Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia: development by concerted actions of BMP signaling

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Erratum

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Various errors in this article were not corrected before going to press.

The title should read:

Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling

Corrected author names are YiPing Chen and Ryuma Haraguchi.

We apologise to the authors and readers for these mistakes.

Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia: development by concerted actions of BMP signaling

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Summary

Extra-corporal fertilization depends on the formation of copulatory organs: the external genitalia. Coordinated growth and differentiation of the genital tubercle (GT), an embryonic anlage of external genitalia, generates a proximodistally elongated structure suitable for copulation, erection, uresis and ejaculation. Despite recent progress in molecular embryology, few attempts have been made to elucidate the molecular developmental processes of external genitalia formation.

Bone morphogenetic protein genes (Bmp genes) and their antagonists were spatiotemporally expressed during GT development. Exogenously applied BMP increased apoptosis of GT and inhibited its outgrowth. It has been shown that the distal urethral epithelium (DUE), distal epithelia marked by the *Fgf8* expression, may control the initial GT outgrowth. Exogenously applied BMP4 downregulated the expression of *Fgf8* and *Wnt5a*, concomitant with increased apoptosis and decreased cell proliferation of the GT mesenchyme. Furthermore, noggin mutants and *Bmpr1a* conditional mutant mice displayed hypoplasia and hyperplasia of the external genitalia respectively. noggin mutant mice exhibited downregulation of *Wnt5a* and *Fgf8* expression with decreased cell proliferation. Consistent with such findings, *Wnt5a* mutant mice displayed GT agenesis with decreased cell proliferation. By contrast, *Bmpr1a* mutant mice displayed decreased apoptosis and augmented *Fgf8* expression in the DUE associated with GT hyperplasia. These results suggest that some of the Bmp genes could negatively affect proximodistally oriented outgrowth of GT with regulatory functions on cell proliferation and apoptosis.

The DUE region can be marked only until 14.0 dpc (days post coitum) in mouse development, while GT outgrowth continues thereafter. Possible signaling crosstalk among the whole distal GT regions were also investigated.

Key words: External genitalia, Genital tubercle, BMP, FGF8, Noggin, WNT5A, Apoptosis, Cell proliferation, Distal urethral epithelium

Introduction

External genitalia are regarded as appendages emerging from the posterior body trunk. External genitalia develop to perform copulation and transfer sperm, as well as for uresis. Within the developmental diversities of different appendages, e.g. between limb and external genitalia, research on their morphogeneses has focused mainly on the development of limbs. In fact, only a few analyses have been performed as to the putatively conserved mechanisms underlying the outgrowth of such appendages. Pioneering studies have suggested that the functions of several developmental genes, e.g. the Hox genes, are conserved in part and play a role in orchestrating both embryonic limb and external genitalia development (Dolle et al., 1991). However, the role of growth factors in the external genital anlage, i.e. genital tubercle (GT) development, remain unexplored.

The GT differentiates into the penis in males and the clitoris in females (Murakami and Mizuno, 1986). Epithelial-mesenchymal interactions play an essential role in regulating a wide variety of developmental processes (Capel, 2000; Hogan, 1999; Johnson and Tabin, 1997; Kurzrock et al., 1999b; Thesleff et al., 1991; Thesleff et al., 1995; Tickle and Eichele, 1994). Such signaling governs many aspects of organogenesis, from the initiation of organ development to differentiation (Dassule and McMahon, 1998; Tucker et al., 1999). The developing limb has long served as a model system for

studying such mechanisms (Duboule, 1993; Duboule, 1994; Tabin, 1991; Tabin, 1995). The distal signaling epithelia of the developing limb (apical ectodermal ridge; AER) is essential for sustained outgrowth and the patterning of a limb through, e.g. fibroblast growth factors (FGFs) (Laufer et al., 1994; Niswander et al., 1993; Niswander et al., 1994; Pizette and Niswander, 1999; Sun et al., 2002; Zuniga et al., 1999).

It has been reported that bone morphogenetic proteins (BMPs) may regulate the regression and function of the limb AER (Pizette and Niswander, 1999). In many cases, these interactions have been associated with the mesenchyme expressing various Bmp genes. A number of epithelial-mesenchymal interactions have been shown to involve FGF and BMP signals, including development of the limbs, teeth, feather buds and lung buds (Martin, 1998; Thesleff et al., 1995; Tickle, 1999; Weaver et al., 2000).

BMPs are released by a variety of source cells and influence neighboring target cells (Hogan, 1996). Expression of *Bmp4* is observed in the developing limb, lung, kidney, hair follicle and tooth bud, where inductive interactions occur between the mesenchyme and adjacent epithelium (Bitgood and McMahon, 1995). The essential role of BMP signaling in organogenesis and the coordinated action of their antagonists have been demonstrated in many developmental processes. The distribution and activity of BMPs is regulated by antagonists such as noggin (NOG), chordin (CHD) and gremlin (CKTSF1B1 – Mouse Genome Informatics) (Khokha et al., 2003; Sasai and De Robertis, 1997). It has been suggested that the interaction between BMP and its antagonist is essential for the development and differentiation of the limb appendage (Brunet et al., 1998; Merino et al., 1999; Niswander, 2003).

BMP signaling is mediated by specific receptors composed of heterodimer/tetramers of two different transmembrane serine/threonine kinase subunits (Liu et al., 1995), type I and type II receptors. Upon binding of the ligand, the type II receptor phosphorylates and activates the type I receptor (Mishina, 2003; Wrana et al., 1992). In the mouse, a single type II subunit (BMPR2) has been identified (Beppu et al., 1997), while at least two type 1 subunits [BMPR1A (Alk3) and BMPR1B (Alk6)] have been reported (ten Dijke et al., 1994).

Apoptosis is an important process for eliminating regressing tissue regions and cells during embryonic development. Several Bmp genes have been reported to be involved in the regulation of apoptosis in various tissues including limbs (Furuta et al., 1997; Ganan et al., 1996; Jernvall et al., 1998; Macias et al., 1997; Zou and Niswander, 1996). Apoptosis has been further implicated in interdigital cell death (Merino et al., 1998; van der Hoeven et al., 1994), and cell death in the AER (Chen and Zhao, 1998; Pizette and Niswander, 1999) and tooth enamel knot (Vaahtokari et al., 1996b), which underlie the regression of such transiently formed embryonic regions (Vaahtokari et al., 1996a).

The external genital anlage develops from the posterior embryonic region as a bud-anlage (Yamada et al., 2003). The first morphological alteration indicative of GT outgrowth occurs at 10.5 days post coitum (dpc) in the mouse and continues throughout the perinatal period (Suzuki et al., 2002). Several growth factors, including FGF proteins, sonic hedgehog (SHH) and WNT have been implicated in the control of external genitalia development in mice (Haraguchi et al., 2000; Haraguchi et al., 2001; Perriton et al., 2002; Yamaguchi et al., 1999). FGF signaling plays a key regulatory component in orchestrating growth and differentiation of the GT (Haraguchi et al., 2000; Morgan et al., 2003) and SHH signaling is required during the initiation of GT outgrowth and subsequent differentiation, particularly during urethra formation (Haraguchi et al., 2001; Perriton et al., 2002). The distal signaling epithelia, the distal urethral epithelium (DUE), has a role in controlling mesenchymal gene expression and the initial outgrowth of the GT for early GT development (Haraguchi et al., 2000; Ogino et al., 2001). In addition to *Fgf8* and *Shh* expression, we noticed that several BMP signaling molecules were expressed in the DUE and in the distal-ventral mesenchyme adjacent to the DUE.

The role of BMP signaling in the caudal-end of the developing urogenital system has been only partially studied so far. *Bmp4* expression in the urogenital sinus has been shown to be important for prostate development (Lamm et al., 2001). However, the regulatory role of BMP systems in external genitalia development is unknown. We report on the roles of BMP signaling in murine external genitalia development by in vitro organ culture and by analyzing *Nog*, *Wnt5a* mutants and *Bmpr1a* conditional mutant mice.

