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BMP signalling inhibits premature neural differentiation in the mouse embryo

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The specification of a subset of epiblast cells to acquire a neural fate constitutes the first step in the generation of the nervous system. Little is known about the signals required for neural induction in the mouse. We have analysed the role of BMP signalling in this process. We demonstrate that prior to gastrulation, Bmp2/4 signalling via *Bmpr1a* maintains epiblast pluripotency and prevents precocious neural differentiation of this tissue, at least in part by maintaining *Nodal* signalling. We find that during gastrulation, BMPs of the 60A subgroup cooperate with Bmp2/4 to maintain pluripotency. The inhibition of neural fate by BMPs is independent of FGF signalling, as inhibition of FGF signalling between 5.5 and 7.5 days post-coitum does not block neural differentiation in the mouse embryo. Together, our results demonstrate that inhibition of BMP signalling has a central role during neural induction in mammals and suggest that FGFs do not act as neural inducers in the post-implantation mouse embryo.

KEY WORDS: Neural induction, Bmpr1a, BMP signalling

INTRODUCTION

Bone morphogenetic proteins (BMPs) are a family of secreted growth factors belonging to the Tgfβ superfamily. They bind to a heterotetrameric receptor complex composed of type I and type II receptors to activate an intracellular pathway that has Smad1/5/8 (Smad8 is also known as Smad9 – Mouse Genome Informatics) and Smad4 as crucial effector molecules (Shi and Massague, 2003). In mouse, although a large number of BMPs have been identified, only Bmp2 and Bmp4 have been shown to have roles during early embryonic development. Analysis of null mutations for Bmp2 and Bmp4 has revealed multiple roles for these proteins during the early post-implantation period, for example in primordial germ cell (PGC) induction (Lawson et al., 1999), mesoderm and node formation, primitive streak morphogenesis (Fujiwara et al., 2001; Winnier et al., 1995), extra-embryonic mesoderm development (Zhang and Bradley, 1996) and left-right patterning (Fujiwara et al., 2001). The fact that embryos carrying mutations in either type I or type II BMP receptors display phenotypes that are more severe than those of Bmp2 or Bmp4, suggests that functional redundancy exits between BMP proteins (Mishina, 2003).

In *Xenopus*, chick and zebrafish, BMP signalling has been shown to have a central role in neural induction (Harland, 2000). Although there is much debate regarding the precise mechanism for this process, two main models have been proposed. The first, based primarily on experiments performed in *Xenopus*, argues that BMP signalling prevents a 'default' neural differentiation of the naïve ectoderm. Central to this model is the idea that secretion of BMP antagonists protects the presumptive neural plate from the action of BMPs and allows neural differentiation to occur (Vonica and Brivanlou, 2006). Experiments in both chick and *Xenopus* embryos have challenged this idea and have led to the proposal that inhibition of BMP signalling alone is not sufficient for neural induction and that other signals are required (Stern, 2005; Wilson and Edlund, 2001).

Recent reports have indicated that fibroblast growth factors (FGFs) may constitute one of these signals, as inhibition of FGF signalling blocks neural induction in chick (Streit et al., 2000; Wilson et al., 2000) and *Xenopus* (Delaune et al., 2005). The emerging picture of how FGFs promote neural differentiation seems to be complex. FGF signalling can repress BMP mRNA expression (Wilson et al., 2000) and downregulate BMP signalling intracellularly (Pera et al., 2003), but, in addition to this, FGF signalling is required for neural induction independently of BMPs (Aubin et al., 2004; Delaune et al., 2005; Linker and Stern, 2004). In zebrafish, it has been proposed that both BMP inhibition and FGF signalling can act as neural inducers, with BMP antagonism inducing anterior and FGFs inducing posterior neural fates (Kudoh et al., 2004).

In the mouse embryo, a role for BMPs in neural induction has not been shown. Single mutations in BMPs or BMP antagonist do not affect the initial size of the neural plate and overlapping activities have been proposed as an explanation for this lack of neural induction phenotype. More surprisingly, double mutants for the BMP antagonists chordin and noggin are able to form a neural plate although fail to maintain forebrain markers at later stages (Bachiller et al., 2000). In mouse embryonic stem (ES) cells, BMP proteins have been implicated in maintaining pluripotency. In ES cells, BMPs are required to induce the expression of the inhibitor of differentiation (Id) genes, an essential step for ES self-renewal (Ying et al., 2003a). Bmp4 has also been shown to be required for ES cell self-renewal by inhibiting mitogen-activated kinase pathways (Qi et al., 2004). The induction of Id genes in ES cells specifically blocks neural differentiation. The role of BMPs in maintaining ES cell self-renewal while blocking neural differentiation suggests that in the embryo, BMP-mediated inhibition of neural fate might be part of a mechanism to maintain epiblast pluripotency. In the mouse embryo, anterior neural markers are not expressed until 7.25 days post-coitum (dpc) (Yang and

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Klingensmith, 2006) and the epiblast remains pluripotent until this stage (Beddington, 1982; Beddington, 1983; Diwan and Stevens, 1976; Lawson et al., 1991). However, little is known about the mechanisms that maintain this pluripotency.

We have analysed the role of BMP signalling in patterning the epiblast of the mouse embryo. Three type I receptors have been shown to act downstream of BMPs: *Acvr1*, *Bmpr1a* and *Bmpr1b* (also known as *Alk2*, *Alk3* and *Alk6*). *Acvr1* expression is restricted to the visceral endoderm at 6.5 dpc. At 7.5 dpc, *Acvr1* is expressed in the extra-embryonic mesoderm, extra-embryonic ectoderm, in the mesoderm and in the visceral endoderm (de Sousa Lopes et al., 2004; Gu et al., 1999; Kishigami et al., 2004; Roelen et al., 1994). Expression of *Bmpr1b* has not been detected prior to 9.5 dpc (Dewulf et al., 1995). The ubiquitous expression of *Bmpr1a* after implantation makes it the only type I BMP receptor expressed in the epiblast (Mishina et al., 1995; Roelen et al., 1994). For this reason, to address the role of BMP signalling in the epiblast we have used a null mutation and an epiblast-specific deletion of *Bmpr1a*.

MATERIALS AND METHODS

Mouse lines and embryo genotyping

Bmpr1a$^{+/-}$ (Mishina et al., 1995) and *Bmpr1a*$^{fx/fx}$ mice (Mishina et al., 2002) were maintained on a 129SvCC genetic background. The *Bmpr1a*$^{+/-}$; *Sox2Cre*$^{+/-}$ mice were kept on a mixed 129SvCC/CBA/J/C57BL6 background. Embryos were genotyped after whole-mount in situ hybridisation or alkaline phosphatase staining as described (Martinez Barbera et al., 2000) using primers that are specific for the *Bmpr1a*-null mutation (Mishina et al., 1995), the *Bmpr1a*$^{fx/fx}$ mutation (Mishina et al., 2002) and *Sox2Cre* transgene (Hayashi et al., 2002).

Immunohistochemistry and whole-mount in situ hybridisation (WISH)

Staining for pSmad1/5/8 (a gift from Ed Laufer, Columbia University, New York, NY) was performed as described (Persson et al., 1998; Yang and Klingensmith, 2006). WISH was carried out following standard procedures (Thomas and Beddington, 1996).

Western blotting

Western blots were carried out following manufacturers conditions for the pSmad1/5/8 antibody (Cell Signaling)

Inhibition of FGF signalling

For inhibition of FGF signalling, embryos from *Bmpr1a*$^{+/-}$ inter-cross litters or from *Bmpr1a*$^{fx/fx}$×*Bmpr1a*$^{+/-}$; *Sox2Cre*$^{+/-}$ crosses were dissected in M2 medium and cultured for 24 or 48 hours in 1:1 DMEM:rat serum plus 80 μM SU5402 (Sigma) or plus DMSO alone at 37°C, 5% CO_2, fixed in 4% paraformaldehyde (PFA), dehydrated through a methanol series and processed for WISH.

