BMP Sustains Embryonic Stem Cell Self-Renewal through Distinct Functions of Different Krüppel-like Factors

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SUMMARY

Bone morphogenetic protein (BMP) signaling exerts paradoxical roles in pluripotent stem cells (PSCs); it sustains self-renewal of mouse embryonic stem cells (ESCs), while it induces differentiation in other PSCs, including human ESCs. Here, we revisit the roles of BMP-4 using mouse ESCs (mESCs) in naive and primed states. SMAD1 and SMAD5, which transduce BMP signals, recognize enhancer regions together with KLF4 and KLF5 in naive mESCs. KLF4 physically interacts with SMAD1 and suppresses its activity. Consistently, a subpopulation of cells with active BMP-SMAD can be ablated without disturbing the naive state of the culture. Moreover, Smad1/5 double-knockout mESCs stay in the naive state, indicating that the BMP-SMAD pathway is dispensable for it. In contrast, the MEK5-ERK5 pathway mediates BMP-4-induced self-renewal of mESCs by inducing Klf2, a critical factor for the ground state pluripotency. Our study illustrates that BMP exerts its self-renewing effect through distinct functions of different Krüppel-like factors.

INTRODUCTION

Pluripotent stem cells (PSCs) harbor core properties of self-renewal and pluripotency (Hackett and Surani, 2014; Martello and Smith, 2014). Mouse embryonic stem cells (mESCs), established from the inner cell mass (ICM) of pre-implantation blastocysts, are classically derived from a permissive 129-strain and kept in serum-containing medium supplemented with leukemia inhibitory factor (LIF). They are considered to possess “naive” pluripotency. Bone morphogenetic proteins (BMPs) have been shown to substitute for serum and sustain self-renewal of naive mESCs together with LIF (Ying et al., 2003). On the other hand, human ESCs, which have characteristics similar to those of mouse epiblast stem cells (mEpiSCs) derived from postimplantation embryos, are more mature and are referred to as being “primed” for differentiation. These cells are LIF independent, and are usually maintained in the presence of fibroblast growth factor 2 (FGF2) and activin A, which stabilize the primed pluripotent state.

Although the serum-free medium supplemented with LIF and BMP-4 is well established, PSCs maintained with BMP-4 are rather exceptional. To derive mESCs from non-ossifying strains, suppression of the differentiation-inducing mitogen-activated protein kinase (MEK1/2) extracellular signal-regulated kinase 1/2 (ERK1/2) pathway is required (i.e. LIF+BMP-4+MEK1/2i) (Batlle-Morera et al., 2008). A cocktail of two inhibitors, i.e. for the MEK1/2 and GSK3 pathways (2i+LIF, or 2iL) has been proved to be sufficient to maintain mESCs with full pluripotency, or a “ground state” (Ying et al., 2008), and LIF supplementation (2i+LIF, or 2iL) strengthens the robustness. In the ground state, the BMP-SMAD pathway has been shown to be less active (Boroviak et al., 2014). Furthermore, BMP-4 induces differentiation of primed pluripotent cells. Thus, clarifying the role of BMP in mESCs from permissive strains will provide important clues for the molecular basis of naive pluripotency.

BMPs are members of the transforming growth factor β (TGF-β) family, and transduce their signals through distinct sets of type I and type II receptors (Derynck and Miyazono, 2008; Miyazono et al., 2010). Activated type I receptors phosphorylate receptor-regulated SMAD proteins (R-SMADs; SMAD1, SMAD5, and SMAD8 in the BMP signaling pathway, and SMAD2 and SMAD3 in the TGF-β/activin pathway), which form heterotrimeric complexes with SMAD4 and translocate into the nucleus. SMAD complexes are recruited to cell-type-specific binding sites through interaction with master transcription factors, controlling the expression of specific target genes (Morikawa et al., 2008).
Figure 1. SMAD1 and SMAD5 Bind to Enhancer Regions Together with KLF4 and KLF5 in mESCs
(A) Upper: Schematic of the mESC differentiation system used to derive EpiSC-like cells (ESD-EpiSCs). Lower: Morphology and alkaline phosphatase (AP) activity (inset) of mESC and ESD-EpiSC. Scale bar, 200 μm.
(B) Transcriptome changes between mESCs and mESD-EpiSCs. Each point represents FPKM (fragments per kilobase of exon per million fragments mapped) values of a gene. Genes with FPKM <0.1 in both conditions were excluded.

