BMP4 promotes mouse iPS cell differentiation to male germ cells via Smad1/5, Gata4, Id1 and Id2

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Abstract

Generation of male germ cells from pluripotent cells could provide male gametes for treating male infertility and offer an ideal model for unveiling molecular mechanisms of spermatogenesis. However, the influence and exact molecular mechanisms, especially downstream effectors of BMP4 signaling pathways, in male germ cell differentiation of the induced pluripotent stem (iPS) cells, remain unknown. This study was designed to explore the role and mechanism of BMP4 signaling in the differentiation of mouse iPS cells to male germ cells. Embryoid body (EB) formation and recombinant BMP4 or Noggin were utilized to evaluate the effect of BMP4 on male germ cell generation from mouse iPS cells. Germ cell-specific genes and proteins as well as the downstream effectors of BMP4 signaling pathway were assessed using real-time PCR and Western blots. We found that BMP4 ligand and its multiple receptors, including BMPR1a, BMPR1b and BMPR2, were expressed in mouse iPS cells. Real-time PCR and Western blots revealed that BMP4 could upregulate the levels of genes and proteins for germ cell markers in iPS cells-derived EBs, whereas Noggin decreased their expression in these cells. Moreover, Smad1/5 phosphorylation, Gata4 transcription and the transcripts of Id1 and Id2 were enhanced by BMP4 but decreased when exposed to Noggin. Collectively, these results suggest that BMP4 promotes the generation of male germ cells from iPS cells via Smad1/5 pathway and the activation of Gata4, Id1 and Id2. This study thus offers novel insights into molecular mechanisms underlying male germ cell development.

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Introduction

In the last few years, several groups have shown that embryonic stem (ES) cells and the induced pluripotent stem (iPS) cells can differentiate into male germ cells (Kee et al. 2009, Easley et al. 2012, Lim et al. 2014, Ramathal et al. 2014). We have previously demonstrated that mouse iPS cells have the potential to give rise to male germ cells in vitro (Yang et al. 2012, Li et al. 2013). Numerous studies have reported that bone morphogenetic proteins (BMPs) have essential function in primordial germ cell (PGC) specification and germ cell lineage commitment from pluripotent cells (Wei et al. 2008, Hiller et al. 2011). However, little is known about the role and mechanisms of BMP4 signaling in generating male germ cells from iPS cells.

BMPs belong to members of transforming growth factor β (TGF-β) superfamily, and they play various kinds of roles in regulating the self-renewal of stem cells and developmental processes, including neural, hematopoietic, cardiomyogenic and hepatic lineage formation (Varga & Wrana 2005, Fei et al. 2010). As examples, BMPs are involved in directing both self-renewal and differentiation of ES cells. BMPs, working with LIF, maintain the self-renewal state of mouse ES cells (Yang et al. 2003, Li et al. 2012). As a mesoderm inducer, BMPs induce both ES and iPS cells to differentiate to PGCs (Wei et al. 2008, Saitou & Yamaji 2010, Panula et al. 2011). Interestingly, BMPs could efficiently coax germ cell generation from human ES or iPS cells (Panula et al. 2011). BMPs can also regulate both spermatogonial proliferation and differentiation in vitro or in vivo. For instance, BMP4 exerts both mitogenic and differentiation effect on undifferentiated spermatogonia (Hu et al. 2004, Itman & Loveland 2008). Collectively, as
a pivotal factor, BMPs might be closely associated with ES or iPS cell differentiation into all three germ layers and eventually germ cells (Li & Chen 2013).

BMPs initiate the signal through binding to their multiple receptors, including transmembrane type I receptors BMPR1a or BMPR1b and type II receptor BMPR2, which phosphorylates receptor-activated Smads (R-Smads, Smad1/5/8) to form a complex with common Smad (co-Smad and Smad4). The Smad complex translocates into the nucleus and regulates downstream target gene expression through other DNA-binding factors and transcription factors in the nucleus (Shi & Massague 2003, Fei et al. 2010). Despite progress in classical BMP4 signaling cascades, the influence and exact molecular mechanisms, especially downstream effectors of BMP4 signaling pathways, in male germ cell differentiation of iPS cells, remain to be elucidated. The generation of male germ cells from iPS cells offers an ideal system for exploring molecular mechanisms underlying germline formation. To better understand the role and mechanisms of BMP4 signaling in regulating the differentiation of iPS cells into male germ cells, here we have for the first time examined the effect of BMP4 and its inhibitor Noggin on the male germ cell lineage commitment of mouse iPS cells. Moreover, we probed the expression, function and signaling transduction pathway of BMP4 during the differentiation of iPS cells into male germ cells.