Blastocyst outgrowth assays and ES cell derivation

Blastocysts were harvested from *Bmpr1a*$^{+/-}$ intercrosses and cultured in gelatin-coated 48-well plates in ES cell medium containing 20% serum, or in serum-free medium with Bmp4 alone (ESGRO Clonal Grade, Chemicon) or supplemented with 200 ng/ml Bmp7 (R&D Systems), 20 ng/ml activin (Sigma), or 200 ng/ml activin for either 5 or 10 days. Blastocysts were then stained for alkaline phosphatase activity according to manufacturer's instructions (Sigma) or trypsinised and used to derive *Bmpr1a*$^{-/-}$ ES cells following standard feeder-free conditions (Nichols et al., 1990). Given that we were not aware that it was possible to derive *Bmpr1a*$^{-/-}$ ES cells in the absence of MAP kinase inhibition prior to our outgrowth experiments, *Bmpr1a*$^{-/-}$ *lacZ* ES cells were generated from parental ES cells homozygous for the floxed *Bmpr1a* allele and heterozygous for the *ROSA26lacZ* allele by infection of Cre-expressing adenovirus in the presence of the SB203580 MAP kinase inhibitor as previously described (Qi et al., 2004).

Generation of chimeric embryos

Chimeras were generated by blastocyst injection as described (Bradley and Robertson, 1986) and stained for *lacZ* expression following standard procedures (Hogan et al., 1994).

RESULTS

Maintenance of epiblast pluripotency in vivo requires BMP signalling

We first wanted to determine to what extent was BMP signalling affected in *Bmpr1a* mutant embryos. A critical step in the BMP signalling pathway is the activation by phosphorylation of Smad1/5/8 by the type I BMP receptors. The phosphorylated form of Smad1/5/8 (pSmad1/5/8) associates with Smad4 and translocates to the nucleus. In wild-type embryos at 5.5 dpc, pSmad1/5/8 expression was observed in the majority of visceral endoderm cells and in a mosaic fashion in the epiblast (Fig. 1A). By 6.5 dpc, pSmad1/5/8 expression had become restricted to the proximal epiblast and proximal visceral endoderm (Fig. 1C) and remained expressed in these cell populations until 7.5 dpc (Fig. 1F). By contrast, we found no expression of pSmad1/5/8 in *Bmpr1a*$^{-/-}$ embryos at 5.5 dpc (Fig. 1B). At 6.5 dpc, pSmad1/5/8 expression could be found in the proximal visceral endoderm of *Bmpr1a*$^{-/-}$ embryos, but was completely absent from the epiblasts of 25% of the mutant embryos (Fig. 1D) and present in a weak and mosaic manner in the epiblast of the rest of the *Bmpr1a*$^{-/-}$ embryos (Fig. 1E). By 7.5 dpc, no pSmad1/5/8 expression could be observed in *Bmpr1a*$^{-/-}$ embryos (Fig. 1G). These results indicate that *Bmpr1a* is the type I receptor required for BMP signalling in the epiblast during the pre- and early gastrulation stages.

A previous characterisation of the *Bmpr1a* mutant phenotype found that mutant embryos fail to gastrulate and do not express the mesoderm marker brachyury (T), suggesting that severe patterning defects are present (Mishina et al., 1995). In ES cells, BMP signalling has been implicated in the maintenance of pluripotency (Qi et al., 2004; Ying et al., 2003a). We therefore studied the state of differentiation of the epiblast of *Bmpr1a* mutants. For this we analysed the expression of *Oct4* (also known as *Pou5f1* – Mouse Genome Informatics), *Nanog* and *Fgf5*. *Oct4* is expressed throughout the epiblast at 5.5 and 6.5 dpc (Rosner et al., 1990). At 5.5 dpc, we observed no difference in the level of *Oct4* expression in *Bmpr1a* mutant embryos when compared with controls (Fig. 2A,B). However, at 6.5 dpc, half of the *Bmpr1a* mutant embryos displayed a clear downregulation of *Oct4* expression (Fig. 2C,D; n=4/8). *Nanog* is required for ES and inner cell mass pluripotency (Chambers et al., 2003; Mitsui et al., 2003). At 6.5 dpc, *Nanog* expression was observed in the proximal-posterior epiblast (Fig. 2E). In all *Bmpr1a*$^{-/-}$ embryos analysed at 6.5 dpc, *Nanog* expression was completely lost from the epiblast (Fig. 2F). *Fgf5* is a primitive-ectoderm marker and is expressed throughout the epiblast at 6.5 dpc (Fig. 2G) (Kaji et al., 2006; Sun et al., 1999). When we examined *Fgf5* expression in *Bmpr1a* mutant embryos we observed a severe downregulation or loss of expression of this gene (Fig. 2H). The downregulation or loss of *Oct4*, *Nanog* and *Fgf5* expression indicates that BMP signalling is required to maintain epiblast pluripotency.

Bmpr1a is required to inhibit premature neural differentiation of the epiblast

The downregulation of pluripotency markers suggests that a premature differentiation of the epiblast is occurring in *Bmpr1a* mutant embryos. In other vertebrates, inhibition of BMP signalling has been proposed as a central step in neural induction (Harland, 2000;

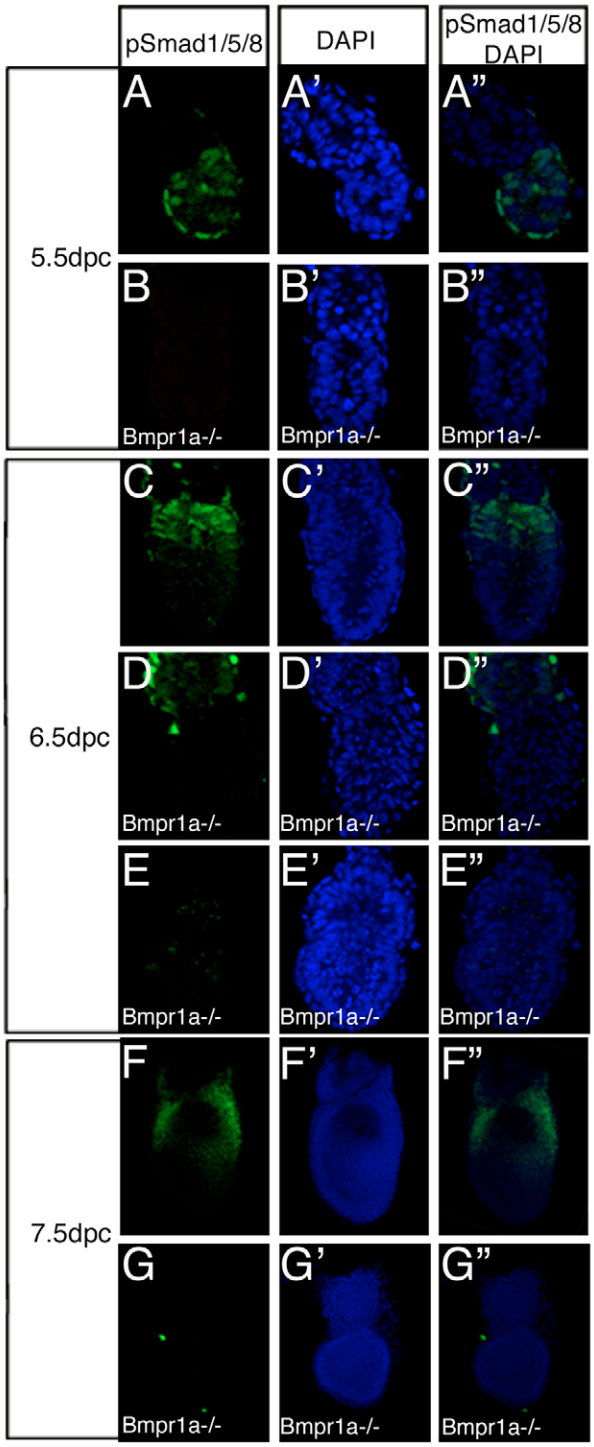

Fig. 1. *Bmpr1a* is essential for BMP signalling in the early mouse embryo. (**A**) At 5.5 dpc, pSmad1/5/8 expression is observed in the majority of visceral endoderm cells and in a mosaic fashion in the epiblast of control embryos, but (**B**) is completely lost in *Bmpr1a* mutant embryos (*n*=4). (**C**) At 6.5 dpc, pSmad1/5/8 is expressed in the proximal epiblast and visceral endoderm of control embryos. (**D**) In *Bmpr1a*$^{-/-}$ embryos, expression is either present in the visceral endoderm and lost from the epiblast (*n*=2) or (**E**) strongly downregulated in the epiblast (*n*=6). (**F**) At 7.5 dpc, pSmad1/5/8 expression can still be observed in the proximal epiblast and visceral endoderm of control embryos, but (**G**) is completely lost in *Bmpr1a* mutant embryos (*n*=4). (**A′-G′**) Nuclear DAPI-stained image; (**A″-G″**) overlay of pSmad1/5/8 and nuclear staining.

