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Fate and function of the ventral ectodermal ridge during mouse tail development

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SUMMARY

In the mouse embryo, the body axis continues to develop after gastrulation as a tail forms at the posterior end of the embryo. Little is known about what controls outgrowth and patterning of the tail, but it has been speculated that the ventral ectodermal ridge (VER), a morphologically distinct ectoderm on the ventral surface near the tip of the tail, is a source of signals that regulate tail development (Grüneberg, H. (1956). *Nature* 177, 787-788). We tested this hypothesis by ablating all or part of the VER and assessing the effects of such ablations on the development of tail explants cultured in vitro. The data showed that the VER produces signals necessary for somitogenesis in the tail and that the cells that produce these signals are localized in the middle and posterior region of the VER. Dye labeling experiments revealed that cells from these regions move anteriorly within the VER and eventually exit it, thereby colonizing the ventral surface ectoderm anterior to the VER. In situ hybridization analysis showed that the genes encoding the signaling molecules FGF17 and BMP2 are specifically expressed in the VER. Assays for gene expression in VER-ablated and control tails were performed to identify targets of VER signaling. The data showed that the VER is required for expression of the gene encoding the BMP antagonist NOGGIN in the tail ventral mesoderm, leading us to speculate that one of the major functions of the VER in tail development is to regulate BMP activity.

Key words: Noggin, Secondary body formation, Somitogenesis, Tail bud, Mesenchyme, Ventral Ectodermal Ridge, VER, Mesoderm

INTRODUCTION

In vertebrate embryos, the body axis develops in two phases, termed primary and secondary body formation (Holmdahl, 1925). During the first phase, the somites and other types of mesoderm found along the length of the anteroposterior (AP) axis are derived from cells that have traversed the primitive streak (in amniotes) or its equivalent (in lower vertebrates). During secondary body formation, the tail bud is the source of somitic mesoderm (Schoenwolf, 1977, 1978; Tam, 1984; Tam and Beddington, 1987; Catala et al., 1995, 1996). In both the mouse and chick, more than half the total number of somite pairs that form during embryogenesis develop from the tail bud (Rugh, 1968; Le Douarin et al., 1998; Kaufman and Bard, 1999). In the mouse embryo, secondary body formation begins on approximately embryonic day (E) 10 (30-31 somites; Wilson and Beddington, 1996) and is completed by ~E13.25 (65 somites; Tam, 1981). Most of the somites produced during the second phase develop into the dermis, muscles and vertebrae of the adult tail. In contrast, in species that do not possess a tail postnatally, such as the chick, the caudalmost somites of the tail die, whereas more rostral tail somites incorporate into the trunk, thus contributing to the caudal region of the body (reviewed by Schoenwolf, 1981; Griffith et al., 1992). At present, the mechanisms that regulate the transition between primary and secondary body formation, tail outgrowth and patterning, and tail remodeling are poorly understood.

During secondary body formation, the somites develop from an undifferentiated, histologically homogeneous stem cell population known as the tail bud mesenchyme (TBM). The process of somite formation in the tail appears to be similar to that in the trunk (Tam, 1986). Epithelial somites segment continuously from the anterior end of the presomitic mesoderm (PSM) at regular intervals, while new somite precursors derived from the TBM incorporate into the posterior end of the PSM. At any given stage, the PSM contains a defined number of somite precursors (6-7 in mouse and 10-12 in chick), which are known as somitomeres (Tam et al., 1982; Packard and Meier, 1983; Tam, 1986; Tam and Trainor, 1994).

The developing tail contains two other mesodermal lineages, the notochord and the lateral mesoderm (see Fig. 1A,B). The latter is positioned ventrally in the tail and will hereafter be referred to as the ventral mesoderm. The notochord is derived from remnants of the node in the tail bud (Catala et al., 1996; Wilson and Beddington, 1996; Le Douarin et al., 1998). The origin of the tail ventral mesoderm is still unclear. It may arise from the TBM (Schoenwolf, 1977) or grow into the tail from

the trunk lateral plate mesoderm (Le Douarin et al., 1998). The tail also contains a postanal gut, known as the tail gut, the dorsal portion of which is derived from node remnants (Catala et al., 1996) and the remainder of which grows into the tail from the hindgut (Schoenwolf, 1978). In addition, there is a neural tube, which develops from a condensed mesenchymal cell population in the tail bud. Unlike the trunk neural tube, which forms by folding of the neural plate, the tail neural tube forms by epithelialization and cavitation, a process known as secondary neurulation (reviewed by Griffith et al., 1992). In organisms that possess a tail postnatally, both the gut and the neural tube are eliminated from the tail by apoptosis during embryogenesis (Nievelstein et al., 1993; Miller and Briglin, 1996).