Materials and methods

Mouse iPS cells and EB culture with BMP4 and/or Noggin

The mouse iPS cell line (Tg-GFP-miPS11.1) was a kind gift from Prof. Ying Jin, Shanghai Jiao Tong University School of Medicine, Shanghai, China. These iPS cells were generated from neural progenitor cells of EGFP transgenic C57BL/6j mice via retroviral transfer of Oct4/Sox2/C-Myc/Klf4 (Li et al. 2009). Notably, this cell line has been demonstrated to be germline competent (Li et al. 2009). iPS cells were cultured in the medium according to the procedure as described previously (Yang et al. 2012). Briefly, iPS cells were co-cultured on feeder cells, namely irradiated mouse embryonic fibroblasts (MEFs), in high glucose DMEM containing 15% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 mg/mL penicillin plus 100 mg/mL streptomycin (Gibco), 2 mM l-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Gibco) and 0.1 mM non-essential amino acids (NEAA; Gibco). The cells were passaged every 2–3 days, and culture was maintained at 37°C in a humidified incubator with 5% CO₂.

Embryoid bodies (EBs) were formed by hanging drop and suspension culture method as follows: iPS cells were digested to become single cells and transferred to low-attachment dishes containing the above culture medium without LIF. EBs were treated without or with 10, 50 and 100 ng/mL BMP4 (R&D) and/or 10, 50 and 100 ng/mL Noggin (R&D) for 7 days.

RNA extraction, RT-PCR and quantitative real-time PCR

Total RNA was extracted from iPS cells and EBs derived from iPS cells using TRIzol (Invitrogen). After DNase treatment to remove potential contamination with genomic DNA, 1 μg of total RNA was transcribed into cDNA using First-Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA). PCR was performed according to the protocol as described previously (Guo et al. 2015, Liu et al. 2015), and quantitative real-time PCR was performed using SYBR Green master mix and a 7500 Fast Real-Time PCR System (Applied Biosystems) pursuant to the method as described previously (Yang et al. 2014). The primer sequences of genes used for PCR and real-time PCR were designed and listed in Tables 1 and 2 respectively. Real-time PCR products were quantified using the comparative C_{t} (threshold cycle) method. The threshold of cycle values was normalized against the threshold value of mouse Gapdh.

Immunocytochemistry

The expression of BMP4 and its receptors as well as germ cell-specific markers in EB cells was obtained by immunocytochemistry according to the procedure described previously (Liu et al. 2015). Briefly, iPS cells were fixed in 4%
parafomaldehyde (PFA) and permeabilized with 0.4% Triton X-100. The cells were blocked with 10% serum and incubated with primary antibodies against BMP4 (Abcam), BMPR1A (Santa Cruz), BMPR1B (Santa Cruz), BMPR2 (Santa Cruz), PRDM1 (Abnova, Taipei, Taiwan) and VASA (Abcam) overnight at 4°C. The detailed information on antibodies was shown in Table 3. The cells were then incubated by goat anti-rabbit Alexa Fluor 594 (red)-labeled secondary antibody (Invitrogen) for 1 h. DAPI was used to label cell nuclei, and images were captured with a fluorescence microscope (Leica).

### Western blots

The EBs derived from iPS cells were lysed using the RIPA buffer (Santa Cruz) containing a cocktail of protease inhibitors (Roche). Cell lysates were cleared by centrifugation at 12,000 g, and the concentrations of total proteins were measured by BCA kit (Dingguo Company, Beijing, China). Twenty micrograms of total protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with 5% BSA and 0.1% Tween20 for 1 h at room temperature, membranes were probed with primary antibodies, including PRDM1 (Abnova), VASA (Abcam), DAZL (Abcam), UCHL1 (Abd Serotec Kidlington, UK), GFRA1 (Santa Cruz), KIT (Abcam), phosho-Smad1/5 (Cell signaling), Smad5 (Cell signaling) and ACTB (beta-actin, Proteintech) overnight at 4°C. The detailed information on antibodies was shown in Table 3. The blots were incubated with HRP-conjugated anti-rabbit or anti-goat IgG polyclonal secondary antibodies (Santa Cruz) at 1:2500 dilution for 1 h at room temperature. After
extensive washes with TBST, the blots were visualized using an enhanced-chemiluminescent detection kit (Santa Cruz).

**Statistical analysis**

All the values were presented as mean ± S.E.M. from at least three independent experiments. Statistical differences were evaluated using the analysis of variance (ANOVA), and \( P < 0.05 \) was considered statistically different.