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et al., 2013). In naive mESCs, SMAD1 has been reported to co-localize with OCT4, SOX2, and NANOG (abbreviated as OSN) and STAT3 on ESC-specific enhancers (Chen et al., 2008). Consistently, the BMP-SMAD target genes id1 and Dusp9 play critical roles in naive mESCs (Li et al., 2012; Ying et al., 2003). However, the existence of Smad4 homozygous mutant mESCs (Sirard et al., 1998) suggests that the SMAD pathway is not required for maintenance of naive pluripotency, leading us to revisit the roles of the BMP-SMAD pathway in PSCs.

In the present study, we have performed both RNA-sequencing and SMAD1/5 genome-wide chromatin immunoprecipitation and sequencing (ChIP-seq) analyses of mESCs in the naive or primed states, and have employed a genome editing method. We show that the BMP-SMAD pathway is dispensable for maintaining naive pluripotency. Instead, BMP utilizes the MEK5-ERK5 pathway, which induces Klf2, an essential factor for the ground state (Yeo et al., 2014). Our study thus sheds important light on the molecular mechanisms underlying naive pluripotency.

RESULTS

BMP Target Genes Are Expressed at Comparable Levels in Both Naive and Primed mESCs

Recent work has shown that naive mESCs can convert into LIF-independent EpiSC-like cells when cultured with FGF2 and activin A, which have been designated mESC-derived (mESD) EpiSCs or mEpiLCs (Hayashi et al., 2011; Zhang et al., 2010). We directly compared mESCs (E14, 129/Ola strain) in the naive and primed states (Figure 1A). Whole transcriptome analysis showed that expression of EpiSC lineage markers, e.g. Fgf5, Dnmt3b, and Lefty1, were upregulated, while genes associated with naive pluripotency, e.g. Klf2, Klf4, Esrrb, and Zfp42 (also known as Rex1), were downregulated in mESD-EpiSCs (Figures 1B, S1A, and S1B). Our analysis also revealed that mESD-EpiSCs possessed a gene expression pattern similar to that of naive mESCs and clustered separately from mESCs (Figure S1C).

In addition, trophoblastic markers, e.g. Cdx2, and mesodermal markers, e.g. T(Brachyury) and Mespt1, were induced in mESD-EpiSCs after BMP-4 treatment, which is not the case in naive mESCs in LIF+BMP-4 (Figure S1A). Importantly, expression levels of BMP target genes, id1 and Dusp9, were comparable or even higher in mESD-EpiSCs treated with BMP-4 (24 hr) than in mESCs (Figures S1A and S1B), suggesting that induction of these genes is not associated with the maintenance or reversion to the naive state.

SMAD1 and SMAD5 Bind to Enhancer Regions Together with KLF4 and KLF5 in Naive mESCs

To address the roles of the BMP-SMAD pathway, we performed ChIP-seq analyses (Figures 1C and S1D). SMAD1/5 were enriched in the promoter regions of Pou5f1 (which encodes OCT4) and Nanog in naive mESCs, as well as a positive control region in the id1 promoter. Consistent with previous findings (Chen et al., 2008), the regions bound by SMAD1/5 were enriched with active enhancer marks (H3K4me1, H3K27ac, and co-activator p300) (Figure 1D). The SMAD1/5 binding regions were co-occupied by KLF4 and KLF5, but not by KLF2, as well as by the core regulators of pluripotency, OSN (Figures 1E and S1E). To investigate whether SMAD1/5-KLF4/5 co-localized with OSN (Chen et al., 2008), we subdivided the SMAD1/5 binding sites into three groups based on overlap with H3K4me1 (one of the enhancer marks) and OSN. KLF4/5 co-localized with SMAD1/5 even in OSN-negative enhancers (group 2, Figure 1F), and binding motifs for KLF4/5 were enriched in group 2 (Figure 1G). Moreover, distinct gene ontologies were enriched in group 2 compared with OSN-positive enhancers (group 1) (Figure 1H).

Motif enrichment analysis also showed that a binding motif for SMAD1/5, i.e. GC-rich SMAD Binding Element (GC-SBE) (Morikawa et al., 2011), and that for SMAD4 and SMAD3, i.e. SMAD Binding Elements (SBE), were not enriched in SMAD1/5 binding sites of mESCs. Intriguingly, these motifs were enriched in those of mESD-EpiSCs.
Thus, it is possible that SMAD1/5 recognize enhancer regions indirectly through KLF4 and KLF5 in naive mESCs, while they recognize their target regions directly in primed mESCs (Figure S1F).