The DUE can be observed only until 14.0 dpc in mouse development, whereas GT outgrowth continues thereafter. Detailed study of gene expression and histological analyses revealed that the DUE is located adjacent to the outer-most epithelial layer aligned with the GT surface ectoderm. With the advent of gene expression analysis on *Bmpr1a* mutant mice, signaling crosstalks among such whole distal GT regions are discussed. These data are consistent with the hypothesis that the actions of ‘positive’ and ‘negative’ signaling involving DUE may be coordinated, depending on the output of BMP signals.

Materials and methods

Mutant mice

Nog and *Wnt5a* mutant mice have been previously described (McMahon et al., 1998; Oishi et al., 2003; Yamaguchi et al., 1999). *Bmpr1a* conditional mutants and the *Brn4*-Cre strain, bcre-32, were generated as described previously (Ahn et al., 2001; Mishina et al., 2002). The genotyping was performed as described (Ahn et al., 2001; McMahon et al., 1998; Yamaguchi et al., 1999).

Mouse genital tubercle (GT) organ culture

Procedures for filter supported organ cultures and a rotating organ culture system for murine GT were previously described (Haraguchi et al., 2000). After 1-3 days in the culture, the GTs were processed for histological analysis. Recombinant human BMP4, BMP7 (R&D, CA) or NOG (R&D, CA) proteins were used at a concentration of 1 or 10 μg/ml. Staurosporine (Sigma) was used at 5-10 nM in the culture. It has been demonstrated that exposure of mouse embryos to staurosporine at such concentrations (5 nM) exerted no general toxicity in embryonic development (Ward et al., 2000). For antibody inoculation, anti-SHH (5E1) antibody (DSHB, USA) or control Ig class-matched antibody (anti-CD90 antibody, Pharmingen) were used at concentrations of 1-5 μg/ml.

In situ hybridization for gene expression

Whole-mount in situ hybridization was performed by standard procedures with probes for *Bmp7* (kindly provided by M. Yoshida), *Bmp4* (Jones et al., 1991), *Bmpr1a* (Mishina et al., 1995), *Bmpr2* (kindly provided by K. Miyazono), *Nog* (McMahon et al., 1998),

cyclin D1 (kindly provided by J. S. Charles), *Bambi* (Grotewold et al., 2001), *Wnt5a* (kindly provided by S. Takada), *Fgf8* and *Bmp2* (kindly provided by B. L. Hogan).

RT-PCR analysis

Total RNA was isolated by using ISOGEN (Nippon Gene). RT-PCR was performed with 100 ng of total RNA using One-Step RT-PCR System (Invitrogen). The primer sequences for *EF1α*: were 5-CTGCTGAGATGGGAAAGGGCTC-3 and 5-ACAGGGACAGTG-CCAATGCCTC-3, and the annealing temperature 61°C, as described for *Fgf8* (Ozawa et al., 1997) and cyclin D1 (Klein et al., 2003). The PCR cycle consisted of a cDNA synthesis step at 50°C for 30 minutes, a pre-denaturation step at 94°C for 2 minutes, a denatureted step at 94°C for 15 seconds, an annealing step for 30 seconds, and a extension step at 70°C for 1 minute by using a DNA thermal cycler (Biometra Co).

Histological and scanning electron microscopy (SEM) analyses

Staining for *lacZ* expression and Phospho-Smad1 immunohistochemical analyses were performed as described (Ahn et al., 2001). Tissues were fixed in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin wax and sectioned. Hematoxylin and Eosin staining was performed by standard procedures (Haraguchi et al., 2001). Mouse GTs were fixed in 4% PFA and dehydrated for SEM analysis (Suzuki et al., 2000).

Preparation of BMP4 protein beads

Recombinant human BMP4 was used at a concentration of 100 μg/ml in phosphate-buffered saline (PBS). Affigel Blue beads (BioRad) were washed with PBS and subsequently soaked in each protein for 1 hour at 37°C. Control beads were treated with PBS containing an equivalent amount of bovine serum albumin (Sigma).

Analysis of cell proliferation and cell death in vitro

Proliferating cells were detected using rabbit anti-phospho-histone H3 (Ser 10) (Cell Signaling Technology). Cells included in equal areas of control and treated GT were counted and average numbers were compared. TUNEL analysis for the detection of apoptotic cells in the GT was performed using the in situ apoptosis detection kit (TAKARA). Nile Blue (Sigma) was used to localize apoptotic cells. Dissected GTs were stained with 0.01% Nile Blue in the medium, then washed with PBS several times and photographed.

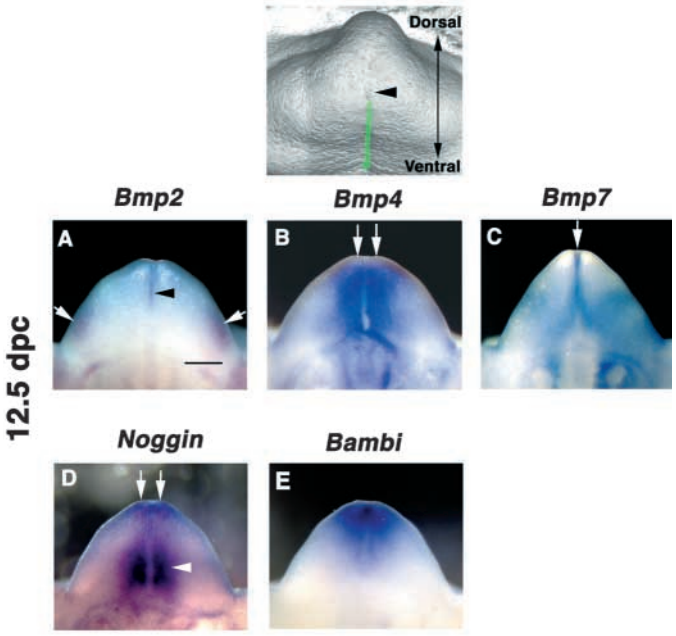

Fig. 1. Expression of Bmp and BMP antagonist genes during mouse genital tubercle (GT) development. (Top) SEM picture of an embryonic GT at 12.5 dpc (green line indicates urethral plate). The arrowhead indicates the distal urethral epithelium (DUE; which is covered with an epithelial layer). (A) *Bmp2* expression was localized to the urethral epithelium (black arrowhead) and proximolateral mesenchyme (white arrows) of the GT at 12.5 dpc. (B) By 12.5 dpc, the expression of *Bmp4* was detected in the mesenchyme along the urethral plate and also around the DUE (white arrows). (C) *Bmp7* was expressed in the DUE (white arrow) and in the urethral epithelium with its expression level moderately higher in the distal and lower in the proximal regions. (D) *Nog* was expressed in the mesenchyme around the DUE (white arrows). Its expression was also localized in the proximal mesenchyme around the urethral plate (white arrowhead). (E) *Bambi* was expressed in the mesenchyme with its expression level moderately higher in the distal and lower in proximal regions. All figures are ventral views. Scale bar: 250 μm.

Results

Expression of Bmp and Bmp antagonist genes in the developing mouse Genital Tubercle (GT)

To gain insight into the roles of Bmp genes in GT formation, we performed an examination of the expression patterns of the *Bmp* genes and their antagonists. Of note is the expression of *Bmp4* in the ventral distal mesenchyme adjacent to the DUE, the putative distal signaling epithelia (Fig. 1B, white arrows). The location of this gene expression adjacent to the DUE, prompted us to investigate the potential regulatory role of BMP4 in GT morphogenesis. Expression of *Bmp4* was also detected in the ventral bilateral mesenchyme along the urethral plate proximodistally. *Bmp4* was also expressed prominently in the dorsal GT mesenchyme from 11.5-14.5 dpc (data not shown).

