripotency in the absence of Nanog (Osorno et al., 2012). Thus, further analysis with EpiSCs could provide clues to the identification of a *Nanog* substitute in the epiblast, which could play an important role in maintaining pluripotency of human ESCs.

Transcriptional regulation of Nanog in the epiblast

A complex array of gene regulatory proteins controls the expression of *Nanog*: some acting as activators including Oct4, Sox2 (Kuroda et al., 2005; Rodda et al., 2005), Esrrb (van den Berg et al., 2008), Brachyury, and Stat3 (Suzuki et al., 2006), and others as repressors including p53 (Lin et al., 2005), GCNF (Gu et al., 2005), Grb2/Mek pathway (Hamazaki et al., 2006), and Tcf3 (Pereira et al., 2006). The concentrations of these regulators are thought to change during development. Particular combination of all the proteins triggers transcription of *Nanog*. A similar mechanism was reported for the complex regulatory region of the *human beta-GLOBIN* gene, which is part of a cluster of *GLOBIN* genes (Stamatoyannopoulos, 2005). Strong activators of *Nanog* are Oct4, Sox2, Esrrb, and Smad2, which bind to Oct/Sox elements and Smad2/Esrrb elements, located around 150 base pairs upstream of the transcriptional start site of *Nanog*. A Two-step switching paradigm of transcription activator complex occurs during peri- and postimplatation development. In ICM cells of preimplantation blastocysts, a large gene regulatory complex containing Oct4/Sox2/Esrrb activates *Nanog* transcription (Festuccia et al., 2012; Kuroda et al., 2005; Rodda et al., 2005). Next, in E5.5 epiblasts briefly after implantation, the *Nanog* regulatory complex changes to Oct4/Sox2/Smad2, due to downregulation of *Esrrb*. Then, during anterior–posterior axis patterning around E6.5, we speculate that the protein complex binding to the *Nanog* promoter further evolves into Oct4/Smad2 based on our observations in Sox2 patterning of E6.5 embryos and immunostaining of E5.5 epiblast under VE-free condition (Fig. 6). However, the precise role of Sox2 in *Nanog* regulation remains inconclusive and requires further analysis. Epigenetic changes at the *Nanog* promoter region during development, from blastocyst to the primitive streak stage, are largely unknown. Furthermore, it is unclear how other regulatory factors

binding to interspersed sequences around *Nanog* interact with various forms of Oct4-centered complexes through control of chromatin condensation or decondensation in embryonic development, as observed for *beta-GLOBIN* (Mahajan et al., 2007). *Nanog* can respond to an enormous number of combinatorial complexes that could induce appropriate level of expression at the correct time to ensure proper development.

Materials and methods

Cell culture

Mouse R1 ES cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako) supplemented with 15% fetal bovine serum (FBS) (Millipore), 10^{-4} M 2-mercaptoethanol, and 1000 U of recombinant LIF (Chemicon)/ml at 37 °C. Mouse EpiSCs were maintained with mouse embryonic fibroblast (MEF)-conditioned medium (CM) (DMEM/F12 HAM (Sigma) supplemented with 20% Knockout serum replacement (KSR) (Invitrogen), L-glutamine, non-essential amino acids, 2-mercaptoethanol, and 10 ng/ml bFGF (Peprotech)). NIH 3T3 cells were cultured in DMEM containing 10% fetal bovine serum.

in vitro culture of the epiblast

Female conditional *Nanog*-knockdown mice (NRi-Tg) were mated with Cre-ER mice (Hayashi and McMahon, 2002; Yamaguchi et al., 2009). To recover E6.5 embryos, females were sacrificed 6 days after vaginal plug was observed. Embryos were dissected in DMEM with FBS. To isolate the epiblasts, embryos were incubated with 0.25% Trypsin and 1 mM EDTA (TE) for 1 min. Following three times washes with phosphate buffered seline (PBS), visceral endoderm was removed by gentle pipetting using mouth pipette with fine-drawn capillary (100 μm in inner-diameter). Extra-embryonic ectoderm was removed with a glass needle.

For *ex-vivo* culture, isolated epiblasts were cut into four to five pieces by glass needle, and seeded onto feeder layer of inactivated MEFs in EpiSC medium (DMEM/F12 HAM) with 15% KSR, L-glutamine, non-essential amino acids, 2-mercaptoethanol, penicillin, streptomycin, 10 ng/ml bFGF, and 20 μg/ml ActivinA (Peprotech). To induce *Nanog* knockdown, 4-hydroxytamoxifen (Sigma) was added at a concentration of 1 μM. The pan-caspase inhibitor Z-VAD-FMK (Biomol) was used at a concentration of 20 μM.