KLF4 Physically Interacts with SMAD1 and Suppresses Its Activity

We next examined the relationship between SMADs and Krüppel-like factors (KLFs). Because of high overlap in binding between KLF4 and KLF5 (Figure 2A), we assumed that KLF4/5 recognized similar binding regions and that different efficiency of the ChIP procedures may explain the difference. We thus performed functional screening for KLFs using a BMP reporter, BRE-luc (Morikawa et al., 2011). Ectopic expression of SMAD1 enhanced the activity of BRE-luc, while co-expression of KLF4 strongly attenuated the effect of SMAD1 (Figure 2B). Similarly, the BMP-4-induced BMP reporter activation was attenuated by KLF4 co-expression (Figure 2B).

Moreover, SMAD1 was co-immunoprecipitated with KLF4 in HEK293T cells (Figure 2C). The interaction with KLF4 was mapped to the MH2 domain of SMAD1 (Figure 2D). We also confirmed that endogenous KLF4 protein was co-immunoprecipitated with SMAD1/5 in naive mESCs (Figure 2E). Thus, KLF4 physically interacts with SMAD1/5 and suppresses transcriptional activity of SMADs, suggesting that the activity of BMP-SMAD is low in naive mESCs.

The BMP-SMAD Pathway Is Dispensable for Maintaining Naive Pluripotency

To evaluate the BMP-SMAD pathway in naive mESCs, we generated artificial reporter constructs that minimized possible contributions of other transcription factors. Previously, we identified and characterized GC-SBE (Figure S2A), a palindromic GGCGCC sequence (abbreviated as GC) that has strong affinity compared with GGAGCC (or GA), both of which exist in SMAD1/5 binding regions in the genome (Morikawa et al., 2011). We thus generated BMP reporter constructs containing GC-SBE (GC/GA) as well as SBE (Figure 3A). The two BMP reporters had different sensitivity to BMP stimulation in cultured cells.
Figure 3. BMP-SMAD Pathway Is Dispensable for Maintaining Naive Pluripotency

(A) Schematic presentation of BMP reporter constructs. Fragments with GGGGCC (GC) or GGAGCC (GA) sequences together with SBE were triplicated and fused with reporter genes. TK, herpes simplex virus type 1 thymidine kinase gene.

(B) A BMP type I receptor inhibitor, DMH1 (5 μM), attenuated GFP expression in Tg(3xGA-GFP) transgenic fish in vivo. Control, head to the right position; DMH1, head to the left position.

(C) Expression of GFP in 3xGA-GFP cells in LIF+serum or LIF+BMP-4. Scale bar, 40 μm.

(D) Negative selection with ganciclovir (GCV) in 3xGA-TK cells. Upper: Phase contrast. Lower: AP staining. Scale bar, 200 μm.

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They also responded faithfully to BMP signaling in transgenic zebrafish in vivo (Figures 3B, S2C, and S2D). To perform lower selection, we chose the 3xGA construct with lower sensitivity, since BMPs are present in serum at concentrations of 2–6 ng/ml (Herrera and Inman, 2009).

We then generated lentiviruses harboring 3xGA-GFP or herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene (Figure 3A), and introduced them into mESCs. As expected, GFP protein expression was regulated by BMP-4 (Figure S2E). When the cells were maintained in LIF+BMP-4, a subpopulation of cells became strongly positive for GFP (Figure 3C). The subpopulation could be abolished by the addition of ganciclovir in 3xGA-TK cells and the remaining cells were alkaline phosphatase (AP) positive (Figure 3D). This suggested that strong activation of the BMP-STAT pathway is not required for the maintenance of naive pluripotency.

To directly examine the roles of the BMP-STAT pathway, we generated Smad1 and Smad5 double-knockout (Smad1/5 dKO) and Smad4 knockout (Smad4 KO) mESCs using CRISPR-Cas9 genome editing (Figure S2F) (Mali et al., 2013). We established two different clones with biallelic mutations around the Cas9 target sites; mutants were screened by western blotting and confirmed by sequencing of the target regions (Figures S2G and S2H, data not shown). Smad1/5 dKO lines and Smad4 KO lines did not respond to BMP-4, or treatment with both BMP-4 and activin A, respectively (Figures 3E and 3S1). Similar to Smad4 KO lines (Figure S2J) (Sirard et al., 1998), Smad1/5 dKO lines formed AP-positive colonies in LIF+serum and LIF+BMP-4 (Figure 3F), with growth rates comparable to those of parental mESCs (Figure 3G). Smad1/5 dKO lines also showed comparable expression levels of pluripotency markers with parental mESCs (Figure 3H). We also examined TFE3 protein localization; TFE3 stays in the nucleus when cells are in the naive state, while it shifts to the cytoplasm when cells start to differentiate (Figure S2K) (Betschinger et al., 2013). TFE3 was localized mainly in the nucleus in Smad1/5 dKO lines in LIF+serum (Figure 3I). Collectively, our observations suggest that the BMP-STAT pathway is dispensable for maintaining naive pluripotency.

