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 induce 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.

Reproduction (2017) **153** 211–220

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 (Ying *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 BMPRIa or BMPRIb and type II receptor BMPRII, 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 leukemia inhibiting factor (LIF; Millipore), 100 U/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 μ g 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%

Table 1 Primers of genes used for RT-PCR.

Genes	Primer sequences	Product size (bp)	T _m (°C)	Number of cycles
<i>Bmp4</i>	Forward 5'-TAGTCCCAAGCATCACCC-3' Reverse 5'-TCTCAGCGGCATCCAC-3'	354	56	35
<i>Bmpr1a</i>	Forward 5'-TCAGGGAGAAACCACAT-3' Reverse 5'-CTGCCATCAAAGAACG-3'	180	50	35
<i>Bmpr1b</i>	Forward 5'-TGGATGGGAAAGTGGC-3' Reverse 5'-GGTGTGGGTGGGATG-3'	456	53	35
<i>Bmpr2</i>	Forward 5'-GGAGCGAAATGAAGGG-3' Reverse 5'-CACCGATGTGAGACCAA-3'	470	53	35
<i>Oct4</i>	Forward 5'-ATGGCATACTGTGGACCTCA-3' Reverse 5'-AGCAGCTTGGCAAACCTGTC-3'	236	58	35
<i>Sox2</i>	Forward 5'-GCGGAGTGGAACTTTTGTCC-3' Reverse 5'-GCGAAGCGTACTTATCCTTCT-3'	156	60	35
<i>Nanog</i>	Forward 5'-TCGCCCTTCTCTGAAGAC-3' Reverse 5'-TGCTTCTGAAACCTGTCCTTGA-3'	195	60	35
<i>Gapdh</i>	Forward 5'-TCTCTGCGACTTCAACA-3' Reverse 5'-TGGTCCAGGGTTTCTTACT-3'	178	55	35

Table 2 Primers of genes used for real-time PCR.

Genes	Primer sequences	Product size (bp)	T _m (°C)	Number of cycles
<i>Blimp1</i>	Forward 5'-CTTCTCTTGGAAAAACGTGTGGG-3' Reverse 5'-TCATATCAGCGTCTCCATGT-3'	203	60	40
<i>Fragilis</i>	Forward 5'-CTCCATCCTTTGCCCTCCAG-3' Reverse 5'-GTAGTTTGGGGGCTGTCCTC-3'	100	60	40
<i>Stella</i>	Forward 5'-GGGAAGTTCAAAGCGCCTTTC-3' Reverse 5'-ACTCATTTCTTCGAGCCTTTT-3'	129	60	40
<i>Vasa</i>	Forward 5'-ATTCTTGGAAAGTCAGAAGCAGAAG-3' Reverse 5'-CTGGTTGACCAATTCTCGAGT-3'	467	60	40
<i>Dazl</i>	Forward 5'-TCTGCCACAACCTCTGAGGC-3' Reverse 5'-CCTGATTTCGGTTTCATCCATCC-3'	168	60	40
<i>Kit</i>	Forward 5'-CCCCACGCAACTTCCTTA-3' Reverse 5'-CGCTTCTGCCTGCTCTTC-3'	385	58	40
<i>Boule</i>	Forward 5'-CCATTCCTATATCTGCAACCTTCT-3' Reverse 5'-GGTCAGAATAAGGCTCTGGAAC-3'	124	58	40
<i>Scp1</i>	Forward 5'-GAGCCAATGAGCAGACTGTAT-3' Reverse 5'-CGCTGGGCTTCAATTATCTTTC-3'	137	58	40
<i>Scp3</i>	Forward 5'-CCAGGTTTCCTCAGATGCTT-3' Reverse 5'-AAGGTGGCTTCCCAGATTTC-3'	123	57	40
<i>Crebbp</i>	Forward 5'-TGTGCATACTCAGCCTCCTG-3' Reverse 5'-TGGGAAGTGGTTTCAGCACT-3'	105	60	40
<i>Gata4</i>	Forward 5'-CAGTTGTGGTGGTGGGTTT-3' Reverse 5'-AGGGCAGGGCTTCTATGTCT-3'	125	60	40
<i>Fos</i>	Forward 5'-GGATTGACTGGAGGTCTGC-3' Reverse 5'-TTGCTGATGCTCTTGACTGG-3'	120	60	40
<i>Jun</i>	Forward 5'-ACAGACAGACGGACGGACTT-3' Reverse 5'-GAAGGGACTCTCCAAATGCTC-3'	147	58	40
<i>Id1</i>	Forward 5'-GCATCTTGTGTCGCTGAGG-3' Reverse 5'-GCGGTAGTGTCTTTCCAGA-3'	118	60	40
<i>Id2</i>	Forward 5'-CCCGATGAGTCTGCTCTACA-3' Reverse 5'-GCAGGATTCCATCTTGGTC-3'	104	59	40
<i>Id3</i>	Forward 5'-GCATGGATGAGCTTCGATCT-3' Reverse 5'-CTCTTCAGGCCACCCAAGT-3'	111	60	40
<i>Id4</i>	Forward 5'-CAGTGCATATGAACGACTGC-3' Reverse 5'-GACTTCTTGTGGGCGGGAT-3'	72	60	40
<i>Gapdh</i>	Forward 5'-AGAACATCATCCCTGCATCC-3' Reverse 5'-CACATTGGGGGTAGGAACAC-3'	110	58	40