Somitogenesis normally ceases at ~E13.25 in the mouse, when the TBM regresses by apoptosis. However, when ~E13 TBM cells are grafted into the primitive streak of the ~E8.5 embryo, they survive and make significant contributions to paraxial mesoderm of the trunk (Tam and Tan, 1992). This observation suggests that TBM survival and perhaps other processes such as cell proliferation, fate determination and/or differentiation are influenced by extrinsic signals. Although any of the tissues in the developing tail might be the source of such signals, it has been suggested that the ventral ectodermal ridge (VER) is a good candidate because of its morphological resemblance to the apical ectodermal ridge (AER) of the limb bud (Grüneberg, 1956), which is known to be the source of signals that control limb bud outgrowth and patterning (reviewed by Martin, 1998). In both chick and mouse, the VER stretches caudally along the AP axis of the tail from the region containing the PSM to the anterior border of the TBM (Grüneberg, 1956, 1963). Like the AER, which is present throughout the period of limb bud development, the VER is present during secondary body formation and regresses just prior to the stage at which somitogenesis ceases in the tail.

Although it has been more than thirty years since Grüneberg speculated that the VER and AER have similar functions in the morphogenesis of the tail and the limb, respectively, this hypothesis has remained untested. In this study, we employed culture conditions that support the development of mouse embryo tail explants in vitro and performed VER ablation experiments similar in principle to those used to analyze AER function in the chick limb (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1981). The results of this analysis have provided insight into the role of the VER in tail development.

MATERIALS AND METHODS

Tail explant culture

Tails were isolated from embryos obtained by mating CD1 (Charles River Laboratories, Hollister, CA) or ARC/S (Animal Resource Centre, Perth, West Australia) outbred albino mice. Noon of the day on which the vaginal plug was detected was considered as ~E0.5 in the timing of embryo collection, but embryos were staged more precisely by counting the number of somites posterior to the genital tubercle, with the somite at the genital tubercle scored as somite 31 (Tam, 1981). All dissections were performed in PB1 (Sturm and Tam, 1993). To produce a tail tissue fragment, embryos with 35-41 somites were transected using electrolytically sharpened wire needles at the anterior border of the second to last somite. The tail fragments were placed in a rotating apparatus (BTC Engineering, Cambridge, UK) and cultured for a total of 25-28 hours in 20%O_2, 5%CO_2, 75%N_2 (Sturm and Tam, 1993).

Ectoderm ablation and replacement

For the ablation of VER or lateral surface ectoderm, a fine glass needle was used to score a rectangle in the ectoderm, thus marking the area to be removed. The ectoderm was peeled away from the underlying mesoderm by pushing the tip of the needle beneath the ectoderm and then shearing along the undersurface of the ectoderm. In about half the cases, the tails were treated briefly with a mixture of enzymes prior to VER ablation to help separate the ectoderm from mesoderm, as described by Sturm and Tam (1993) with the following modifications. A 1:1 mixture of 2.5% pancreatin (Gibco, Grand Island, NY) and 0.25% trypsin (Sigma, St. Louis, MO), 0.02% EDTA was made and then diluted 1:7 in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). The tail fragments were incubated in the enzyme mixture on ice for 2 minutes.

In the VER replacement experiments, embryos of a transgenic line in which a *lacZ* gene containing a nuclear localization signal is ubiquitously expressed (Tam and Tan, 1992) were used as the source of donor tissue. For orthotopic grafts, the donor tissue consisted of a fragment of ectoderm containing part of the VER, spanning ~1/3 of its width across the midline and the posterior ~2/3 of its length along the AP axis. For heterotopic grafts, similar-sized fragments were dissected from the lateral ectoderm of the tail or from the AER of the limb bud. The donor ectoderm fragment was pushed into the mesoderm of a VER-ablated host tail explant. VER-ablated tails with an ectodermal graft and those without (as controls) were placed in static culture for 45 minutes to 2 hours to allow the graft to incorporate into the host tissues, then were transferred to rotating culture. The graft-derived tissues were identified by their β-galactosidase activity (Tam and Tan, 1992).

Fate-mapping of the VER

To track VER cell fate, cells were labeled by microinjecting the carbocyanine dyes DiI and DiO (Molecular Probes, Eugene, OR) using a Leitz micromanipulation system in conjunction with a Leitz Fluovert fixed-stage inverted microscope. Groups of cells within the anterior, middle or posterior thirds of the VER were labeled by delivering approximately 0.05 μl of dye (0.5 mg/ml) to the midline of the ridge. After culture, the tails were washed in PBS and analyzed in whole mount using a Leica DLMB fluorescence microscope. The specimens were examined using rhodamine (for DiI) and FITC (for DiO) excitation and transmission filter sets. The images were captured by a CCD camera with intensifier (Panasonic WV-CL700/A0) and processed digitally using Leica Q500MC image processing and analysis software.