**Results**

**BMP4 and its receptors were expressed in mouse iPS cells**

To examine whether BMP4 signals via an autocrine or a paracrine pathway in mouse iPS cells, RT-PCR and immunocytochemistry were performed to detect the expression of BMP4 and its multiple receptors. As shown in Fig. 1A, the mRNA of Bmp4 and its receptors, including Bmpr1a, Bmpr1b and Bmpr2, were detectable in iPS cells. Immunocytochemistry further showed both BMP4 ligand (Fig. 1B) and its three receptors, i.e. BMPR1a, BMPR1b and BMPR2 were expressed in iPS cells (Fig. 1C). These results implicate that BMP4 acts via an autocrine or paracrine manner in mouse iPS cells.

**BMP4 affected male germ cell differentiation of iPS cells**

To determine the role of BMP4 in regulating the differentiation of mouse iPS cells, expression levels of germ cell-specific genes in iPS cells, EBs without treatment (Control), EBs treated with 100 ng/mL BMP4 or BMP4 antagonist Noggin were compared using RT-PCR. Pluripotency markers, e.g. Oct4, Sox2 and Nanog, were detected in the undifferentiated mouse iPS cells (Fig. 2A). As shown in Fig. 2B, the expression of germ cell-specific markers, including Stella and Blimp1, and hallmarks of primordial germ cells (PGCs) (Saitou et al. 2005, Wei et al. 2008, Hayashi & Surani 2009), was...
enhanced in BMP4-treated EBs compared to the untreated EBs and Noggin-treated EBs. Transcripts of male germ cell-specific gene Vasa (Silva et al. 2009) and differentiating spermatogonial marker Kit (He et al. 2007) and meiotic germ cell marker Scp3 (West et al. 2006) were upregulated in BMP4-treated EBs compared with the untreated EBs and Noggin-treated EBs. However, expression of haploid germ cell marker Prm1 (Kerr & Cheng 2010) was undetected in BMP- or Noggin-treated EBs and -untreated EBs.

To further examine the expression of major germ cell lineage markers in iPS cell-derived cells, iPS cell-derived EBs were treated with BMP4 and assessed for the expression of germ cell-specific genes and proteins. Real-time PCR revealed that the expression levels of PGC markers Blimp1 and Stella were remarkably upregulated in EBs treated with 10–100 ng/mL BMP4 compared to the control (Fig. 3A). Similarly, the transcripts of male germ cell-specific genes, including Vasa and Kit, and meiotic germ cell marker Scp1 were expressed at highest levels in EBs with 100 ng/mL BMP4 treatment (Fig. 3A). In addition, the transcription of Dazl, Boule and Scp1, markers of early germ cells and meiotic germ cells, was significantly upregulated in EBs treated with 50 ng/mL BMP4 compared to the control (Fig. 3A).

We next probed the impact of BMP4 on the expression of germ cell-specific proteins in EBs derived from iPS cells. Western blots showed that exposure of iPS cells to BMP4 at 10–100 ng/mL led to higher expression levels of PGC marker (PRDM1), germ cell hallmarks (VASA and DAZL) and spermatogonial markers (UCHL1, GFRA1 and KIT) (Kwon et al. 2004, He et al. 2007) in a dose-dependent manner compared with the control (Fig. 3B and C). Collectively, these data, together with real-time PCR results, suggest that BMP4 stimulated the differentiation of iPS cells into male germ cells phenotypically.

Noggin signaling blocked the differentiation of iPS cells into male germ cells

To further determine the effect of BMP4 on male germ cell differentiation of iPS cells, iPS cell-derived EBs were treated without or with different doses of BMP4 antagonist, namely Noggin (10, 50 and 100 ng/mL) to assess the expression changes of germ cell-specific genes and proteins. Real-time PCR showed that the expression of Stella, Dazl and Scp3 were significantly reduced in EBs...
treated with 100 ng/mL Noggin (Fig. 4A). Meanwhile, no obvious change was observed in the expression of other germ cell-specific genes, e.g. Blimp1, Fragilis, Vasa, Kit, Boule and Scp1 (Fig. 4A).

We also evaluated the effect of Noggin on protein expression of germ cell markers in EBs. Western blots displayed that the translation of almost all germ cell-specific markers, including VASA, DAZL, UCHL1, GFRA1 and KIT was significantly decreased in EBs treated with 100 ng/mL Noggin compared to the control without Noggin (Fig. 4B and C). Therefore, 100 ng/mL Noggin was chosen as a standard dosage in further experiments. Together, these results reflect that Noggin blocks BMP4 signaling in promoting the differentiation of mouse iPSCs into male germ cells.