paraformaldehyde (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).

Table 3 Detailed information on the antibodies used for immunocytochemistry and Western blots.

Antibodies	Sources (product number)	Dilutions
BMP4	Abcam (ab39733)	1:200 (Immunocytochemistry)
BMPR1A	Santa Cruz (sc-20736)	1:200 (Immunocytochemistry)
BMPR1B	Santa Cruz (sc-25455)	1:200 (Immunocytochemistry)
BMPR2	Santa Cruz (sc-20737)	1:200 (Immunocytochemistry)
PRDM1	Abnova (PAB13234)	1:200 (Immunocytochemistry)
VASA	Abcam (ab13840)	1:200 (Immunocytochemistry)
DAZL	Abcam (ab34139)	1:1000 (Western blots)
UCHL1	AbD Serotec (AHP739)	1:1000 (Western blots)
GFRA1	Santa Cruz (sc-10716)	1:1000 (Western blots)
KIT	Abcam (ab62154)	1:1000 (Western blots)
phospho-Smad1/5	Cell signaling (mAb #9516)	1:1000 (Western blots)
Smad5	Cell signaling (mAb #12534)	1:1000 (Western blots)
ACTB	Proteintech (20536-1-AP)	1:2000 (Western blots)

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 12,000g, 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), phospho-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 $\pm$ 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

Figure 1 Expression of BMP4 and its receptors BMPR1a, BMPR1b and BMPR2 in mouse iPS cells. (A) RT-PCR displayed the transcripts of *Bmp4*, *Bmpr1a*, *Bmpr1b* and *Bmpr2* in iPS cells. *Gapdh* served as the loading control of total RNA. (B and C) Immunohistochemistry revealed the translation of BMP4 (B), BMPR1a, BMPR1b and BMPR2 (C) in iPS cells. The expression of EGFP in these cells was also shown. Scale bars in (B) and (C) = 50 μ m.

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

Figure 2 BMP4-induced male germ cell differentiation of mouse iPS cells. (A) RT-PCR showed mRNA expression of *Oct4*, *Sox2* and *Nanog* in iPS cells, EBs without treatment (Control), EBs treated with 100 ng/mL BMP4 or Noggin, and mouse testis. (B) RT-PCR revealed mRNA expression of *Stella*, *Blimp1*, *Vasa*, *Kit*, *Scp3* and *Prm1* in iPS cells, EBs without treatment (Control), EBs treated with 100 ng/mL BMP4 or Noggin and testis. (C) Immunohistochemistry showed PRDM1 and VASA expression in EBs treated with 100 ng/mL BMP4. Scale bars = 50 μ m.

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 with BMP4 treatment were stained with PGC hallmark PRDM1 and male germ cell-specific marker VASA. Immunocytochemistry showed that PRDM1- and VASA-positive cells were observed in 30–40% of iPS cell-derived EBs (Fig. 2C), suggesting that iPS cells could differentiate into germ cells.