In addition to *Bmp4*, the expression patterns of other Bmp genes were examined. We found that *Bmp7* was expressed in the DUE (Fig. 1C, white arrow) (Morgan et al., 2003) and in the urethral epithelium with expression moderately higher in the distal regions and lower in the proximal regions. *Bmp2* expression was localized to the urethral epithelium (Fig. 1A, black arrowhead) and in the proximolateral mesenchyme of the GT at 12.5 dpc (Fig. 1A, white arrows). BMP signaling is mediated by specific receptors that are heterodimer/tetramers of two different transmembrane serine/threonine kinase subunits (Massague, 1998). Expression of *Bmpr1a* and *Bmpr2* was rather ubiquitous in the developing epithelium and mesenchyme of the GT (data not shown) (Morgan et al., 2003).

BMP signaling can be antagonized by the activity of several secreted proteins including NOG (Brunet et al., 1998; Hirsinger et al., 1997; McMahon et al., 1998; Merino et al., 1998; Zimmerman et al., 1996). NOG is known to bind and antagonize BMP2, BMP4 and BMP7. In developing organs, Bmp gene- and *Nog*-expressing cells are often detected in adjacent domains (Brunet et al., 1998; Reshef et al., 1998). Given the complexity of the expression patterns of several Bmp genes during GT formation, we also examined the expression of Bmp antagonist genes. *Nog* was expressed in the mesenchyme surrounding the DUE (Fig. 1D, white arrows). Its expression was also detected in the proximal mesenchyme of the ventral GT (Fig. 1D, white arrowhead).

Recently, a transmembrane protein that can attenuate BMP signaling, termed BAMBI (BMP and activin membrane-bound inhibitor) was identified in *Xenopus* (Onichtchouk et al., 1999). It has been shown that the spatiotemporal expression pattern of *Bambi* closely matches with that of *Bmp4* during mouse embryonic development (Grotewold et al., 2001). *Bambi* was found to be prominently expressed in the distal mesenchyme adjacent to the DUE at 12.5 dpc (Fig. 1E). These dynamic expression patterns of several Bmp genes and their antagonists has prompted us to examine their roles during murine GT development.

BMP4 and BMP7 promote apoptosis while NOG inhibits apoptosis in vitro

Apoptosis was normally observed in the distal epithelium and less prominently in the distal mesenchyme of the GT at 11.5 dpc and it was confined mainly to the distal mesenchyme between 12.5 dpc and 13.5 dpc (data not shown) (Haraguchi et al., 2001). At 14.5 dpc, the number of apoptotic cells decreased in both the epithelium and mesenchyme (data not shown). Of note is the fact that the spatiotemporal pattern of apoptosis in both the distal mesenchyme and the DUE is correlated with the pattern of *Bmp4* and *Bmp7* expression. This prompted us to investigate the effect of several BMP(s) on apoptosis. BMP4 beads implanted into the murine GT increased mesenchymal apoptosis (Fig. 2C). It was also found that GT outgrowth was inhibited on the side of the GT implanted with BMP4 beads, compared with the opposite side implanted with control BSA beads (Fig. 2A; see also Fig. 4). Inhibition of GT outgrowth by BMP4 was restored by the addition of NOG and BMP4 beads induced *Nog* expression in GT mesenchyme (K.S. and G.Y., unpublished).

BMP7 is also a potent inducer of apoptosis in several organs, such as developing limbs (Macias et al., 1997). As shown in Fig. 2D-F, specimens treated with BMP4 or BMP7 in the medium displayed increased apoptosis mainly in the distal mesenchyme compared with the control specimens by using a rotating culture. By contrast, a clear reduction in the number of apoptotic cells within the distal GT mesenchyme was observed after 24 hours of exposure to NOG (Fig. 2G,H). Likewise, suppression of BMP signaling by a *Bmpr1a* conditional mutation resulted in reduced apoptosis in the GT (see below). Overall, these observations suggested that Bmp genes may be involved in temporally and spatially regulated apoptosis during GT development.

Role of apoptosis in the regulation of GT outgrowth

It has been recently reported that reagents with augmenting activities for apoptosis have revealed a role, in part, in limb development (Sanz-Ezquerro and Tickle, 2000). To determine the role of apoptosis in GT development, we used staurosporine (a protein kinase inhibitor known to induce apoptosis) to achieve ectopic apoptosis (Jacobson et al., 1997). The number of apoptotic cells increased in the distal epithelium and its adjacent mesenchyme following its treatment (Fig. 3C-F), which was accompanied by retarded GT outgrowth (Fig. 3A,B).

Fgf8 was expressed in the DUE, and it has been suggested as one of the signaling molecules for the control of initial GT outgrowth. The GT outgrowth was prominent between 10.5 dpc and 14.5 dpc, whereas *Fgf8* was expressed in the DUE and maintained until 14.0 dpc. Its expression was reduced after 14.5 dpc (Haraguchi et al., 2000; Perriton et al., 2002). Thus, the onset and disappearance of growth factor gene expression, e.g. *Fgf8*, and the presence of DUE show a correlation with the initial outgrowth of the GT.

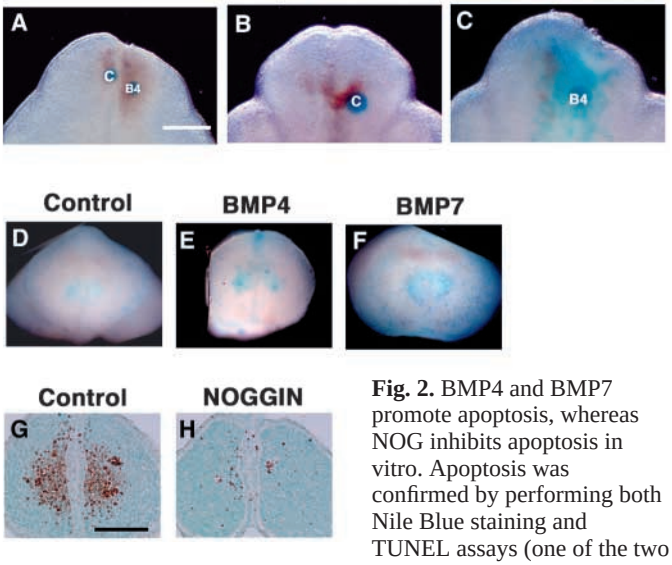

Fig. 2. BMP4 and BMP7 promote apoptosis, whereas NOG inhibits apoptosis in vitro. Apoptosis was confirmed by performing both Nile Blue staining and TUNEL assays (one of the two assays was shown). (A-C) GT explants were micro-dissected from 12.0 dpc embryos, implanted with BMP4 (B4) or control (c) BSA beads, and cultured for 24 hours. BMP4 beads inhibited GT outgrowth when compared with the opposite side of the same specimen implanted with control BSA beads (A). Implanted BMP4 beads (C) in the murine GT mesenchyme increased apoptosis compared with control beads (B). (D-F) BMP-treated GT displayed increased apoptosis in the distal GT region compared with the control (D, control; E,F, BMP4- and BMP7-treated GT, respectively). (G,H) TUNEL analysis was performed on NOG treated specimens. A clear reduction in the number of apoptotic cells within the distal GT mesenchyme was observed by the NOG application (G, control section; H, NOG treated section). (A-C) Ventral views. (D-F) Frontal views. (G,H) Coronal sections. The upper side is dorsal and bottom side is ventral. Scale bars: in A, 125 μm in A-C and 250 μm in D-F; in G, 50 μm in G,H.