Vonica and Brivanlou, 2006). For this reason we investigated the possibility that the epiblast of *Bmpr1a*$^{-/-}$ embryos is undergoing neural differentiation. At 7.5 dpc, markers of the anterior neural ectoderm are expressed in a discrete patch of the anterior epiblast (Yang and Klingensmith, 2006). We analysed the expression of the anterior neural markers *Hesx1* and *Six3* and of the pan-neural marker *Sox1* and found that at 7.5 dpc, these genes are expressed throughout the epiblast of *Bmpr1a* mutant embryos (Fig. 2J,L,N; *n*=7/8, 4/6 and 7/8), indicating that ectopic neural differentiation of the epiblast is occurring in these embryos. *Foxg1* is a forebrain marker that is first expressed at the three-somite stage (Xuan et al., 1995). At 7.5 dpc, a distinct patch of *Foxg1* expression could be observed in *Bmpr1a*$^{-/-}$ embryos at the boundary between the embryonic and extra-embryonic regions (Fig. 2P; *n*=4/6), indicating that, at least to some degree, the ectopic neural tissue is regionalised in *Bmpr1a* mutants. We next analysed the expression of midbrain marker engrailed 1 (Davis and Joyner, 1988). In *Bmpr1a*$^{-/-}$ embryos, no engrailed 1 expression was observed at 7.5 dpc (data not shown, *n*=7), suggesting that there is only premature expression of anterior neural genes in these embryos.

Given that at 6.5 dpc we had found downregulation of pluripotency markers, we wanted to test whether neural differentiation of the epiblast was evident as early as 6.5 dpc. Interestingly, we found *Hesx1* to be expressed throughout the epiblast of 3/10 *Bmpr1a*$^{-/-}$ embryos, and *Six3* throughout the epiblast of 3/5 mutant embryos analysed at 6.5 dpc (Fig. 2R,L); this is a similar proportion to that of mutant embryos showing no pSmad/1/5/8 at this stage. The precocious expression of anterior neural gene markers coupled with the downregulation of pluripotent epiblast markers observed in *Bmpr1a*$^{-/-}$ embryos suggests that BMP signalling is required to prevent premature neural differentiation of the epiblast prior to gastrulation.

Ectopic neural induction is accompanied by suppression of mesoderm in *Bmpr1a*$^{-/-}$ embryos

A necessary step for neural induction to occur is the suppression of mesoderm and mesoderm-inducing signals in the prospective neural plate region. To test whether the loss of pluripotency in the epiblasts of *Bmpr1a*$^{-/-}$ embryos leads to any mesoderm differentiation we analysed the expression of *Fgf8*, *T* and eomesodermin. At 6.5 dpc, *Fgf8* is expressed in the posterior epiblast and visceral endoderm (Fig. 3A) (Crossley and Martin, 1995) and both eomesodermin and *T* are expressed in the posterior epiblast and extra-embryonic ectoderm (Fig. 3C,E) (Perea-Gomez et al., 2004; Russ et al., 2000). In *Bmpr1a* mutant embryos, *Fgf8* expression was lost from the epiblast, but could still be observed in the visceral endoderm (Fig. 3B) and expression of both eomesodermin and *T* was lost from the epiblast and strongly downregulated in the extra-embryonic ectoderm (Fig. 3D,F).

Two signalling molecules that have been shown to be essential for mesoderm specification are Nodal (Brennan et al., 2001) and Wnt3 (Liu et al., 1999). At 6.5 dpc, *Nodal* and the *Nodal* co-receptor *Cripto* (also known as *Tdgf1* – Mouse Genome Informatics) (Gritsman et al., 1999) were found to be expressed in the posterior epiblast (Fig. 3G,I), but expression of both genes was lost in *Bmpr1a* mutants (Fig. 3H,J). *Wnt3* is normally expressed in the posterior epiblast and adjacent visceral endoderm (Fig. 3K), but in embryos lacking *Bmpr1a*, expression of *Wnt3* was strongly downregulated or lost from both these tissues (Fig. 3L). The lack of *Fgf8*, eomesodermin, *T*, *Nodal*, *Cripto* and *Wnt3* expression in *Bmpr1a*$^{-/-}$ embryos indicates that the ectopic neural differentiation observed in these embryos is accompanied by a suppression of mesoderm and mesoderm-inducing signals.