For floating culture, epiblasts dissected from *Nanog*-GFP transgenic mouse (Okita et al., 2007) were cultured in MEF-conditioned medium in low-cell binding U-bottom 96-well plates (Nunc). Nodal (R&D systems) was added at a concentration of 50 μg/ml for 12 h.

Quantitative reverse transcription PCR

Total RNA was extracted from epiblasts and cultured cells using RNeasy mini Kit (Qiagen) and TRIzol (Invitrogen), respectively, according to the manufacturer's instructions. DNase I (Roche Diagnostics)-treated RNA was reverse-transcribed using random primers and Superscript III Reverse Transcriptase (Invitrogen). Amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions with gene-specific primer sets (Supplemental Table 1). All reactions were carried out in duplicate and gene expression levels were normalized to *Gapdh*. Relative expression of each gene was quantified from threshold cycles for amplification using the ΔΔCt or ΔCt method.

Immunohistochemistry

For whole-mount indirect immunofluorescence analysis, dissected embryos and epiblasts were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, washed three times with 0.1% Triton X-100 in PBS (PBST) and blocked with 3% Bovine serum albumin (BSA; Sigma) in PBST overnight. Embryos were then incubated with anti-Nanog (1:500; CosmoBio), anti-Oct4 (1:50; Santa Cruz), and anti-Sox2 (1:500; gift from S. Yamanaka) for 4 days, washed four times with PBST, incubated overnight with secondary antibodies and 4,6-diamidino-2-phenylindole (DAPI), washed four times with PBST and mounted in Slow Fade Gold (Invitrogen) for observation by confocal microscopy (Olympus).

Micro-chromatin immunoprecipitation

Micro-chromatin immunoprecipitation (μChIP) analysis was performed as described previously with minor modifications (Dahl and Collas, 2008). Mouse ESCs and EpiSCs were dissociated into single-cell suspensions, and 1×10^5 cells were cross-linked with 1% formaldehyde for 8 min. DNA was fragmented by sonication using seven 30-s pulses at 4 °C (Astrason). Immunoprecipitation was performed using Protein G dynabeads (Invitrogen) bound with anti-Oct4 (Santa Cruz), anti-Smad2/3 (Cell Signaling), or anti-Esrrb (R&D systems). Precipitated DNA was purified and analyzed by quantitative PCR (qPCR) using the primer sets listed in Supplemental Table 2.

ex vivo μChIP with epiblasts was performed as reported previously (Dahl and Collas, 2008). Epiblasts were collected from 20 to 25 E6.5 embryos (C57BL/6). Single cells dissociated from epiblasts with TE were mixed with 1×10^6 MEFs followed by cross-linking in PBS containing 1% formaldehyde, 10 mM Dimethyl 3,3′-dithiopropionimidate dihydrochloride (DTBP) (Sigma), and 2.5 mM Dithiobis[succinimidyl propionate] (DSP) (Sigma) for 10 min (Brown et al., 2011). Precipitated DNA was purified and analyzed by nested qPCR using the primer sets listed in Supplemental Table 1. Genomic DNA was used as reference to verify linear amplification. Briefly, DNAs were amplified for 15, 20, or 25 cycles of PCR followed by purification using Qiaquick PCR Purification Kit (Qiagen). The DNA was then diluted and subjected to qPCR.

Reporter vectors and luciferase reporter assays

Construction of the luciferase reporter vector containing *Nanog* promoter region (−332 bp to +50 bp) was previously described (Kuroda et al., 2005). Oligonucleotide-directed mutations were introduced into the Oct4, Smad2/3, and/or Esrrb binding elements by PCR as previously reported (Zheng et al., 2004) using primers containing nucleotide replacements listed in Supplemental Table 3.

Mouse ESCs (5.0×10^5), NIH 3T3 (2.5×10^5), and rho-associated protein kinase (ROCK) inhibitor-treated EpiSCs (1.0×10^6) were incubated in six-well tissue culture plates for 24 h. Each reporter construct (1.25 pmol) was co-transfected with vector phRL-TK (0.125 pmol) (Promega) as an internal control using Lipofectamine 2000 (Invitrogen). Cell extracts were prepared 48 h after transfection, and luciferase activities were evaluated using a dual-luciferase assay system (Promega). The luciferase activity of each construct was referenced to that of control vector pGL3-Basic. All conditions were performed in triplicate, and the promoter activities were reported as mean ± standard deviation.