The MEK5-ERK5 Pathway Mediates BMP-4-Induced Self-Renewal of mESCs
What mechanisms can explain the role of BMP in maintaining naive pluripotency, if the BMP-STAT pathway is dispensable? In addition to the SMAD proteins, BMP/TGF-β type I receptors activate non-SMAD signaling pathways, e.g. ERK and p38 mitogen-activated protein (MAP) kinase pathways (Derynck and Miyazono, 2008; Miyazono et al., 2010). Using mESCs in LIF+BMP-4, we screened a set of kinase inhibitors targeting non-SMAD pathways. In addition to the extracellular BMP antagonist NOGGIN, inhibitors for MEK5 (BIX 02189) and ERK5 (XMD 8–92) suppressed cell growth and promoted differentiation of mESC (Figures 4A and S3A); the effects of MEK5i on differentiation was weaker than ERK5i, which may be caused by off-target effects of MEK5i on MEK1/2. Consistently, BMP-4 treatment induced ERK5 phosphorylation in both mESCs and Smad1/5 dKO mESCs (Figures 4B and S3B). Furthermore, we introduced either the constitutively active S311D/T315D mutant of MEK5 (MEK5DD), or the dominant-negative S311A/T315A mutant (MEK5AA) (Figure 4C) (Spiering et al., 2009). Among known target genes, e.g. ld1 (Spiering et al., 2009) and Klf/2/4 (Sunadome et al., 2011), MEK5DD induced Klf2 in mESCs (Figures 4D and 4E). Intriguingly, MEK5AA suppressed and MEK5DD stimulated cell growth (Figure 4F). MEK5DD-expressing mESCs maintained ES-like morphology, while cells with the constitutively active S218D/S222D mutant of MEK1 (MEK1DD) underwent differentiation (Figure 4G).

To further confirm the roles of the MEK5-ERK5 pathway in self-renewal of naive mESCs, we used mESCs from the C57BL/6 mouse strains (BL6-mESCs), which cannot be kept in the feeder-free LIF+BMP-4 condition (Balle-Morera et al., 2008). Again, MEK5DD induced both mRNA and protein of Klf2 in BL6-mESCs (Figures 4D and 4E). In addition, similar to overexpression of KLF2 which can replace MEK1/2i (Yeo et al., 2014), MEK5DD-expressing mESCs maintained undifferentiated colony morphology in serum-free LIF+GSK3i (Figures 4H, 4I, S3C, and S3D). The phenotype was mediated by the MEK5-ERK5 pathway, since it was disrupted by MEK5i and ERK5i (Figures 4I, S3C, and S3D). Thus, our study revealed an important role of the MEK5-ERK5 pathway to maintain naive pluripotency (Figure S3E).

(E) qRT-PCR analysis to evaluate BMP responsiveness. Cells were stimulated with 50 ng/ml BMP-4 or 50 ng/ml activin A for 1.5 hr. Data represent means ± SEM of three independent experiments. ***p < 0.001 versus parental mESCs. S15 dKO, Smad1/5 double knockout.
(F) Morphology (left) and AP activity (right) of Smad1/5 dKO lines in LIF+serum and LIF+BMP-4. Scale bar, 200 μm.
(G) Proliferation and doubling time (Td) of parental mESCs and Smad1/5 dKO cell lines maintained in LIF+serum. A representative growth curve data is shown. Td (mean ± SEM) was calculated using three independent experiments.
(H) qRT-PCR analysis of Smad1/5 dKO cell lines cultured in LIF+serum. Data represent means ± SEM of three independent experiments. *p < 0.05, **p < 0.01 versus parental mESCs.
(I) Expression and localization of TFE3 (green), DNA/DRAQ5 (blue), and their merge in Smad1/5 dKO lines in LIF+serum. Scale bar, 20 μm. See also Figure S2.
Figure 4. The MEK5-ERK5 Pathway Mediates BMP-4-Induced Self-Renewal of mESCs