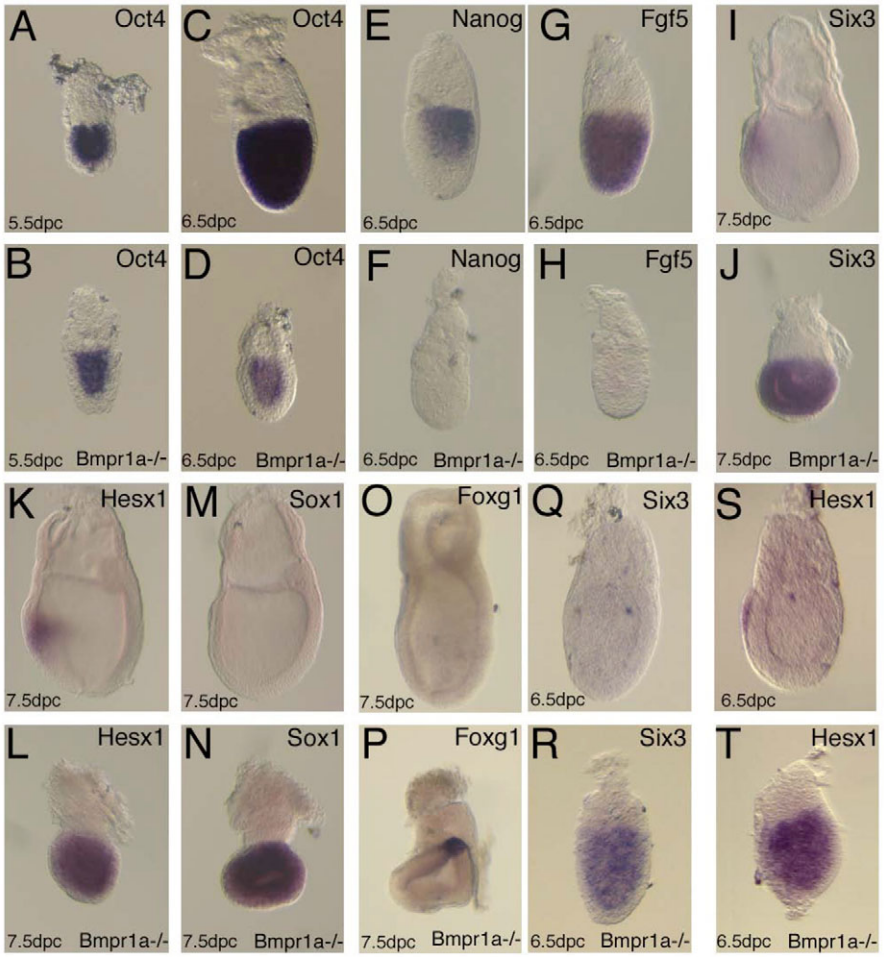

Fig. 2. Premature neural differentiation of the epiblast occurs in *Bmpr1a*$^{-/-}$ embryos. (**A**,**B**) At 5.5 dpc, *Oct4* expression is observed throughout the epiblast of control and *Bmpr1a* mutant mouse embryos (*n*=5). (**C**,**D**) At 6.5 dpc, *Oct4* is expressed in the epiblast of controls but its expression is downregulated in 50% of *Bmpr1a*$^{-/-}$ embryos (*n*=4/8). (**E**,**F**) *Nanog* expression is observed in the proximal posterior epiblast at 6.5 dpc in controls, but not in *Bmpr1a* mutant embryos (*n*=6). (**G**,**H**) At 6.5 dpc, *Fgf5* is expressed throughout the epiblast of controls but is strongly downregulated or absent in *Bmpr1a*$^{-/-}$ embryos (*n*=5). (**I-L**) At 7.5 dpc, *Six3* and *Hesx1* are expressed in the anterior neural ectoderm of controls, but in *Bmpr1a* mutant embryos expression of both these genes is observed throughout the epiblast (*n*=7/8 and *n*=4/6, respectively). (**M**,**N**) *Sox1* expression is not observed at 7.5 dpc in control embryos, but in *Bmpr1a*$^{-/-}$ embryos expression is observed in the entire epiblast (*n*=7/8). (**O**,**P**) No *Foxg1* expression is observed in control embryos at 7.5 dpc, but in *Bmpr1a*$^{-/-}$ embryos *Foxg1* is expressed in a discrete patch of cells at the boundary between the embryonic and extra-embryonic region (*n*=4/6). (**Q**,**R**) At 6.5 dpc, *Six3* is not expressed in control embryos but is present throughout the epiblast of two-thirds of *Bmpr1a*$^{-/-}$ embryos (*n*=3/5). (**S**,**T**) *Hesx1* is expressed in the anterior visceral endoderm at 6.5 dpc, but in a third of *Bmpr1a* mutant embryos expression is observed throughout the epiblast (*n*=3/10).

BMP signalling is required in the epiblast to inhibit neural differentiation

Bmpr1a is expressed in all three tissues of the early post-implantation embryo: the epiblast, the visceral endoderm and the extra-embryonic ectoderm (Mishina et al., 1995; Roelen et al., 1994). To test whether the defects observed in *Bmpr1a*$^{-/-}$ embryos are due to requirements of BMP signalling in the epiblast, we deleted *Bmpr1a* specifically from this tissue. We used *Bmpr1a*$^{fx/fx}$ mice that carry a conditional mutation in *Bmpr1a* (Mishina et al., 2002), and the epiblast-specific Cre-driver mouse line *Sox2Cre*$^{+/-}$ (Hayashi et al., 2002).

We first analysed how BMP signalling is affected in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos by looking at pSmad1/5/8 expression. At 5.5 dpc, we found pSmad1/5/8 to be slightly reduced in the epiblast of

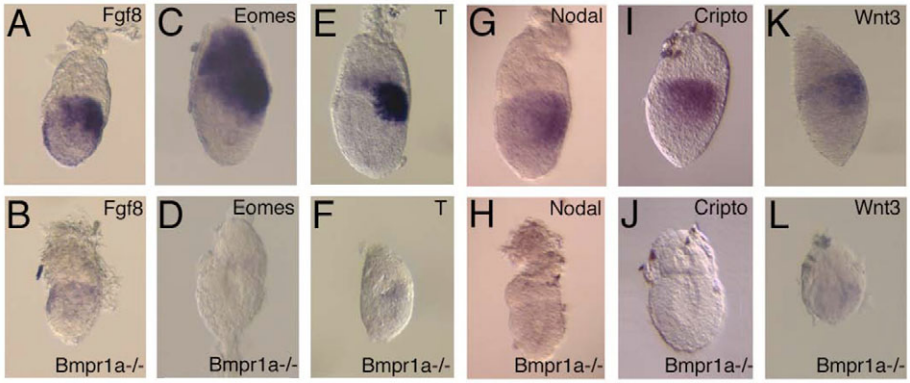

Fig. 3. Suppression of mesoderm in *Bmpr1a* mutant mouse embryos. (**A**,**B**) At 6.5 dpc, *Fgf8* is expressed in the posterior epiblast and visceral endoderm of controls, but is lost from the posterior epiblast of *Bmpr1a* mutant embryos (*n*=5). (**C-F**) eomesodermin and brachyury (*T*) are expressed at 6.5 dpc in the posterior epiblast and extra-embryonic ectoderm of controls, but in *Bmpr1a*$^{-/-}$ embryos their expression is lost from the posterior epiblast and is downregulated in the extra-embryonic ectoderm (*n*=9 and *n*=4). (**G-J**) *Nodal* and *Cripto* are expressed in the posterior epiblast at 6.5 dpc in controls, but are lost in *Bmpr1a* mutant embryos (*n*=5 and *n*=4, respectively). (**K**,**L**) At 6.5 dpc, *Wnt3* is expressed in the posterior epiblast and posterior visceral endoderm of the control but is strongly downregulated in *Bmpr1a*$^{-/-}$ embryos (*n*=4). All are lateral views, anterior to the left.