Nuclear extraction and co-immunoprecipitations (coIP)

The nuclear protein was extracted according to the method previously reported (Dyer and Herzog, 1995). CoIP was carried out as previously described (Vallier et al., 2009) with minor modifications. Nuclear protein was diluted with HEMG110 buffer to a concentration of 2.5 mg/ml. In each IP assay, 0.5 mg nuclear protein and 8 μg antibody (anti-Smad2/3 (Cell Signaling), anti-Oct4 (Santa Cruz), anti-Nanog (ReproCell)) were used and incubated for 3 h at 4 °C. Protein G Dynabeads (50 μl) (Invitrogen) was added followed by 1 h of incubation at 4 °C. The beads were then washed with HEMG110 at five times, and then proteins were eluted by Laemmli sample buffer at 70 °C for 15 min. Protein samples were separated by 10% SDS-PAGE, and transferred onto a PVDF membrane (Millipore). Membranes were probed with anti-Oct4 (1:200) (Santa Cruz), anti-Smad2 (1:1000) (Cell Signaling), and anti-Nanog (1:1000) (ReproCell) antibodies at 4 °C overnight. The membranes were incubated with HRP-conjugated anti-rabbit IgG (1:1000) (Abcam) for 1 h. Signals were visualized using ECL Western Blotting Detection Kit (GE Healthcare).

Acknowledgments

L.T.S. was funded by Whitaker International Program administered by the Institute of International Education (IIE).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.ydbio.2014.06.002.