SHH signaling has previously been shown to be required for the initiation of GT outgrowth (Haraguchi et al., 2001). We next examined whether *Fgf8* expression in GT explants was affected by treatment with the anti-SHH antibody, 5E1. GT explants treated with 5E1 showed a reduction in *Fgf8* expression after 24 hours of culture (Fig. 3G,H). These results may suggest that SHH signaling is necessary for *Fgf8* expression, not only for the initiation of GT outgrowth as shown previously, but also during the outgrowth phase. The treatment of 5E1 antibody retarded GT outgrowth later, at 12.5 dpc with an increase of apoptosis (Fig. 3I,J). This raises a possibility that regulation of apoptosis during GT formation through various signaling outputs may constitute an important mechanism affecting outgrowing phase of the development.

Exogenous BMP4 down-regulates the expression of *Fgf8* and *Wnt5a* and suppresses cell proliferation

Fgf8 is expressed in the DUE and has been implicated in the regulation of initial GT outgrowth (Haraguchi et al., 2000; Morgan et al., 2003; Perriton et al., 2002). Recently, it has been

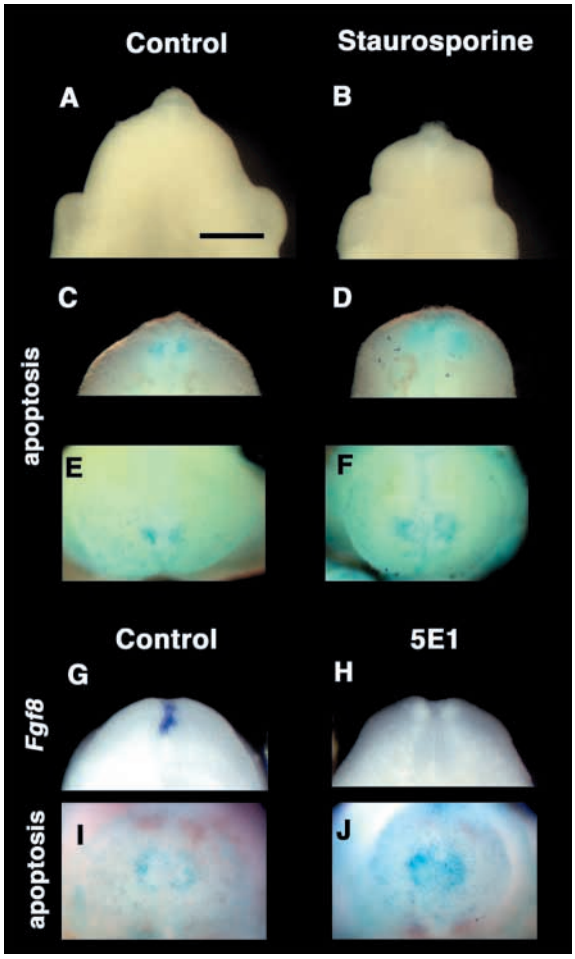

Fig. 3. Retarded GT outgrowth associated with an increase of apoptosis. (A-F) GTs at 12.0 dpc were cultured for 3 days (A,B) or for 24 hours (C-F). Apoptotic cells were increased in the GT epithelium and its adjacent mesenchyme after the staurosporine treatment, which was associated with retarded GT outgrowth proximodistally. (G,H) GT explants between 11.5 dpc and 12.0 dpc treated with anti-SHH antibody (5E1) showed a reduction in *Fgf8* expression after 24 hours of culture. (I,J) An increase of apoptosis was observed by the 5E1 treatment. The number of Nile Blue-positive cells was significantly increased by 5E1 treatment. (A-D,G,H) Ventral views; (E,F,I,J) frontal views. Scale bar: 250 μm.

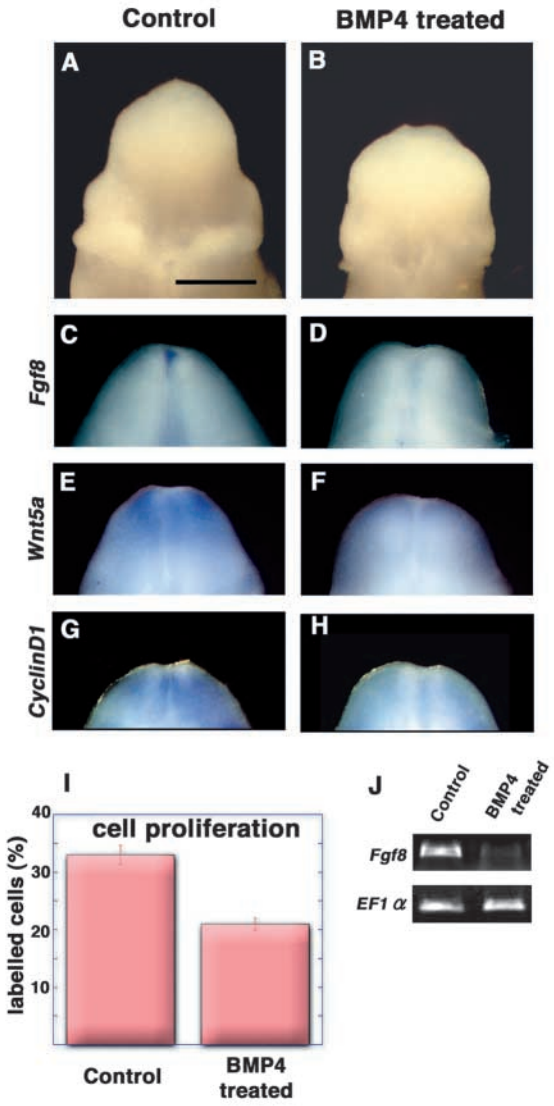

Fig. 4. Exogenous BMP4 downregulates the expression of *Fgf8*, *Wnt5a* and cyclin D1 and suppresses cell proliferation. GTs from 12.0 dpc embryos were cultured for 3 days (A,B) or for 24 hours (C-H) with BMP4 protein or BSA in the medium. (A,B) BMP4-treated GT displayed retarded GT outgrowth. (C-H) *Fgf8*, *Wnt5a* and cyclin D1 expression were suppressed by BMP4 treatment. *Shh* expression was unaltered by BMP4 treatment indicating specific effects by BMP4 (data not shown). (I) Reduced cell proliferation of BMP4-treated GT. The number of phospho-histone H3 immunopositive cells decreased to 60% compared with those of control GT explants. (J) Alteration of *Fgf8* expression was observed 6.5 hours after the BMP4 treatment by RT-PCR analysis. (A-H) Ventral views. Scale bar: 250 μm in A,B; 325 μm in C-H.

shown that *Wnt5a* is expressed prominently in the distal region of the GT mesenchyme (Oishi et al., 2003; Yamaguchi et al., 1999). Subsequently, it was shown that *Wnt5a* mutant mice exhibit proximodistally affected hypoplasia of the external genitalia and limbs affecting various appendage mesenchymes.