BMP4 signaling promoted male germ cell differentiation of iPS cells

To better understand the effect of BMP4 on male germ cell differentiation of iPS cells, iPS cell-derived EBs were treated without or with different concentrations of BMP4 (10, 50 and 100 ng/mL) to compare the expression changes 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

Figure 3 The effect of various concentrations of BMP4 signaling on the differentiation of mouse iPS cells into male germ cells. (A) Real-time PCR showed the transcripts of *Blimp1*, *Fragilis*, *Stella*, *Vasa*, *Dazl*, *Kit*, *Boule*, *Scp1* and *Scp3* in EBs treated without or with 10, 50 and 100 ng/mL BMP4. (B) Western blots displayed PRDM1, VASA, DAZL, UCHL1, GFRA1 and KIT protein expression in EBs treated with 0, 10, 50 and 100 ng/mL BMP4. ACTB was used as the control of loading proteins. (C) Quantification of PRDM1, VASA, DAZL, UCHL1, GFRA1 and KIT protein expression relative to ACTB in EBs treated with different doses of BMP4. Relative band intensities were calculated by densitometry normalized to ACTB. Data were presented with mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$ represented significant differences in BMP4-treated EBs compared to the control.

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 iPS cells into male germ cells.

BMP4 induced iPS 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 iPS cell differentiation into male germ cells.

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

To determine which transcription factors were involved in the BMP4-induced male germ cell generation from iPS 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 iPS cells.

Id1 and Id2 were the targets of BMP signaling in iPS 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 iPS 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 iPS cells.

Figure 4 The influence of different concentrations of Noggin signaling on the differentiation of mouse iPS cells into male germ cells.

(A) Real-time PCR revealed *Blimp1*, *Fragilis*, *Stella*, *Vasa*, *Dazl*, *Kit*, *Boule*, *Scp1* and *Scp3* transcripts in EBs treated with 0, 10, 50 and 100 ng/mL Noggin. (B) Western blots showed PRDM1, UCHL1, VASA, DAZL, GFRA1 and KIT protein expression in EBs treated with 0, 10, 50 and 100 ng/mL Noggin. ACTB served as the control of loading proteins. (C) Quantification of PRDM1, VASA, DAZL, UCHL1, GFRA1 and KIT protein expression in EBs treated with different doses of Noggin. Relative band intensities were calculated by densitometry normalized to ACTB. Data were presented with mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$ represented significant differences in Noggin-treated EBs compared to the control.

Figure 5 Expression of Smad1/5 phosphorylation, *Gata4*, *Crebbp*, *Fos* and *Jun* transcription as well as *Id1*, *Id2*, *Id3* and *Id4* in EBs derived from mouse iPS cells. (A) Western blots revealed the expression of Smad1/5 phosphorylation in EBs treated with 100 ng/mL BMP4 or 100 ng/mL Noggin. Smad5 and ACTB were used as a loading control of total proteins. (B) Quantification of phos-Smad1/5 expression in EBs treated with BMP4 or Noggin. Relative band intensities were calculated by densitometry normalized to ACTB. (C) Real-time PCR displayed the transcription of *Gata4*, *Crebbp*, *Fos* and *Jun* in EBs treated with BMP4 or Noggin. (D) Real-time PCR showed the expression of *Id1*, *Id2*, *Id3* and *Id4* in EBs exposed to BMP4 or Noggin. Data were presented with mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$ represented significant differences in BMP4- or Noggin-treated EBs compared with the control.

Discussion

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

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

Figure 6 The schematic diagram illustrated the transduction of BMP4 signaling pathway in the differentiation of mouse iPS cells into male germ cells. 'P' indicated 'phosphorylate'.

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.

Funding

This work was supported by grants from National Science Foundation of China (31201109, 31671550, 31171422, 31401250), key grants from National Nature Science

Foundation of China (31230048) and Chinese Ministry of Science and Technology (2016YFC1000606, 2014CB943101), The Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning (2012.53), Shanghai Municipal Education Commission-Gaofeng Clinical Medicine Grant Support (20152511), a grant from Shanghai Hospital Development Center (SHDC12015122), a key grant from the Science and Technology Commission of Shanghai Municipality (12JC1405900) and Key Discipline and Specialty Foundation of Shanghai Municipal Commission of Health and Family Planning.

Acknowledgements

The authors thank Prof. Ying Jin, Shanghai Jiao Tong University School of Medicine, China, for providing mouse iPS cell line.

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Received 31 May 2016

First decision 4 July 2016

Revised manuscript received 27 October 2016

Accepted 18 November 2016