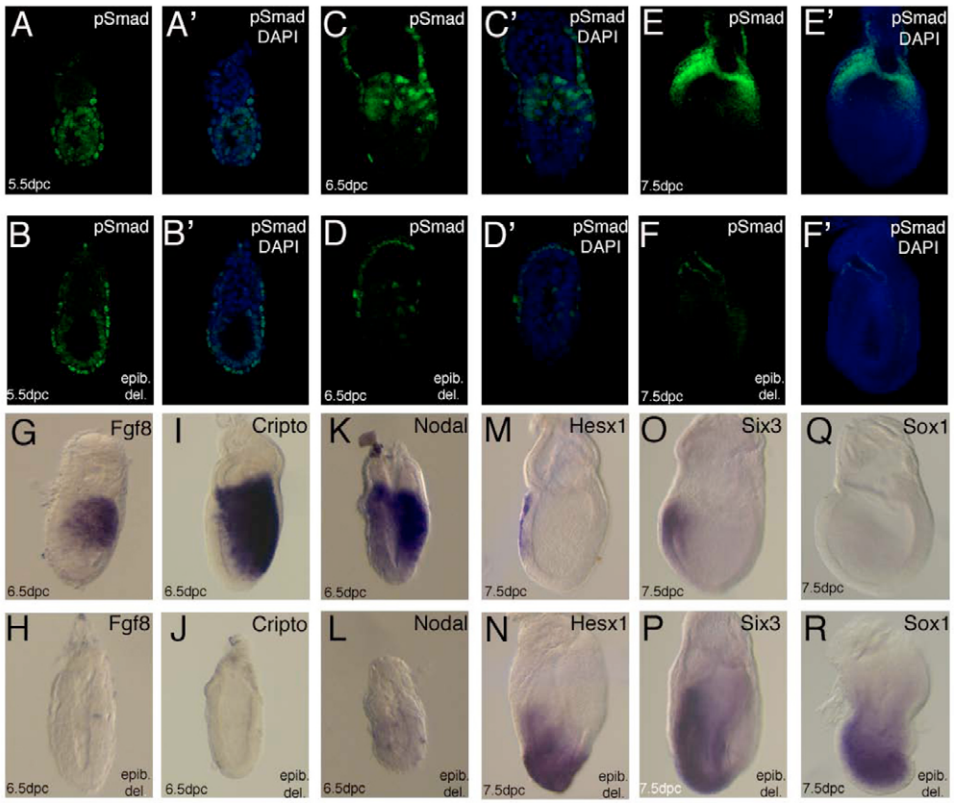

Fig. 4. BMP signalling is required in the epiblast for mesoderm specification and to inhibit neural differentiation. (**A**,**B**) At 5.5 dpc, pSmad1/5/8 expression is slightly decreased in the epiblast of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ mouse embryos (*n*=4/7) as compared with controls. (**C**,**D**) At 6.5 dpc, in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos pSmad1/5/8 expression is severely reduced in the epiblast (*n*=4/7) and (**E**,**F**) by 7.5 dpc is almost lost (*n*=4/4), although some expression in the visceral endoderm remains at both these stages. (**A′-F′**) Overlay of pSmad1/5/8 and nuclear staining. (**G**,**H**) At 6.5 dpc, *Fgf8* is expressed in the posterior epiblast of controls but its expression is reduced or lost in the majority of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos (*n*=4/6). (**I**,**J**) *Cripto* is expressed in the posterior epiblast of controls at 6.5 dpc but is lost in a proportion of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos (*n*=3/9). (**K**,**L**) At 6.5 dpc, *Nodal* is expressed in the posterior epiblast of controls, but when *Bmpr1a* is deleted from the epiblast *Nodal* expression is reduced or lost (*n*=3/4). (**M**,**N**) At 7.25 dpc, *Hesx1* is expressed in the anterior definitive endoderm and anterior neural ectoderm of controls. A large expansion in the domain of *Hesx1* expression is observed in the epiblast of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos (*n*=4/6). (**O**,**P**) *Six3* is a marker of the anterior neural ectoderm at 7.5 dpc. In *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos, *Six3* expression is greatly expanded (*n*=7/8). (**Q**,**R**) At 7.5 dpc, *Sox*1 is not expressed in control embryos but can be found throughout the epiblast of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos. All are lateral views, anterior to the left.

Bmpr1a$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos when compared with controls (Fig. 4B; *n*=4/7). This expression in the epiblast of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos was drastically reduced at 6.5 dpc in the majority of embryos analysed (Fig. 4D; *n*=4/7) and almost absent by 7.5 dpc in all embryos studied (Fig. 4E; *n*=4/4). Therefore, in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos there is a loss of BMP signalling; however, this loss occurs later than in *Bmpr1a*-null mutants.

We next studied mesoderm specification and posterior epiblast patterning in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos by analysing the expression of *Fgf8*, *Nodal* and *Cripto*. At 6.5 dpc, we observed either a complete loss of expression or a downregulation of these markers in the majority of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos analysed (for *Fgf8* *n*=4/6, Fig. 4H; *Cripto* *n*=3/9, Fig. 4J; *Nodal* *n*=3/4, Fig. 4L). To determine whether ectopic neural differentiation is also occurring in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos, we analysed the expression of *Hesx1*, *Six3* and *Sox1*. At 6.5 dpc, no expression of either *Hesx1* (*n*=10, data not shown) or *Six3* (*n*=13, data not shown) was detected, but by 7.5 dpc a large expansion in the domain of expression of both these markers could be seen in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos (*Hesx1* *n*=4/6, Fig. 4N and *Six3* *n*=8/10 Fig. 4P). Similarly, *Sox1* was also expanded at 7.5 dpc in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos (Fig. 4R; *n*=2/2). These observations indicate that BMP signalling is required in the epiblast to inhibit neural differentiation. Furthermore, given that BMP signalling is lost later in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos than in *Bmpr1a*$^{-/-}$ embryos, and that this later loss is correlated with a later onset of expression of ectopic neural markers, one possibility is that BMP inhibition of neural differentiation is occurring continually in the epiblast rather that at one specific time-point. Alternatively, it is also possible that BMP signalling might regulate the expression in the visceral endoderm of other secreted factors that are also required to maintain pluripotency.

Bmp2/4 signal via *Bmpr1a* to maintain pluripotency

We next asked which BMPs signal via *Bmpr1a* to maintain pluripotency. In *Drosophila*, the *Bmpr1a* homologue *thickveins* preferentially transduces *dpp* signals (reviewed by Podos and Ferguson, 1999), and in vertebrates the Dpp homologues Bmp2 and Bmp4 bind with high affinity to *Bmpr1a* (ten Dijke et al., 1994). We therefore tested whether *Bmpr1a* is required for Bmp2/4 signalling in the early embryo. We first analysed the ability of Bmp4 to stimulate the phosphorylation of Smad1/5/8 in vitro. We found that in wild-type ES cells, Bmp4 can stimulate phosphorylation of Smad1/5/8. By contrast, in *Bmpr1a* mutant ES cells, Bmp4 was unable to simulate Smad1/5/8 phosphorylation (Fig. 5A). We further tested the ability of Bmp2/4 to signal via *Bmpr1a* by using an in vitro blastocyst outgrowth assay. Wild-type and *Bmpr1a*$^{-/-}$ blastocysts were cultured on gelatin-coated dishes in serum-free conditions in the presence of Bmp4 and assayed for alkaline phosphatase activity, a characteristic marker of pluripotent cells. When grown for 10 days in these conditions, wild-type and heterozygous blastocysts had formed large outgrowths that displayed strong levels of alkaline phosphatase activity (Fig. 5G and Fig. 6I). By contrast, *Bmpr1a*$^{-/-}$ blastocysts showed hardly any proliferation during the 10 days in culture and exhibited variable levels of alkaline phosphatase activity (Fig. 5H and Fig. 6J), indicating that Bmp4 was unable to sustain self-renewal of the inner cell mass in these embryos. The loss of

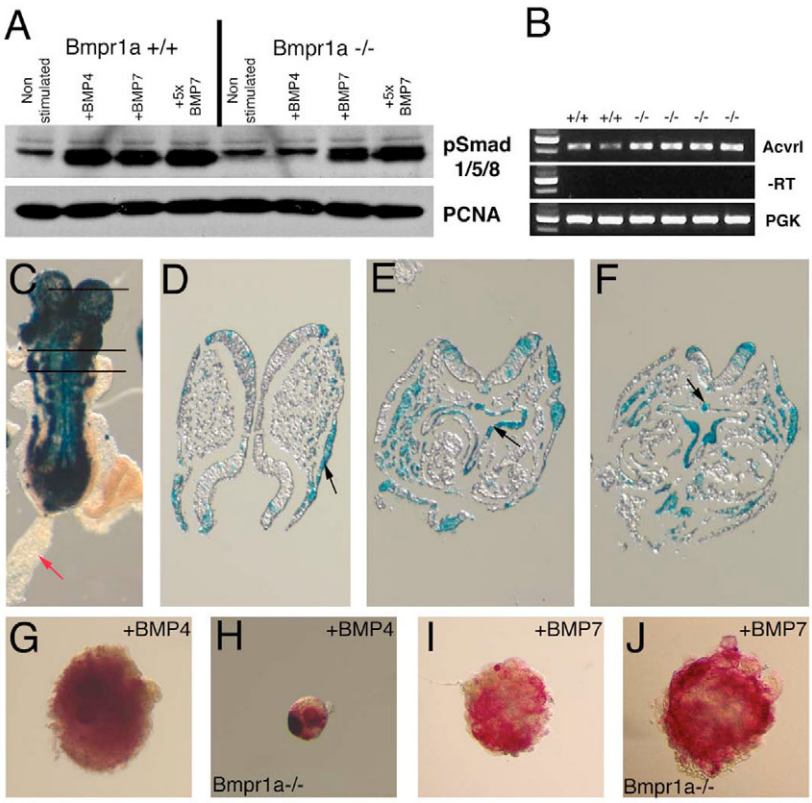

Fig. 5. Bmp7 signalling via *Acvr1* cooperates with Bmp2/4 signalling to maintain pluripotency. (**A**) Western blot showing pSmad1/5/8 expression (upper) and loading control (lower) after Bmp4 and Bmp7 stimulation of control and *Bmpr1a*$^{-/-}$ ES cells. (**B**) Upregulation of *Acvr1* is observed in *Bmpr1a*$^{-/-}$ cells by RT-PCR. (**C**) Ventral view of a medium-contribution chimera generated with *Bmpr1a*$^{-/-}$ *lacZ* ES cells. Red arrow, allantois. (**D-F**) Transverse sections of a medium-contribution chimera. Black arrows mark (D) the surface ectoderm, (E) the endoderm-derived gut and (F) the mesodermally derived notochord. (**G**,**H**) *Bmpr1a*$^{-/-}$ embryos display self-renewal defects when cultured in serum-free conditions and Bmp4. (**I**,**J**) The self-renewal defects are rescued when *Bmpr1a*$^{-/-}$ embryos are cultured in serum-free conditions plus Bmp7.

pSmad1/5/8 expression observed in *Bmpr1a*$^{-/-}$ mutant embryos at 5.5 dpc, coupled with the inability of Bmp4 to signal in *Bmpr1a* mutant embryos and ES cells, indicates that Bmp2/4 signalling via *Bmpr1a* is the BMP pathway that maintains pluripotency prior to gastrulation.