Given the functions of Bmp genes in GT formation shown in this study, it is tempting to speculate that Bmp genes may function in concert with other growth factor systems. Thus, we analyzed the expression of several genes to determine the molecular basis for outgrowth inhibition. The BMP4-treated specimen displayed retarded GT outgrowth (Fig. 4A,B), with a reduction in *Fgf8* and *Wnt5a* expression (Fig. 4C-F). Alteration of *Fgf8* expression was observed 6.5 hours after the BMP4 treatment by RT-PCR analysis (Fig. 4J), suggesting that such alteration was elicited by direct or by indirect mechanisms including few cascades in between. By contrast, downregulation of the *Wnt5a* was not observed at 12 hours but detected after 24 hours of the BMP4 treatment (Fig. 4E,F; data not shown). To examine the role of BMPs in regulating cell proliferation during GT outgrowth, explants were incubated with BMP4 protein. The number of phospho-histone H3-immuno-positive cells decreased to 60% compared with control GT explants (Fig. 4I). To analyze further the effects of BMP4 on cell proliferation of GT, cyclin D1 expression was

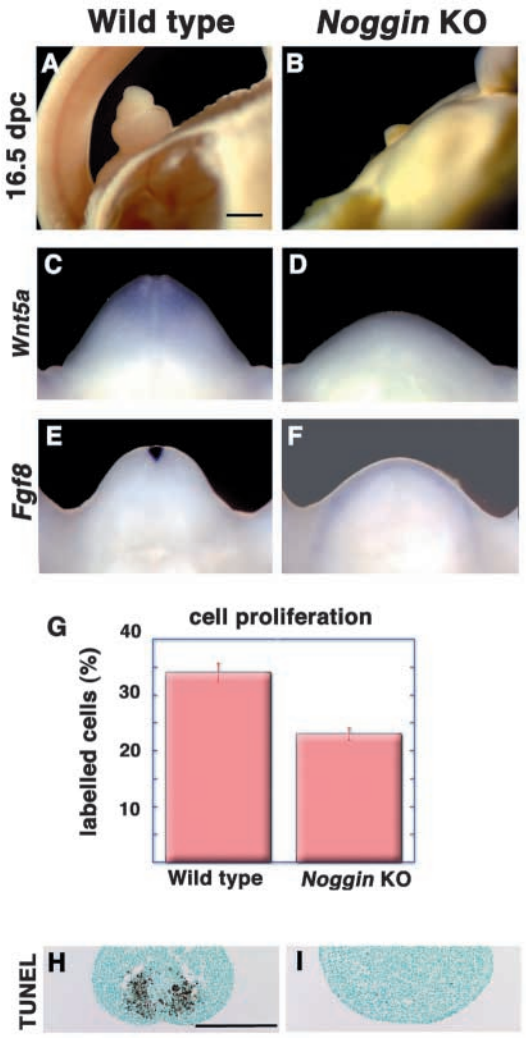

Fig. 5. GT hypoplasia of *Nog* mutant mice. (A,B) *Nog* mutant mice showed GT hypoplasia at 16.5 dpc. (C-F) A reduction of *Wnt5a* and *Fgf8* expression in the developing external genitalia region of *Nog* mutants compared with wild type at 12.5 dpc (for *Wnt5a*) and at 12.0 dpc (for *Fgf8*). (G) Reduced cell proliferation of *Nog* mutant mice GT. The number of phospho-histone H3 immunopositive cells decreased to 60% compared with wild type. (H,I) TUNEL analysis was performed on *Nog* mutant at 12.5 dpc. Apoptotic-cells were not detected in distal mesenchyme of *Nog* mutants. (A,B) Lateral views; (C-F) ventral views; (H,I) coronal sections. Scale bars: 650 μm in A,B; 250 μm in C-F; 200 μm in H,I.

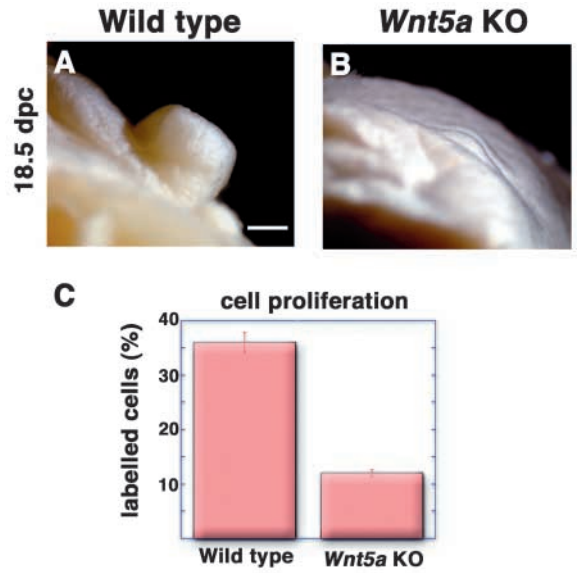

Fig. 6. GT agenesis of *Wnt5a* mutant mice. (A,B) *Wnt5a* mutant mice displayed external genitalia agenesis at 18.5 dpc. (C) The number of phospho-histone H3 immunopositive cells decreased to 40% compared with wild type at 10.5 dpc. Scale bar: 550 μm in A,B.

analyzed. CyclinD1 regulates the G1 phase of the cell cycles through the control of cyclin-dependent kinases. Reduction of cyclin D1 expression was observed 24 hours after the treatment (Fig. 4G,H) and its reduction was initially observed 12 hours after the BMP4 treatment (data not shown). These results imply that inhibition by BMP4 may be mediated either by the action of BMP(s) on cells per se and/or by the modulation of the expression of factors such as *Fgf8* and *Wnt5a* (see Discussion).

Nog mutant mice display GT hypoplasia with decreased *Wnt5a, Fgf8* expression and cell proliferation

Although current observations in vitro demonstrate that exogenously altering BMP signaling could influence GT outgrowth, cell proliferation and apoptosis, they do not necessarily address their roles in vivo.

Mice lacking *Nog* have previously been characterized with regard to defects in somite, forebrain and skeletal formation (Bachiller et al., 2000; Brunet et al., 1998; McMahon et al., 1998). To investigate the possible role of BMP signaling in murine external genitalia development, we analyzed the phenotype of *Nog* mutant mice external genitalia. *Nog* mutant mice displayed GT hypoplasia and decreased *Wnt5a* and *Fgf8* expression (Fig. 5A-F), which is consistent with the hypothesis that BMP signaling may exert an inhibitory effect on GT outgrowth. Some *Nog* mutant mice displayed milder phenotypes, probably owing to the phenotypic variations by strain backgrounds (less than 50%; data not shown). To gain insight into the cellular basis of GT hypoplasia, cell proliferation and apoptosis in *Nog* mutant mice were examined. Consistent with the reduced cell proliferation of the organ culture system (Fig. 4I), *Nog* mutant mice also displayed significant reduction in cell proliferation judged by phospho-histone H3 staining assay (Fig. 5G; ~60% compared with wild type).

Analyzing the extent of apoptosis in the *Nog* mutant mice GT was also performed. In contrast to the expectation based on observations in vitro, a reduction of the distal mesenchymal apoptosis of the *Nog* mutant GT was observed (Fig. 5H,I). *Nog* mutants show anomalies including cloacal regions already at early stages (data not shown). Analyses will be required including a possibility for the alteration of distal mesenchyme already at early stages by *Nog* mutation. Alternatively, apoptotic regulation by NOG in vitro (Fig. 2G,H) might reflect, in part, overexpression effects.

External genital agenesis and reduced cell proliferation of *Wnt5a* mutant mice

To further examine the plausible genetic cascade composed by BMP signaling with *Wnt5a*, the external genitalia of *Wnt5a*

mutant mice were examined. *Wnt5a* mutant mice displayed prominent external genitalia agenesis, around mid-embryogenesis (Fig. 6A,B) (Yamaguchi et al., 1999). It was revealed that *Wnt5a* mutant mice external genitalia displayed marked reduction of cell proliferation (~60%), consistent with the observations for the cell proliferation of *Nog* mutant mice (Fig. 6C).