(A) Screening for the effects of low molecular weight kinase inhibitors which target components of non-SMAD pathways using colony-forming assays. Cells were maintained in LIF+BMP-4 supplemented with indicated inhibitors for 4–5 days. Left: Data represent

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DISCUSSION

BMP ligands induce the outgrowth of ICM during the derivation of mESCs (Di-Gregorio et al., 2007; Ying et al., 2003). Inactivation of either Bmp4 or Bmpr1a (which encodes ALK3) in mice results in early embryonic lethality due to impaired growth of the epiblast as early as embryonic day 6.5 (E6.5). However, Smad1- and/or Smad5-deficient mice exhibit abnormalities in extra-embryonic tissue development and angiogenesis from E7.5 or later (Derynck and Miyazono, 2008). In addition, Bmpr1a KO mESCs show self-renewal defects, while BMP-7 can rescue the defects through ALK2 activation (Di-Gregorio et al., 2007). Interestingly, activin/ALK4 can partially rescue the self-renewal defects of Bmpr1a KO mESCs (Di-Gregorio et al., 2007), suggesting that BMP and activin commonly activate non-SMAD pathway(s) and regulate the proliferative activity of epiblast at an early stage of development. Consistently, Smad1/5 dKO mESCs and Smad4 KO mESCs exhibit characteristics of naive pluripotency when maintained in LIF+serum (Figures 3 and S2) (Sirard et al., 1998).

KLFs are zinc finger proteins that belong to the Specificity protein (Sp)/KLF family of transcription factors. Among them, Sp1 has been shown to cooperate with SMAD transcription factors (Derynck and Miyazono, 2008). KLF2/4/5 contain conserved zinc finger DNA binding domain in the C-terminal regions and are functionally redundant in naive mESCs (Jiang et al., 2008). However, recent reports described that KLF4/5 are targets of LIF-STAT3 and NANOG, while KLF2 protein is a direct target of ERK2 MAP kinase (Aksoy et al., 2014; Hall et al., 2009; Yeo et al., 2014), suggesting that KLF4/5 and KLF2 have distinct functions. In addition, we revealed that KLF4, but neither KLF2 nor KLF5, physically interacts with SMAD1 and suppresses the transcriptional activity of SMAD complex (Figure 2). Thus, KLF4 has additional properties by interacting with SMAD1 possibly through the N-terminal region, which is divergent among KLF2/4/5.

To conclude, we have dissected the roles of BMP signaling in self-renewal of naive mESCs and have illustrated that BMP exerts its self-renewing effect through distinct functions of different KLFs. BMP can both strengthen naive pluripotency through ERK5-KLF2 and induce differentiation via other pathways (Figure S3E). The balance between the two pathways determines the effect of BMP signaling in mESCs; suppression of the SMAD pathway through LIF-STAT3-KLF4 and application of the MEK1/2 inhibitor can help to sustain the naive pluripotency. Our findings demonstrate an important role of the MEK5-ERK5 pathway in mESCs. Future studies will aim to elucidate the roles of ERK5, which can be activated by BMP, TGF-β/activin and FGF2, in PSCs of mouse and other species.

EXPERIMENTAL PROCEDURES

Cell Culture
Feeder-free E14 mESCs were maintained in media containing 15% fetal bovine serum or in the serum-free LIF+BMP-4 condition. Differentiation and maintenance of ESD-EpiSCs were essentially as described by Zhang et al. (2010). C57BL/6-background mESCs were maintained in the feeder-free 2iL condition. For details see Supplemental Experimental Procedures.

Statistical Analysis
All data are reported as the mean ± SEM. The difference between experimental groups were analyzed using Student’s t test, with p < 0.05 being considered significant.

Zebrafish Experiments
All zebrafish experiments were carried out in compliance with the guidelines at the Zebrafish Technology Platform, Science for Life Laboratory, Uppsala University.
ACCESION NUMBERS
The accession number for sequencing data described in this report is GEO: GSE70581.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.12.004.

AUTHOR CONTRIBUTIONS
M.M. conceived the study. M.M. performed most of the experiments, with the assistance of A.S., D.K., A.M., N.K., S.T., and K.H. T.W. provided important materials. M.M. and D.K. wrote the original draft. H.A., C.-H.H., and K.M. supervised the project and wrote the manuscript.

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