References

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., Lovell-Badge, R., 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 17, 126–140.
Beck, S., Le Good, J.A., Guzman, M., Ben Haim, N., Roy, K., Beermann, F., Constam, D.B., 2002. Extraembryonic proteases regulate Nodal signalling during gastrulation. Nat. Cell Biol. 4, 981–985.
Beddington, R.S., Robertson, E.J., 1999. Axis development and early asymmetry in mammals. Cell 96, 195–209.
Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E.J., Constam, D.B., 2006. The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev. Cell 11, 313–323.
Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., Gifford, D.K., Melton, D.A., Jaenisch, R., Young, R.A., 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122, 947–956.
Brennan, J., Lu, C.C., Norris, D.P., Rodriguez, T.A., Beddington, R.S.P., Robertson, E.J., 2001. Nodal signalling in the epiblast patterns the early mouse embryo. Nature 411, 965–969.
Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., Vallier, L., 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448, 191–195.
Brown, S., Teo, A., Pauklin, S., Hannan, N., Cho, C.H., Lim, B., Vardy, L., Dunn, N.R., Trotter, M., Pedersen, R., Vallier, L., 2011. Activin/Nodal signaling controls divergent transcriptional networks in human embryonic stem cells and in endoderm progenitors. Stem Cells 29, 1176–1185.
Camus, A., Perea-Gomez, A., Moreau, A., Collignon, J., 2006. Absence of nodal signaling promotes precocious neural differentiation in the mouse embryo. Dev. Biol. 295, 743–755.
Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., Smith, A., 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113, 643–655.
Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Grotewold, L., Smith, A., 2007. Nanog safeguards pluripotency and mediates germline development. Nature 450, 1230–1234.
Conlon, F.L., Lyons, K.M., Takaesu, N., Barth, K.S., Kispert, A., Herrmann, B., Robertson, E.J., 1994. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 120, 1919–1928.
Dahl, J.A., Collas, P., 2008. A rapid micro chromatin immunoprecipitation assay (microChIP). Nat. Protoc. 3, 1032–1045.
Dyer, R.B., Herzog, N.K., 1995. Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. BioTechniques 19, 192–195.
Festuccia, N., Osorno, R., Halbritter, F., Karwacki-Neisius, V., Navarro, P., Colby, D., Wong, F., Yates, A., Tomlinson, S.R., Chambers, I., 2012. Esrrb is a direct Nanog target gene that can substitute for Nanog function in pluripotent cells. Cell Stem Cell 11, 477–490.
Ginsburg, M., Snow, M.H., McLaren, A., 1990. Primordial germ cells in the mouse embryo during gastrulation. Development 110, 521–528.
Greber, B., Wu, G., Bernemann, C., Joo, J.Y., Han, D.W., Ko, K., Tapia, N., Sabour, D., Sterneckert, J., Tesar, P., Scholer, H.R., 2010. Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. Cell Stem Cell 6, 215–226.
Gu, P., LeMenuet, D., Chung, A.C., Mancini, M., Wheeler, D.A., Cooney, A.J., 2005. Orphan nuclear receptor GCNF is required for the repression of pluripotency genes during retinoic acid-induced embryonic stem cell differentiation. Mol. Cell. Biol. 25, 8507–8519.
Hamazaki, T., Kehoe, S.M., Nakano, T., Terada, N., 2006. The Grb2/Mek pathway represses Nanog in murine embryonic stem cells. Mol. Cell. Biol. 26, 7539–7549.
Hart, A.H., Hartley, L., Ibrahim, M., Robb, L., 2004. Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. Dev. Dyn.: Off. Publ. Am. Assoc. Anat. 230, 187–198.
Hatano, S.Y., Tada, M., Kimura, H., Yamaguchi, S., Kono, T., Nakano, T., Suemori, H., Nakatsuji, N., Tada, T., 2005. Pluripotential competence of cells associated with Nanog activity. Mech. Dev. 122, 67–79.
Hayashi, S., McMahon, A.P., 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev. Biol. 244, 305–318.
Hoffman, J.A., Wu, C.I., Merrill, B.J., 2013. Tcf7l1 prepares epiblast cells in the gastrulating mouse embryo for lineage specification. Development 140, 1665–1675.
Hough, S.R., Clements, I., Welch, P.J., Wiederholt, K.A., 2006. Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog. Stem Cells 24, 1467–1475.
Kimura-Yoshida, C., Nakano, H., Okamura, D., Nakao, K., Yonemura, S., Belo, J.A., Aizawa, S., Matsui, Y., Matsuo, I., 2005. Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm. Dev. Cell 9, 639–650.
Kumar, A., Novoselov, V., Celeste, A.J., Wolfman, N.M., ten Dijke, P., Kuehn, M.R., 2001. Nodal signaling uses activin and transforming growth factor-beta receptor-regulated Smads. J. Biol. Chem. 276, 656–661.
Kuroda, T., Tada, M., Kubota, H., Kimura, H., Hatano, S.Y., Suemori, H., Nakatsuji, N., Tada, T., 2005. Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. Mol. Cell. Biol. 25, 2475–2485.
Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P., Hogan, B.L., 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev. 13, 424–436.
Lin, T., Chao, C., Saito, S., Mazur, S.J., Murphy, M.E., Appella, E., Xu, Y., 2005. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat. Cell Biol. 7, 165–171.
MacArthur, B.D., Sevilla, A., Lenz, M., Muller, F.J., Schuldt, B.M., Schuppert, A.A., Ridden, S.J., Stumpf, P.S., Fidalgo, M., Ma'ayan, A., Wang, J., Lemischka, I.R., 2012. Nanog-dependent feedback loops regulate murine embryonic stem cell heterogeneity. Nat. Cell Biol. 14, 1139–1147.
Mahajan, M.C., Karmakar, S., Weissman, S.M., 2007. Control of beta globin genes. J. Cell. Biochem. 102, 801–810.
Mesnard, D., Guzman-Ayala, M., Constam, D.B., 2006. Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. Development 133, 2497–2505.
Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., Yamanaka, S., 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113, 631–642.
Nichols, J., Smith, A., 2009. Naive and primed pluripotent states. Cell Stem Cell 4, 487–492.
Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S.C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., Surani, M.A., 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 436, 207–213.
Okita, K., Ichisaka, T., Yamanaka, S., 2007. Generation of germline-competent induced pluripotent stem cells. Nature 448, 313–317.
Osorno, R., Tsakiridis, A., Wong, F., Cambray, N., Economou, C., Wilkie, R., Blin, G., Scotting, P.J., Chambers, I., Wilson, V., 2012. The developmental dismantling of pluripotency is reversed by ectopic Oct4 expression. Development 139, 2288–2298.
Perea-Gomez, A., Vella, F.D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H., Behringer, R.R., Ang, S.L., 2002. Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. Dev. Cell 3, 745–756.
Pereira, L., Yi, F., Merrill, B.J., 2006. Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal. Mol. Cell. Biol. 26, 7479–7491.
Qi, X., Li, T.G., Hao, J., Hu, J., Wang, J., Simmons, H., Miura, S., Mishina, Y., Zhao, G.Q., 2004. BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. Proc. Natl. Acad. Sci. USA 101, 6027–6032.