GT of *Bmpr1a* mutant mice displays augmented outgrowth, up-regulation of *Fgf8* expression, reduced apoptosis and the enlarged DUE

To examine the possible effects of ablated BMP signaling during external genitalia formation, we then analyzed *Bmpr1a* conditional mutant mice. A null mutation of the *Bmpr1a* results in embryonic lethality indicating its essential role during gastrulation (Mishina et al., 1995). We used the Cre gene driven by the *Brn4* promoter to achieve tissue-specific gene mutation of the *Bmpr1a* during external genitalia development (Ahn et al., 2001). To determine the spatial and temporal expression of the Cre gene, we crossed the *Brn4*-Cre strain (bcre-32) (Ahn et al., 2001) with the ROSA reporter strain (Soriano, 1999). *Brn4*-Cre-mediated expression of *lacZ* was detected in the surface GT ectoderm and in the outer-most epithelial layer adjacent to the DUE, but not in the DUE itself at 12.5 dpc (Fig. 7A). *lacZ* expression was already detected at 10.5 dpc before GT outgrowth in the cloacal regions (Fig. 7B). To assess the state of BMP signaling upon the *Bmpr1a* mutation, immunohistochemistry was performed using an antibody for the phosphorylated form of Smad1 (pSMAD1) (Ahn et al., 2001). Smad1 is a transcription factor that is phosphorylated and activated by type I BMP receptors (Kretzschmar et al., 1997). We observed that pSMAD1 immunopositive cells were detected in the outer-most epithelial layer as well as in GT surface ectoderm of the wild type, which overlapped with the *lacZ* pattern in Fig. 7A, and was not detected in the mutant GT at E12.5 dpc (Fig. 7C). Prominent reduction (~80%) of pSMAD1 signals were already detected at 10.5 dpc before GT formation (Fig. 7C). Thus, conditional mutation of the *Bmpr1a* resulted in a reduction in BMP signaling in the distal region of the GT including the outer-most epithelial layer and GT surface ectoderm (see below).

Bmpr1a mutant mice displayed augmented GT outgrowth proximodistally with augmented expression of *Fgf8* (Fig. 7D-G). Such augmentation of *Fgf8* expression was observed at 10.5 dpc, before the hyperplasia phenotype (data not shown). It was also observed by SEM analysis that the DUE region of *Bmpr1a* mutant mice was enlarged concomitant with such upregulation (Fig. 7D,E, inset).

We next examined whether the apoptosis of GT was affected by the conditional *Bmpr1a* mutation. A marked reduction of apoptosis in the distal GT mesenchyme in the mutants was observed compared with wild-type mice at 12.5 dpc (Fig. 7H,I). These results are consistent with the hypothesis that Bmp genes may regulate the outgrowth of the GT, at least in part, through the regulation of apoptosis and/or by the regulation of expression of growth-promoting genes.

Questions remain, however, as to how such ablation of BMP signaling affects *Fgf8* expression in the DUE where Cre

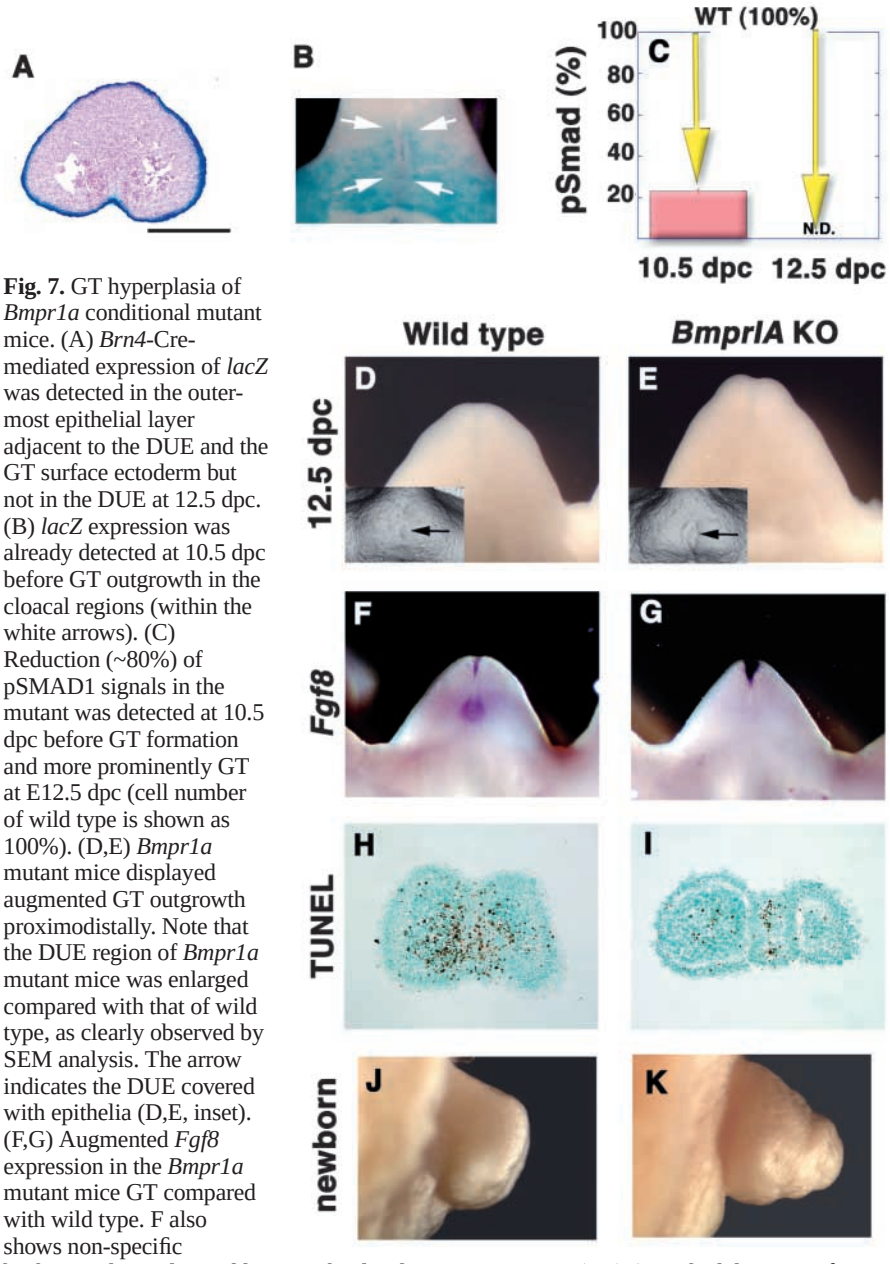

Fig. 7. GT hyperplasia of *Bmpr1a* conditional mutant mice. (A) *Brn4*-Cre-mediated expression of *lacZ* was detected in the outer-most epithelial layer adjacent to the DUE and the GT surface ectoderm but not in the DUE at 12.5 dpc. (B) *lacZ* expression was already detected at 10.5 dpc before GT outgrowth in the cloacal regions (within the white arrows). (C) Reduction (~80%) of pSMAD1 signals in the mutant was detected at 10.5 dpc before GT formation and more prominently GT at E12.5 dpc (cell number of wild type is shown as 100%). (D,E) *Bmpr1a* mutant mice displayed augmented GT outgrowth proximodistally. Note that the DUE region of *Bmpr1a* mutant mice was enlarged compared with that of wild type, as clearly observed by SEM analysis. The arrow indicates the DUE covered with epithelia (D,E, inset). (F,G) Augmented *Fgf8* expression in the *Bmpr1a* mutant mice GT compared with wild type. F also shows non-specific background signals in addition to the distal DUE expression. (H,I) A marked decrease of apoptosis was found in the distal GT region of *Bmpr1a* mutants. (J,K) GT hyperplasia was prominently observed in *Bmpr1a* mutant newborns. (A,H,I) Coronal sections; (B,D-G) ventral views; (J,K) lateral views. N.D., not detected. Scale bar: 200 μm in A; 300 μm in B; 600 μm in D,E; 750 μm in F,G; 100 μm in H,I; 950 μm in J,K.