Bmp7 signalling via *Acvr1* cooperates with Bmp2/4 signalling during gastrulation

Are Bmp2/4 the only BMPs required to maintain pluripotency? As a first approach to address this question, we analysed in chimeras the developmental potential of *Bmpr1a*$^{-/-}$ ES cells that are unable to respond to Bmp2/4 signalling. We found that although high-contribution chimeras generated with *Bmpr1a*$^{-/-}$ ES cells recapitulated the phenotype of the epiblast-specific deletion of *Bmpr1a* mutant embryos (data not shown), in low-contribution chimaeras *Bmpr1a*$^{-/-}$ cells were able to contribute efficiently to cell types, except the allantois, formed from all three germ layers (Fig. 5C-F). This suggests that BMP signalling via other type I receptors is also required to maintain pluripotency.

Bmp7 is a BMP of the 60A subgroup that is first expressed in mouse at the start of gastrulation (Solloway and Robertson, 1999). In both *Drosophila* and vertebrates, BMPs of the 60A subgroup have been shown to signal preferentially via the type I BMP receptor *Acvr1* (Podos and Ferguson, 1999; ten Dijke et al., 1994). For this reason, we first tested whether Bmp7 could signal in *Bmpr1a*$^{-/-}$ cells. We found that in contrast to Bmp4, Bmp7 was capable of stimulating phosphorylation of Smad1/5/8 in *Bmpr1a*$^{-/-}$ ES cells (Fig. 5A). Interestingly, the ability of Bmp7 to signal in *Bmpr1a* mutant ES cells correlated with an upregulation of *Acvr1* (Fig. 5B), but not of *Bmpr1b*, in these cells (data not shown), suggesting that Bmp7 was signalling preferentially via this type I receptor.

Given the ability of Bmp7 to signal in *Bmpr1a* mutant ES cells, we analysed whether Bmp7 could rescue the defects of *Bmpr1a*$^{-/-}$ cells in vivo. For this we carried out blastocyst outgrowth assays. When *Bmpr1a*$^{-/-}$ blastocysts were cultured in the presence of Bmp7 they formed outgrowths that were equivalent both in size and alkaline phosphatase activity to control outgrowths (Fig. 5I,J). Together, these results suggest that prior to gastrulation, Bmp2/4 signalling via *Bmpr1a* is the main source of BMP signalling that maintains epiblast pluripotency, but as gastrulation commences, signalling by BMPs of the 60A subgroup via *Acvr1* cooperates with Bmp2/4 signalling to inhibit neural differentiation of the epiblast.

activin/*Nodal* signalling can rescue the pluripotency defects of *Bmpr1a* mutant embryos

Nodal signalling has been shown to be required to maintain pluripotency and inhibit neural differentiation in the mouse embryo (Camus et al., 2006; Mesnard et al., 2006). To determine whether at least part of the defects of *Bmpr1a*$^{-/-}$ embryos are due to an absence of *Nodal* signalling, we analysed how early *Nodal* expression is lost in *Bmpr1a* mutant embryos. At 4.5 dpc, *Nodal* is expressed in the epiblast and primitive endoderm (Takaoka et al., 2006) and at 5.5 dpc in the epiblast and overlying visceral endoderm (Varlet et al., 1997). In *Bmpr1a* mutant embryos, we found no difference in the expression of *Nodal* at 4.5 dpc (Fig. 6A,B), but a clear downregulation relative to wild-type embryos at 5.5 dpc (Fig. 6C,D). We next analysed the expression of the *Nodal* co-receptor *Cripto* and of the Tgfβ *Gdf3* at 5.5 dpc. At this stage, both these markers are normally expressed throughout the epiblast (Chen et al., 2006; Ding et al., 1998). Interestingly, although expression of *Gdf3* was normal, *Cripto* expression was downregulated in *Bmpr1a*$^{-/-}$ embryos (Fig. 6F,H). These observations indicate that epiblast patterning defects are present in *Bmpr1a* mutants as early as 5.5 dpc, and that BMP signalling is required to maintain *Nodal* signalling in the early embryo.

We next tested whether *Nodal* signalling was capable of rescuing the pluripotency defects of *Bmpr1a*$^{-/-}$ embryos. For this, we cultured *Bmpr1a*$^{-/-}$ blastocysts in serum-free conditions in the presence of 20

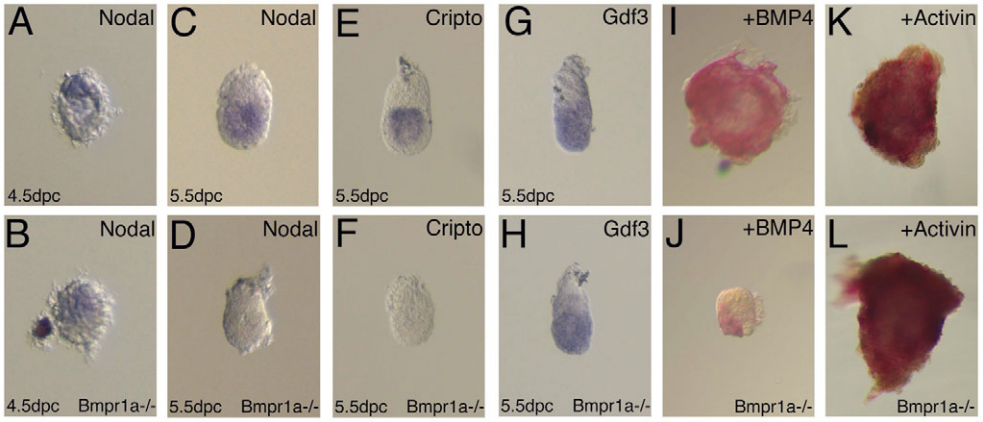

Fig. 6. *Nodal* signalling can resue the pluripotency defect of *Bmpr1a* mutant mouse embryos. (**A**,**B**) At 4.5 dpc, *Nodal* is expressed in the epiblast and primitive endoderm in both control and *Bmpr1a*$^{-/-}$ mouse embryos (*n*=5/5). (**C**,**D**) At 5.5 dpc, *Nodal* is expressed in the epiblast and visceral endoderm of controls but is severely downregulated in a proportion of *Bmpr1a* mutant embryos (*n*=2/3). (**E**,**F**) *Cripto* is expressed in the epiblast at 5.5 dpc of controls, but is lost in a proportion of *Bmpr1a*$^{-/-}$ embryos (*n*=4/7). (**G**,**H**) At 5.5 dpc, *Gdf3* expression is observed throughout the epiblast of control and *Bmpr1a*$^{-/-}$ embryos (*n*=5/5). (**I**,**J**) *Bmpr1a*$^{-/-}$ embryos display self-renewal defects when cultured in serum-free conditions and Bmp4. (**K**,**L**) 20 ng/ml activin can rescue the self-renewal defects of *Bmpr1a*$^{-/-}$ embryos (*n*=5/7).

ng/ml or 200 ng/ml activin (also known as inhibin) for 10 days. Interestingly, we found that although 200 ng/ml activin did not rescue the proliferation or pluripotency defects of *Bmpr1a*$^{-/-}$ embryos (*n*=0/6; data not shown), 20 ng/ml activin did rescue both these defects (*n*=5/7; Fig. 6L). Therefore, *Nodal* signalling can, at least in part, rescue the pluripotency defects of *Bmpr1a*$^{-/-}$ embryos.