**BMP4 induced iPSC cell differentiation via the Smad1/5 pathway**

We further evaluated the level of phosphorylated Smad1/5 in EBs exposed to 100 ng/mL BMP4 or 100 ng/mL Noggin. As shown in Fig. 5A and B, Smad1/5 phosphorylation was remarkably enhanced in EBs with 100 ng/mL BMP4 treatment compared to the control without treatment. In contrast, Smad1/5 phosphorylation was obviously decreased in EBs with 100 ng/mL Noggin treatment compared to BMP4-untreated control. Notably, Noggin blocked the increase of Smad1/5 phosphorylation induced by BMP4 (Fig. 5A and B). Considered together, these data indicate that BMP4 signals via the activation of Smad1/5 pathway in mouse iPSC cell differentiation into male germ cells.

**Gata4 transcription was activated during the differentiation of iPSC cells to male germ cells**

To determine which transcription factors were involved in the BMP4-induced male germ cell generation from iPSC cells, we further determined the mRNA levels of several transcription factors in EBs treated with 100 ng/mL BMP4 or Noggin. As shown in Fig. 5C, BMP4 significantly enhanced the expression of Gata4, whereas Noggin counteracted the increases of Gata4. There was no obvious change with mRNA of Fos, whereas BMP4 diminished the transcripts of Crebbp and Jun (Fig. 5C). Therefore, transcription factor Gata4 might be involved in the BMP4-induced male germ cell differentiation of iPSCs.

**Id1 and Id2 were the targets of BMP signaling in iPSC cells**

Id family genes might be the targets of BMPs signaling pathway (Hollnagel et al. 1999), we finally asked whether BMP4 induced the transcripts of Id family genes during the differentiation of iPSC cells into male germ cells. As shown in Fig. 5D, the transcripts of Id1, Id2, Id3 and Id4 were increased in EBs exposed to 100 ng/mL BMP4 compared to those in EBs without treatment. Notably, Noggin blocked the expression of Id1 and Id2 but enhanced the expression of Id4. Meanwhile, no significant difference of Id3 expression was observed between the control and Noggin-treated EBs. Taken together, these data implicate that Id1 and Id2 might be the targeting genes of BMP4 in inducing male germ cell formation from iPSCs.
Germ cell generation from iPS cells by BMP4

In the past decades, numerous studies have shown the generation of male germ cells from ES cells and iPS cells (West et al. 2010, Hayashi et al. 2011, Panula et al. 2011). Despite the potential tumor-forming risks and genetic aberration, iPS cells hold a great promise to shed novel insights into germ cell development due to fewer ethical issues and sufficient cells (Singh et al. 2015). However, little is known about the molecular mechanisms underlying male germ cell differentiation from pluripotent stem cells. BMP4 has been suggested to be involved in regulating self-renewal and inducing germ cell differentiation of both ES cells and iPS cells, but the exact roles and signaling transduction pathway of BMP4 in directing male germ cell formation from these cells are still unclear. In this study, mouse iPS cells were used as a model to examine the function and molecular mechanisms of BMP4 signaling in controlling male germ cell development. We found that the transcripts and protein of BMP4 and its three receptors were present in mouse iPS cells, suggesting that BMP4 acts via an autocrine pathway in these cells, which is similar to mouse ES cells (Ying et al. 2003).

To further uncover the function of BMP4 in regulating male germ cell differentiation of mouse iPS cells, expression levels of germ cell genes and proteins at different developmental stages were evaluated in BMP4- or Noggin-treated EBs. We found that BMP4 could enhance the expression of PGC-specific markers, including Blimp1, Fragilis, Stella and PRDM1, reflecting that BMP4 induced the generation of PGCs from mouse iPS cells, which was consistent with previous findings (Park et al. 2009, Panula et al. 2011). Interestingly, the expression levels of a number of hallmarks for germ cells, spermatogonia and spermatocytes, including VASA, DAZL (Kee et al. 2009), KIT (Pellegrini et al. 2003, West et al. 2010, Barrios et al. 2012), UCHL1 (He et al. 2010), GFRA1 (He et al. 2007), Boule (Kee et al. 2009), Scp1 and Scp3 (Yang et al. 2014), were increased by BMP4 in a dose-dependent manner, implicating that BMP4 was able to stimulate male germ cell formation of mouse iPS cells.