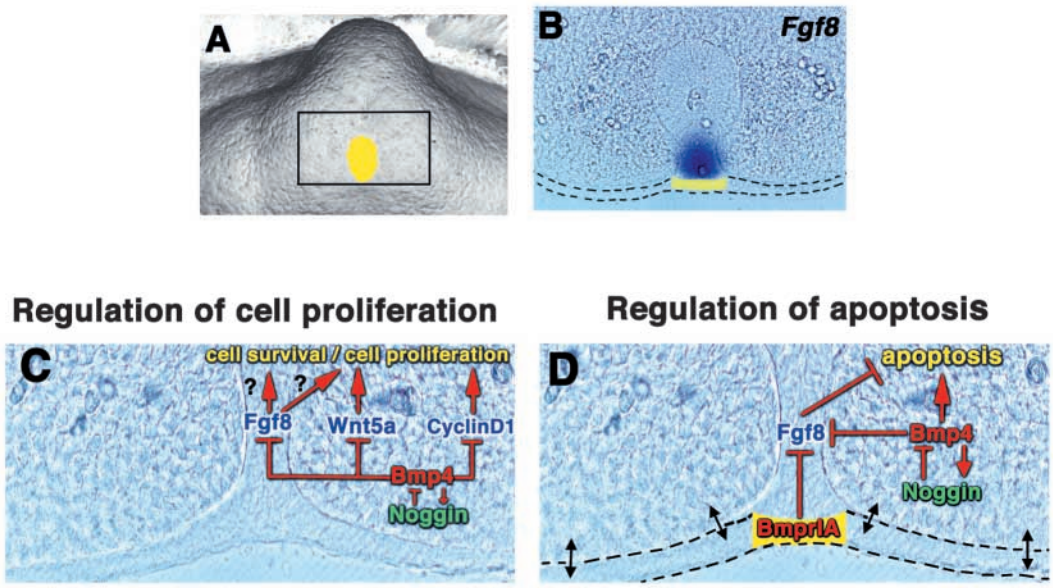

Fig. 8. Possible signaling crosstalks in the whole distal GT region including the DUE. (A) SEM picture of an embryonic genital tubercle (GT) at 12.5 dpc. The yellow region shows the location of the DUE. (B) Coronal sections of the boxed region in A. DUE, which expresses *Fgf8*, locates adjacent to the outer-most epithelial layer (yellow region) aligned with normal GT ectoderm (region between black broken lines). (C) Possible crosstalks in the distal GT mesenchymes including BMP4, WNT5A may underlie the alteration of cell survival or of cell proliferation. Previous studies suggested Fgf8 as a growth stimulatory factor (Haraguchi et al., 2000). This study indicated a possibility that it could also work as a survival factor. (D) Ablation of BMP signaling in the *Bmpr1a* mutant distal GT region is shown, which may be modulated by distal GT epithelia (double-headed arrows; possibly also by distal-dorsal epithelia) and GT mesenchyme. Epithelial derived (either from the outer-most epithelial layer or from the adjacent GT ectoderm) signals may affect *Fgf8* expression in the DUE and consequently affect apoptosis. Putative positive signaling factors are indicated by blue characters and negative signaling factors are indicated by red characters. The antagonist, noggin, is shown in green.

activity was not detected. Roles of possible signaling crosstalks within the whole distal GT epithelia are discussed in relation to the structure and expression status (see below; Fig. 8).

GT proximodistal hyperplasia was also observed in the later stages of development, e.g. in newborn samples (Fig. 7J,K). Thus, it seems likely that the GT hyperplasia of *Bmpr1a* mutant mice may possibly not, at least for late-staged phenotypes, be attributed to abnormal signaling in the DUE, because DUE is not observed after mid-gestation in normal embryogenesis. Possible signaling output through the whole distal GT regions is discussed (see below; Fig. 8).

Discussion

The role of BMP signaling in controlling GT outgrowth, apoptosis and cell proliferation

Regulation of apoptosis during development is one of the characteristic requirements for proper outgrowth of embryonic anlagen. Limb morphogenesis in amniote embryos, for example, has drawn considerable attention. A balance between cell proliferation and apoptosis has been suggested as important for limb bud outgrowth (Ferrari et al., 1998).

External genital anlage, genital tubercle (GT), is one of the organs outgrowing from the posterior body trunk. GT initiates from the cloaca region forming as a bud-anlage from 10.5 dpc and continues to differentiate throughout the perinatal period (Suzuki et al., 2002; Yamada et al., 2003). A coordinated mode of development of the caudal embryonic region including the cloaca, tail gut and VER (ventral ectodermal ridge) has been reported previously (Goldman et al., 2000; Miller and Briglin, 1996). Regulated apoptosis has been described as underlying such posterior-embryonic development, e.g. tail gut regression. Of note, the emergence and presence of apoptosis during GT development was observed in parallel with the onset and maintenance of the expression of Bmp genes. In this study, series of gain-of-function studies by organ culture and loss-of-function studies with several knockout mice indicated an importance of the regulation of apoptosis and cell proliferation during GT formation. Apoptosis in the distal region of GT was markedly reduced in *Bmpr1a* conditional mutants or by NOG treatment in vitro. By contrast, apoptosis was increased mainly in the distal GT mesenchyme by BMP, staurosporine and anti-SHH antibody treatment suggesting an importance of apoptotic regulation depending on several signaling outputs.

The activity of BMP has also been associated with the developmentally regulated onset of apoptosis (Coucouvanis and Martin, 1999; Furuta et al., 1997). Bmp genes are responsible for the regulated outgrowth of the limb (Macias et al., 1997; Merino et al., 1999). In the case of urogenital development, *Bmp4* is normally expressed in the mesenchyme adjacent to the cloaca and induced ectopically in the affected cloaca-like region with augmented apoptosis in *Shh* mutant embryos, which show GT agenesis (Haraguchi et al., 2001). Taken together, the output of BMP signaling may affect GT outgrowth, in part, through a mechanism including apoptosis.

In addition to the regulation of apoptosis, the clear reduction of GT cell proliferation, shown by organ culture studies or by *Nog* and *Wnt5a* mutant mice analyses, suggested a plausible regulation of cell proliferation by the BMP system. The role of *Wnt5a* in regulating cell proliferation as a possible downstream gene of *Bmp4* is suggested. In limb morphogenesis, *Wnt5a* is expressed in the distal limb mesenchyme close to AER. *Wnt5a* mutant mice showed decreased cell proliferation in such regions (Yamaguchi et al., 1999). But they showed increased cell proliferation of lungs suggesting tissue-dependent variability in the role of WNT5A signaling. We found a significant decrease of cell proliferation in *Wnt5a* mutant GTs associated with GT agenesis.

The current data might imply plausible differences in between cell survival factors and growth stimulatory factors (mitotic factors) because increased apoptotic signals were not detected in the hypoplasic *Wnt5a* mutant GTs without alteration of *Fgf8* expression (data not shown). Thus,*Wnt5a* might work as a cell mitotic (growth stimulatory) factor rather than as a cell survival factor. It may not be primarily responsible as a 'close' downstream effector of BMP signaling because its expression was not altered after 12 hours of BMP4 treatment. *Bmpr1a* mutant mice hyperplasic GTs did not show alteration of cell proliferation nor alteration of *Wnt5a* expression (data not shown). However they showed clear upregulation of *Fgf8* expression and loss of apoptosis. It has been also suggested that FGF8 might be one of the survival factors because its downregulation induced augmented apoptosis for limb formation (Sun et al., 2002). BMP4 treatment in vitro altered both *Fgf8* and *Wnt5a* expression leading to augmented apoptosis and decreased cell proliferation (Figs 2, 4). Reduction of cell proliferation in the *Nog* mutant mice with loss of *Fgf8* and *Wnt5a* expression may also suggest a possibility of alteration of both factor systems. The two activities described above, however, can not be strictly distinguished as mutually distinct pathways and further analysis on the relationships for both processes, will be required.