Inhibition of FGF signalling does not block neural differentiation in the post-implantation mouse embryo

FGFs have been proposed as inducers of neural fate in both chick (Streit et al., 2000; Wilson et al., 2000) and *Xenopus* embryos (Delaune et al., 2005), and in mouse have been shown to act antagonistically to BMP signalling during mesoderm development (Miura et al., 2006). We tested wether FGF signalling is responsible for the precocious and ectopic neural induction observed in *Bmpr1a* mutant embryos. In agreement with previous studies (Corson et al., 2003), we found that 80 μM SU5402 could efficiently inhibit FGF signalling within 1 hour in cultured 6.0 dpc embryos (data not shown). Given that *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos do not show precocious neural marker expression at 6.5 dpc, we used these embryos to test whether inhibition of FGF signalling during gastrulation could block the expression of the anterior neural markers. 6.5 dpc control and *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos were cultured for 24 hours with DMSO or 80 μM SU5402 and assayed for *Hesx1* expression. After 24 hours of culture, only a small proportion of DMSO-treated controls (2/30) or *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ (2/7) embryos had initiated *Hesx1* expression in the epiblast (Fig. 7A,C). Surprisingly, although SU5402 inhibition did not significantly change the proportion of control embryos expressing *Hesx1* (5/26; Fig. 7B), it led to a drastic increase in the proportion of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos displaying ectopic *Hesx1* expression (11/13; Fig. 7D). These results indicate that inhibition of FGF signalling during gastrulation stages does not inhibit neural specification in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos and suggest that BMP and FGF signalling might act synergistically to inhibit neural differentiation at these stages.

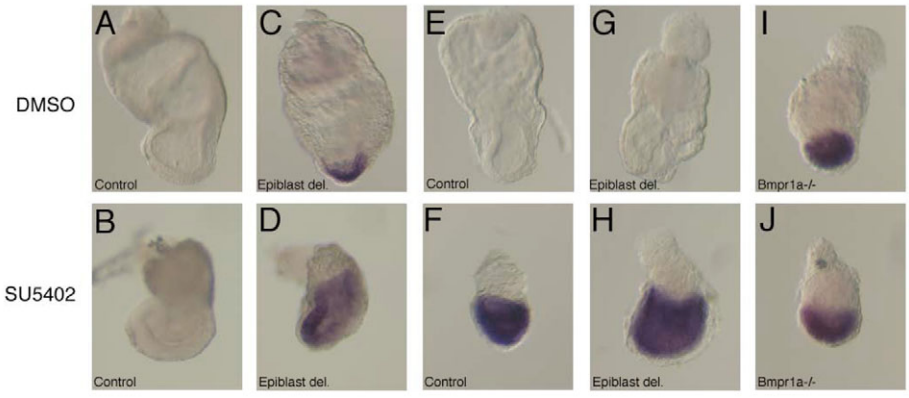

Fig. 7. Inhibition of FGF signalling does not block neural specification in *Bmpr1a*$^{-/-}$ mouse embryos. (**A**,**B**) No *Hesx1* expression is observed in 6.5 dpc control embryos treated with either DMSO or 80 μM SU5402 and cultured for 24 hours. (**C**,**D**) *Hesx1* is expressed in 6.5 dpc *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos after 24 hours culture in DMSO or SU5402. (**E-H**) *Hesx1* expression is observed in 5.5 dpc control and *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos after 48 hours culture with SU5402, but not when cultured with DMSO. (**I**,**J**) 5.5 dpc *Bmpr1a*$^{-/-}$ embryos show similar levels of *Hesx1* expression after 48 hours culture in DMSO or SU5402.

To test whether FGF signalling may be acting prior to gastrulation to induce neural fate, we inhibited FGF signalling by culturing 5.5 dpc *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ and *Bmpr1a*$^{-/-}$ embryos for 48 hours in the presence of SU5402. No control (0/33) or *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ (0/6) embryos and only 55% of *Bmpr1a*$^{-/-}$ embryos (5/9) showed *Hesx1* expression after 48 hours culture in DMSO (Fig. 7E,G,I). By contrast, 29% of control (15/52), 80% of *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ (4/5) and 90% of *Bmpr1a*$^{-/-}$ embryos (10/11) displayed ectopic *Hesx1* expression after 48 hours culture with SU5402 (Fig. 7F,H,J). Interestingly, the extra-embryonic region of these SU5402-treated embryos was much reduced compared with controls, most probably owing to the effects of FGF inhibition on the trophoblast stem cells (Tanaka et al., 1998). These observations indicate that the ectopic neural induction seen in *Bmpr1a* mutants is not mediated by FGF signalling and also suggest that FGFs are not acting as direct neural inducers in the early post-implantation mouse embryo.

DISCUSSION

The role of BMPs in patterning the early vertebrate embryo and inducing differentiation of a number of cell types is well established. For example, in the early mouse embryo Bmp4 plays a crucial role in PGC induction, mesoderm and node formation and left-right asymmetry. In contrast to these findings, we have identified a role for BMP signalling in maintaining epiblast pluripotency and have shown that loss of BMP signalling leads to ectopic neural induction in the mouse embryo.

The role of BMP signalling in maintaining epiblast pluripotency

Strong evidence exists that right up to 7.5 dpc, a proportion of cells from the epiblast remain pluripotent (Beddington, 1982; Beddington, 1983; Diwan and Stevens, 1976; Lawson et al., 1991). The maintenance of this progenitor population is crucial for the allocation of cells to the three germ layers: mesoderm, endoderm and ectoderm. A limited number of factors have been identified as required to maintain epiblast pluripotency, including *Oct4* (Nichols et al., 1998), *Nanog* (Chambers et al., 2003; Mitsui et al., 2003), *Sox2* (Avilion et al., 2003), *Foxd3* (Hanna et al., 2002) and *Fgf4* (Feldman et al., 1995). However, given that mutations in these factors cause lethality at peri-implantation stages, it has not been possible to determine whether these genes affect pluripotency after implantation.

A number of observations indicate that BMP signalling via *Bmpr1a* plays a crucial role in maintaining epiblast pluripotency. *Bmpr1a* is expressed in inner cell mass-derived ES cells (Ying et al., 2003a) and was highly enriched in a screen aimed at identifying stem cell markers (Sato et al., 2003). In *Bmpr1a*$^{-/-}$ embryos, we find a downregulation or loss of expression of genes required for epiblast pluripotency, such as *Oct4*, *Nanog*, *Nodal* and *Cripto*. Furthermore, we show that *Bmpr1a*$^{-/-}$ embryos exhibit self-renewal defects in blastocyst outgrowth assays. In ES cells, BMP signalling cooperates with the Lif/Stat pathway to drive self-renewal (Ying et al., 2003a). BMP signalling is therefore required both in vitro and in vivo to maintain pluripotency.

Inhibition of BMP signalling and neural induction

A key aspect of the role of BMPs in maintaining epiblast pluripotency is the inhibition of precocious neural differentiation. In ES cells, Bmp4 maintains pluripotency by specifically blocking the neural differentiation pathway (Ying et al., 2003a). Similarly, we find that *Bmpr1a* mutant embryos show precocious and ectopic expression of anterior neural markers coupled to the loss of pluripotency markers. Inhibition of BMP signalling has been shown to be a central step for neural induction in a number of vertebrate species (Harland, 2000). Mainly based on experiments in *Xenopus*, a model has been proposed in which BMPs act prior to gastrulation to inhibit a 'default' neural differentiation of the ectoderm. According to this model, neural specification occurs when the onset of expression of BMP antagonists allows differentiation to take place in the prospective neural ectoderm (Vonica and Brivanlou, 2006). The precocious expression of neural markers observed in *Bmpr1a*$^{-/-}$ embryos indicates that as in *Xenopus*, inhibition of BMP signalling in the mouse embryo is occurring prior to gastrulation; therefore, a crucial step in neural induction must be the relief of BMP inhibition.