BMPs antagonist Noggin can be activated with BMP treatment in many cell types, e.g. neural precursor cells and mouse pre-osteoblastic cells, to counteract BMP signaling (Wang et al. 2010, Krause et al. 2011). To further explore the role of BMP4 signaling in mediating germ cell differentiation of pluripotent stem cells, iPS cell-derived EBs were treated with different concentrations of Noggin. Our data showed that the transcripts of several germ cell-specific genes, e.g. Stella, Dazl and Scp3 were decreased by Noggin, and notably, the translation of numerous germ cell-specific markers, including VASA, DAZL, KIT, UCHL1 and GFRA1 were also reduced by Noggin. These results clearly implicate

**Discussion**

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that BMP signaling was blocked by Noggin in mouse iPS cells and further verify that BMP4 plays an essential role in promoting male germ cell differentiation of these cells.

It remains unclear about how BMP signaling regulates cell fate decisions through downstream effectors. Smad1 and Smad5 (Smad1/5) have been suggested to be downstream effectors of BMP pathway (Zeng et al. 2010, Bragdon et al. 2011). We observed that Smad1/5 phosphorylation was upregulated in BMP4-treated EBs but downregulated in Noggin-treated EBs compared to the control, suggesting that BMP4 acts through the Smad1/5 signaling pathway. Strikingly, phosphorylation of Smad1/5 was also observed in the control group without exogenous BMP4, indicating the presence of endogenous BMP4 signaling in EBs derived from iPS cells. Several studies have reported that transcription factors Gata4 (Rojas et al. 2005, Klaus et al. 2012), Crebbp (Vlacic-Zischke et al. 2011), Fos (Wu et al. 2008) and Jun (Hollnagel et al. 1999) are involved in BMP signaling pathway. We employed real-time PCR to identify candidate transcription factors required for male germ cell differentiation of iPS cells. We found that Gata4 transcription was increased by BMP4 in mouse iPS cells. Notably, the increase of Gata4 transcript by BMP4 was blocked by Noggin, suggesting that BMP4 activates Gata4 transcription in male germ cell differentiation of iPS cells. GATA4 belongs to the GATA family of zinc finger transcription factor, which plays crucial roles in heart, reproductive system, gastrointestinal system, respiratory system and carcinogenesis (Suzuki 2011). Previous studies have shown that Gata4 is a downstream transcription factor for BMP4 signaling (Rojas et al. 2005, Guemes et al. 2014), which is in agreement with the observations in our study. In addition, we identified that Fos, Crebbp and Jun were not the targeting transcription factors of BMP4 in mouse iPS cells.

The members of Id gene family have been demonstrated to be dominant targets of BMP/Smad signaling (Hollnagel et al. 1999, Ying et al. 2003, Li et al. 2012). Significantly, we found that the transcripts of Id1 and Id2 were remarkably enhanced by BMP4 and suppressed by Noggin, which suggests that Id1 and Id2 are the targeting genes involved in BMP4-induced male germ cell differentiation of mouse iPS cells. However, the expression pattern of Id gene family in this study was different from those of previous findings (Hollnagel et al. 1999, Ruzinova & Benezra 2003), which may be due to the cell-type-specific manner of Id genes in response to BMP treatment. Interestingly, Id4 expression displayed a distinct pattern from those of Id1, Id2 and Id3, implying that the molecular pathways regulated by Id4 are not similar to those regulated by Id1, Id2 and Id3, which was consistent with previous observations (Patel et al. 2015, Sharma et al. 2015). Further investigation on the role of GATA4 and ID family members in regulating the differentiation of mouse and human iPS cells into male germ cells needs to be clarified.

Conclusion

In conclusion, we have for the first time demonstrated that mouse iPS cells express BMP4 and its multiple receptors and BMP4 promotes male germ cell differentiation of iPS cells via Smad1/5 pathway and the activation of Gata4, Id1 and Id2 pathway. The signaling transduction pathway of BMP4 has been illustrated in Fig. 6. Briefly, BMP4 initiates signaling by binding to BMPR1a, BMPR1b and BMPR2, which activates Smad1 and 5. The phosphorylated Smad1 and 5 translocates into the nucleus to upregulate the transcripts of transcription factors Gata4 and target genes Id1 and Id2. This study thus provides novel insights into molecular mechanisms underlying male germ cell differentiation of pluripotent stem cells. It would be interesting to probe whether BMP4 stimulates the differentiation of human iPS cells and human ES cells into male germ cells through the Smad1/5/Gata4/Id1/Id2 pathway.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Germ cell generation from iPSCs by BMP4