Possible crosstalk among the genes for growth factors

In the mouse, *Bmp4* is frequently expressed adjacent to Fgf-expressing regions and is involved in the regulation of cell proliferation and differentiation (Hogan, 1996). Several Bmp genes are expressed in the mouse dorsal forebrain and facial primordia (Barlow and Francis-West, 1997; Furuta et al., 1997). Ectopic application of BMP4 reduces the expression of both *Shh* and *Fgf8* (Ohkubo et al., 2002) and represses anterior neural gene expression promoting apoptosis in mouse forebrain explants (Graham et al., 1994). *Bmp4* and *Fgf10* are often expressed in adjacent domains during organogenesis (Weaver et al., 2000). During limb morphogenesis, BMP-FGF crosstalk has been suggested as functioning during, for example, the apical ectodermal ridge (AER) formation or differentiation of the inter-digit necrotic zone. Inactivation of BMP signaling results in the loss of *Fgf8* expression in the AER of the limb (Ahn et al., 2001; Pizette et al., 2001). In addition, it has been reported that syndactyly of heterozygous Fused toes (*Ft*) mice (van der Hoeven et al., 1994), correlates with an imbalance in *Bmp4* and *Fgf8* expression (Heymer and Ruther, 1999). Apart from limbs, developmental budding processes are often influenced by the interaction of positive growth regulators (e.g., the FGF family or SHH) and negative growth regulators (such as BMP2, BMP4 and BMP7) (Hogan, 1999; Jung et al., 1998). The BMP and FGF signaling pathways are known to interact antagonistically in many developmental contexts, including branchial arch development (Neubuser et al., 1997; St Amand et al., 2000).

BMP-FGF crosstalk may also function during GT development. Downregulation of the *Fgf8* expression after 6.5 hours of the BMP4 treatment suggested a close, but not necessarily direct, relationship between *Bmp4* and *Fgf8* by this study. Later, *Bmp4* and *Fgf10* are both expressed during bilateral mesenchymal differentiation adjacent to the midline urethral plate epithelium during urethra formation (Haraguchi et al., 2001). During GT development, the expression pattern of *Bmp7* in the DUE overlapped, at least in part, with that of *Fgf8* (Morgan et al., 2003). Combinatorial BMP-FGF crosstalk in the whole distal GT region, such as BMP7-FGF8, BMP4-FGF8 or with BMP antagonists remains to be tested.

In *Drosophila*, Decapentaplegic (DPP) is a downstream target gene of HH signaling. There is also evidence that BMP4 may be a downstream target of SHH (Ingham and McMahon, 2001; McMahon et al., 2003) or located upstream of SHH by regulating its expression in the mouse dental epithelium and limb bud (Ahn et al., 2001; Zhang et al., 2000). It has been shown that SHH possess some outgrowth-promoting activities by GT organ cultures, because blocking SHH signaling induced the GT apoptosis and downregulated the *Fgf8* expression of DUE (this study) (Haraguchi et al., 2000; Haraguchi et al., 2001). Further studies are necessary for BMP(s)-SHH interaction during GT formation.

It has been reported that BMP-WNT signaling pathways may be interactive in limb and lung morphogenesis (Barrow et al., 2003; Li et al., 2002; Soshnikova et al., 2003) and have antagonistic functions in the specification of the trunk neural crest (Jin et al., 2001). BMP4 treatment reduced *Wnt5a* expression in vitro and *Nog* mutant mice showed decreased *Wnt5a* expression with GT hypoplasia. The current study based on gain-or-loss-of-function assays showed marked alterations of gene expression profiles of the DUE region, suggesting its orchestrating functions in early GT development. Detailed analysis of genes related to DUE regions of *Bmpr1a* mutant mice indicated additional aspects of modulation of BMP signaling through whole distal GT regions (see below).

DUE formation and BMP signaling during GT development: the DUE as part of the signaling cascades

The AER, a transient specialized distal epithelium, is essential for vertebrate embryonic limb outgrowth along the proximodistal (PD) axis. The SHH/FGF feedback signaling loop that operates between the polarizing region and the AER, may coordinate growth and patterning for the limb (Haramis et al., 1995; Niswander et al., 1994; Zuniga et al., 2002). Members of the Bmp and Fgf gene families have been suggested as regulating various epithelial-mesenchymal interactions during limb development (Martin, 1998) including opposite roles during limb outgrowth depending on the context of the PD development (Ganan et al., 1996; Niswander and Martin, 1993).

As for external genitalia formation, the DUE has roles in controlling mesenchymal gene expression and initial GT outgrowth (Haraguchi et al., 2000; Haraguchi et al., 2001). Our current analysis of the DUE revealed dynamic and complex gene expression including *Fgf8* and *Bmp4*. Several BMP signaling molecules were found to be dynamically expressed in and adjacent to the DUE. In addition to the *Fgf8* expressing DUE region, the outer-most epithelial layer and aligned GT surface ectoderm regions were identified (Fig. 8B; the yellow region and black broken surface epithelial region, respectively). It was found that *Fgf8*, *Bmp4*, *Bmp7* and *Shh* were not detected in the above two regions (Fig. 8B; data not shown). This may represent an intriguing structural contrast with other signaling epithelia, e.g. the AER, which is composed of distinct apical ectodermal epithelia. Although the current analysis agrees with the hypothesis of an essential

function of the distal GT region orchestrating initial GT outgrowth as a whole, this study also raised a question of the composition of whole distal GT regions and the functions of each included region (Fig. 8C,D). Down- or upregulation of the *Fgf8* expression observed in gain-or-loss-of-function studies are consistent with the idea of the importance of the DUE for early GT development. Alteration of the pSMAD1 expression, however, suggested that the primary effect of *Bmpr1a* conditional mutation resides in the outer-most epithelial layer (Fig. 8D, yellow region) and aligned GT surface ectoderm regions (Fig. 8D; such influences shown by black double-headed arrows may be mediated also by dorsal epithelial regions). The enlargement of the DUE associated with upregulation of *Fgf8* appears to reflect an indirect consequence of altered BMP signaling and cellular alterations being relayed from whole distal GT epithelia, thereby eliciting GT dysmorphogenesis (Fig. 7, Fig. 8D). To what extent could the GT hyperplasia phenotype of *Bmpr1a* mutants be derived from DUE and/or from distal GT epithelia? Although current analyses showed a clear alteration of marker gene expression in the DUE by both gain-or-loss-of-function assays, this study does not yet answer this question. Normal *Fgf8* expression in the DUE remains until ~14 dpc (Haraguchi et al., 2000), while GT outgrowth continues further later. *Bmpr1a* mutant GT hyperplasia was observed in newborn samples. This might reflect DUE-less-dependent GT outgrowth for late-staged GTs. In this aspect previous findings suggesting the importance of normal GT ectoderm for proper GT development would be intriguing, albeit based on broad ectoderm-mesenchymal recombination assays available at that time (Murakami and Mizuno, 1986). Another recent related observation is that ectodermal overexpression of *Nog* by a *K14*-transgene, results in the prominent GT hyperplasia observed for similarly late-staged GT specimens (C.-M. Chuong, personal communication). During other appendage development, it has been shown that Bmp genes are expressed in the early ventral limb ectoderm and that BMP signaling is required for dorsoventral (DV) patterning in limb buds (Ahn et al., 2001; Pizette et al., 2001). Both DV patterning and growth of the limb require signals from the limb ectoderm to the underlying mesenchyme (Chen and Johnson, 1999). Further studies on whole distal GT epithelial regions related to the PD, DV aspect of molecular cascades, will be required.

It remains to be investigated as to what extent outer-most epithelial layer and DUE could be derived from surface ectoderm and/or endoderm origin, respectively, because of the lack of detailed molecular and cellular analyses available (Kurzrock et al., 1999a). The DUE region has received some attention as a unique epithelia related as the cloacal plate epithelium for early stages, and the solid urethral plate for later stages, although its molecular nature has remained completely elusive (Kurzrock et al., 2000; Penington and Hutson, 2002). This study also indicates, for the first time, the possibility of complex signaling in the whole distal GT region.

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