Our results also suggest that BMP inhibition of neural fate is required continuously in the epiblast rather than at a single specific stage of development. Support for this comes from the comparison of *Bmpr1a*$^{-/-}$ and *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ mutant phenotypes. In *Bmpr1a*$^{-/-}$ embryos, a complete loss of pSmad1/5/8 expression occurs in the epiblast as early as 5.5 dpc and this correlates with the precocious and ectopic expression of neural markers at 6.5 dpc. By contrast, a significant decrease in BMP signalling only occurs in *Bmpr1a*$^{-/fx}$; *Sox2Cre*$^{+/-}$ embryos from 6.5 dpc and ectopic neural markers are not seen until 7.5 dpc. Therefore, the timing of the loss of BMP signalling correlates with the timing of the onset of neural differentiation in two separate mutations. This observation suggests that in the wild-type embryo, BMP signalling is required to maintain epiblast pluripotency from the time of implantation until 7.5 dpc, when neural differentiation takes place. It is likely that this neural differentiation occurs when BMPs are antagonised (Fig. 8), first by

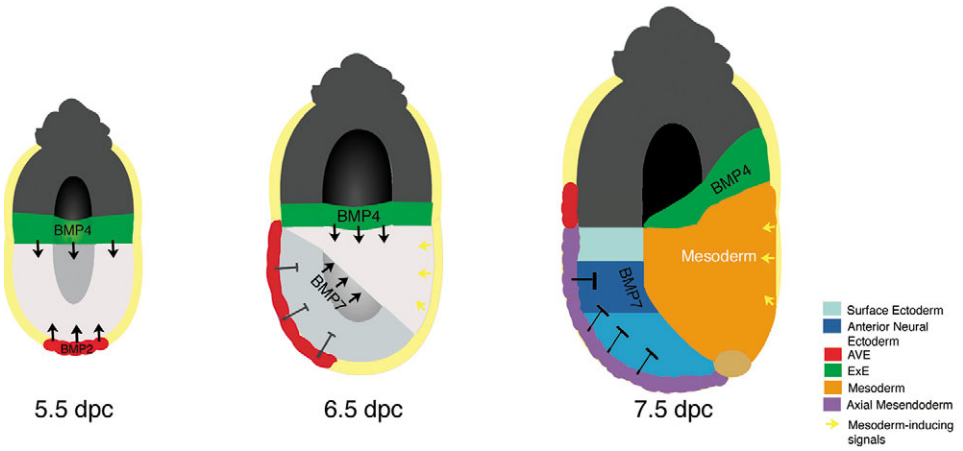

Fig. 8. Model for how Bmp2/4 and BMPs of the 60A subgroup cooperate to maintain epiblast pluripotency in mouse. Prior to gastrulation, Bmp2 and Bmp4 maintain pluripotency and inhibit neural differentiation of the epiblast. As gastrulation commences, BMPs of the 60A subgroup cooperate with Bmp2 and Bmp4 to reinforce the inhibition of neural fate. Neural differentiation occurs in the anterior epiblast when BMPs are antagonised, first by factors secreted by the AVE and later by the axial mesendoderm. AVE, anterior visceral endoderm; ExE, extra-embryonic ectoderm.

factors secreted by the anterior visceral endoderm (AVE) and later by the axial mesendoderm, the two signalling centres required for neural specification (Beddington and Robertson, 1999).

Which BMPs can inhibit neural fate? In *Xenopus* embryos, ubiquitous neural induction throughout the ectoderm only occurs after knock-down of four BMP family proteins: BMP2, BMP4, BMP7 and ADMP (Reversade and De Robertis, 2005). By contrast, we find in mouse that Bmp2/4 signalling via *Bmpr1a* is the main source of BMP signalling that inhibits neural fate prior to gastrulation. The loss of pSmad1/5/8 expression in *Bmpr1a*$^{-/-}$ embryos coupled with the fact that Bmp2/4 cannot signal in *Bmpr1a*$^{-/-}$ ES cells but other BMPs can, supports this view. Interestingly, in mouse it is only as gastrulation commences that signalling by BMPs of the 60A subgroup via *Acvr1* cooperates with Bmp2/4 signalling to reinforce this inhibition of neural differentiation (Fig. 8). Two observations support this. First, members of the 60A subgroup only start to be expressed at the onset of gastrulation (Solloway and Robertson, 1999). Secondly, we find that Bmp7 can rescue the self-renewal defects of *Bmpr1a*$^{-/-}$ embryos in blastocyst outgrowth assays. Therefore, in mouse, although Bmp2 and Bmp4 signalling are initially sufficient to inhibit neural fate, as development proceeds multiple BMPs signalling via different receptor complexes are required to block neural differentiation.

One candidate pathway for acting downstream of BMP signalling is the *Nodal* signalling pathway. BMPs have been shown to act upstream of *Nodal* during left-right patterning (Fujiwara et al., 2002; Piedra and Ros, 2002; Schlange et al., 2002) and to amplify *Nodal* expression in the epiblast (Ben-Haim et al., 2006). *Nodal* mutant embryos display similar pluripotency defects and precocious neural differentiation to those we observe in *Bmpr1a* mutant embryos (Camus et al., 2006; Mesnard et al., 2006). In *Bmpr1a* mutant embryos, we find a loss of expression of both *Nodal* and its co-receptor *Cripto* just prior to the time when we observe the precocious expression of neural markers. Furthermore, we find that activin/*Nodal* signalling can rescue the pluripotency defects of *Bmpr1a* mutant embryos in blastocyst outgrowth assays. These lines of evidence suggest that BMP signalling might be regulating the levels of *Nodal* signalling in the early mouse embryo and that, at least in part, the defects in *Bmpr1a* mutant embryos are owing to the lack of *Nodal* signalling activity.

Are FGFs neural inducers in the mouse embryo?

Recent experiments in both chick and *Xenopus* embryos have challenged the idea that inhibition of BMP signalling is sufficient for neural induction and have suggested that FGF signalling might be an inductive signal during this process. These studies found that inhibition of FGF signalling by the small molecule SU5402 blocks neural induction in both chick (Streit et al., 2000; Wilson et al., 2000) and *Xenopus* (Delaune et al., 2005) embryos and that this role of FGFs is, at least in part, independent of antagonising BMP signalling. However, in contrast to these observations, we find that FGF signalling is not responsible for the ectopic neural induction observed in *Bmpr1a*$^{-/-}$ embryos. Inhibition of FGF signalling using SU5402 24 hours before gastrulation did not inhibit the expression of *Hesx1* in the epiblast of either *Bmpr1a*$^{-/fx}$ or *Bmpr1a*$^{-/fx}$*; Sox2Cre*$^{+/-}$ embryos. Furthermore, inhibition of FGF signalling led to an expansion of anterior neural tissue in a proportion of wild-type embryos. Given that an expansion of neural tissue is also observed in *Fgf8*-null mutants (Sun et al., 1999) and in *Fgfr1* chimeras (Ciruna et al., 1997) and that Fgf2 has been shown to inhibit neural fate in mouse ectoderm explants (Burdsal et al., 1998), it seems unlikely that the primary role of FGF signalling in the early post-implantation mouse embryo is to act as a direct neural inducer, but instead is required to promote mesoderm formation and migration. In ES cells, inhibiting FGF signalling using SU5402 has been shown to block neural differentiation (Ying et al., 2003b). It is therefore possible that FGFs are required during the pre-implantation period for a maturation step in the epiblast that makes this tissue competent for neural specification, but once this step has taken place it is inhibition of BMP signalling that is the main event required for the induction of neural marker expression. It is also possible that, as has been suggested in *Xenopus* (Pera et al., 2003), a role for FGF signalling during early post-implantation development could be to attenuate BMP signalling and drive neural specification in this way.

In conclusion, we have found that Bmp2/4 signalling via *Bmpr1a* is inhibiting neural differentiation of the epiblast prior to gastrulation and that these BMPs cooperate with members of the 60A subgroup during gastrulation. We have also shown that, in contrast to what seems to occur in chick, in the early mouse post-implantation embryo inhibition of FGF signalling does not block neural specification and that it is relief of BMP inhibition that is the critical step for neural induction to occur.

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