Rivera-Perez, J.A., Magnuson, T., 2005. Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. Dev. Biol. 288, 363–371.
Rodda, D.J., Chew, J.L., Lim, L.H., Loh, Y.H., Wang, B., Ng, H.H., Robson, P., 2005. Transcriptional regulation of Nanog by Oct4 and Sox2. J. Biol. Chem. 280, 24731–24737.
Saitou, M., Barton, S.C., Surani, M.A., 2002. A molecular programme for the specification of germ cell fate in mice. Nature 418, 293–300.
Saitou, M., Kagiwada, S., Kurimoto, K., 2012. Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development 139, 15–31.
Sakaki-Yumoto, M., Liu, J., Ramalho-Santos, M., Yoshida, N., Derynck, R., 2013. Smad2 is essential for maintenance of the human and mouse primed pluripotent stem cell state. J. Biol. Chem. 288, 18546–18560.
Schier, A.F., 2003. Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol. 19, 589–621.
Silva, J., Nichols, J., Theunissen, T.W., Guo, G., van Oosten, A.L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I., Smith, A., 2009. Nanog is the gateway to the pluripotent ground state. Cell 138, 722–737.
Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., Rogers, D., 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336, 688–690.
Soares, M.L., Haraguchi, S., Torres-Padilla, M.E., Kalmar, T., Carpenter, L., Bell, G., Morrison, A., Ring, C.J., Clarke, N.J., Glover, D.M., Zernicka-Goetz, M., 2005. Functional studies of signaling pathways in peri-implantation development of the mouse embryo by RNAi. BMC Dev. Biol. 5, 28.
Stamatoyannopoulos, G., 2005. Control of globin gene expression during development and erythroid differentiation. Exp. Hematol. 33, 259–271.
Suzuki, A., Raya, A., Kawakami, Y., Morita, M., Matsui, T., Nakashima, K., Gage, F.H., Rodriguez-Esteban, C., Izpisua Belmonte, J.C., 2006. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. Proc. Natl. Acad. Sci. USA 103, 10294–10299.
Tam, P.P., Loebel, D.A., 2007. Gene function in mouse embryogenesis: get set for gastrulation. Nat. Rev. Genet. 8, 368–381.
Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R. L., McKay, R.D., 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448, 196–199.
Theunissen, T.W., Silva, J.C., 2011. Switching on pluripotency: a perspective on the biological requirement of Nanog. Biol. Sci. 366, 2222–2229.
Vallier, L., Mendjan, S., Brown, S., Chng, Z., Teo, A., Smithers, L.E., Trotter, M.W., Cho, C.H., Martinez, A., Rugg-Gunn, P., Brons, G., Pedersen, R.A., 2009. Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. Development 136, 1339–1349.
van den Berg, D.L., Zhang, W., Yates, A., Engelen, E., Takacs, K., Bezstarosti, K., Demmers, J., Chambers, I., Poot, R.A., 2008. Estrogen-related receptor beta interacts with Oct4 to positively regulate Nanog gene expression. Mol. Cell. Biol. 28, 5986–5995.
Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., Gough, N.M., 1988. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336, 684–687.
Xu, R.H., Sampsell-Barron, T.L., Gu, F., Root, S., Peck, R.M., Pan, G., Yu, J., Antosiewicz-Bourget, J., Tian, S., Stewart, R., Thomson, J.A., 2008. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell 3, 196–206.
Yamaguchi, S., Kimura, H., Tada, M., Nakatsuji, N., Tada, T., 2005. Nanog expression in mouse germ cell development. Gene Expr. Patterns 5, 639–646.
Yamaguchi, S., Kurimoto, K., Yabuta, Y., Sasaki, H., Nakatsuji, N., Saitou, M., Tada, T., 2009. Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells. Development 136, 4011–4020.
Yamamoto, M., Saijoh, Y., Perea-Gomez, A., Shawlot, W., Behringer, R.R., Ang, S.L., Hamada, H., Meno, C., 2004. Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo. Nature 428, 387–392.
Zheng, L., Baumann, U., Reymond, J.L., 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic Acids Res. 32, e115.