Testing the effect of protein factors by bead implants

Beads to be implanted in tail explants were soaked overnight or for up to one week at 4°C in recombinant proteins in PBS alone or with 15-20% heat-inactivated fetal calf serum (FCS). They were washed briefly in PB1 immediately prior to implantation. FGF4 beads were prepared as previously described (Niswander et al., 1993), using human recombinant FGF4 (1 μg/μl, Sigma). To prepare BMP2 beads, Affigel blue agarose beads (Bio-Rad, Hercules, CA) were washed in PBS with 15% FCS and then soaked in human BMP2 (generously provided by Genetics Institute, Cambridge, MA) at concentrations of 0.25 μg/μl, 0.125 μg/μl, 0.05 μg/μl, or 0.025 μg/μl. To prepare NOGGIN beads, Affigel blue agarose beads were washed in PBS and then soaked in 0.5 μg/μl *Xenopus* NOGGIN (generous gift of Richard Harland, UC Berkeley). Activity of the NOGGIN protein was confirmed by incubating *Xenopus* animal caps in 1 μg/μl NOGGIN and assaying for cement gland formation (Lamb et al., 1993). Beads were inserted into a slit in either the VER or the ectoderm at the tip of the tail, or into the mesoderm after VER ablation. The tails were allowed to recover in stationary culture for

45 minutes to 2 hours after bead implantation and then were transferred to rotating culture.

Histological analysis and assays for cell proliferation and apoptosis

Tail tissue was embedded in paraffin wax or in plastic resin (JB-4 catalyzed resin, Polysciences, Inc., Warrington, PA) according to the manufacturer's protocol, and sectioned at 5 μm using standard procedures. Parasagittal sections of the tail were stained with an anti-Proliferating Cell Nuclear Antigen monoclonal antibody (Vector Laboratories, Burlingame, CA) as described by Anderson et al. (1997), and counterstained with Nuclear Fast Red. Proliferative activity was assessed by scoring the number of antibody-labeled and non-labeled nuclei in a defined area that spanned the TBM and the posterior region of the PSM. At least two non-adjacent sections were counted for 6 specimens each of VER-ablated and non-ablated groups. The percentage of labeled (proliferating) cells was calculated for each tail and the data were analyzed using a z-test of proportions and a Wilcoxon rank sum test. TUNEL assays for cell death were performed on sections of the cultured tail explants using an 'In Situ Cell Death Detection-POD' kit (Boehringer-Mannheim), essentially following the manufacturer's instructions. Quantification of apoptotic (dying) cells was performed as described above for cell proliferation.

RNA in situ hybridization analysis

Whole-mount RNA in situ hybridization analysis was performed on intact embryos or on tail explants cultured in vitro. A detailed description of the protocol is provided by Goldman (1999). Digoxigenin-labeled riboprobes were prepared from plasmids described in references cited for each gene. For sectioning after staining, the embryos were embedded in plastic resin, as described above. Some sections were counterstained with 0.1% Nuclear Fast Red.

RESULTS

VER morphology and gene expression

We analyzed VER morphology in mouse embryos at various stages of development (Fig. 1A,B, and data not shown). As previously reported (Grüneberg, 1956), the VER is first found in the surface ectoderm ventral to the posterior neuropore of embryos at ~E9.0 (17-18 somites). By ~E10.0 (30-31 somites), when secondary body formation begins, the VER has thickened and extended posteriorly to almost the tip of the developing tail (Gofflot et al., 1997). By ~E10.5 or shortly thereafter (34-40 somites), the VER reaches its maximum thickness and length along the AP axis, spanning from the anterior end of the segmental plate to near the tip of the tail (Fig. 1A,B). Subsequently, it decreases in both thickness and length, and finally disappears by ~E13.25 (65 somites), coinciding with the cessation of tail elongation.

Gene expression was studied in the VER of ~E10.5 embryos by whole-mount in situ hybridization using probes for genes encoding secreted signaling molecules and their antagonists (Fig. 1C-J, and data not shown). We were especially interested to determine whether the VER expresses members of the FGF gene family, which are thought to be responsible for the signaling function of the AER (reviewed by Martin, 1998). Of the four FGF genes known to be expressed in the mouse AER, *Fgf4* (Niswander and Martin, 1992), *Fgf8* (Heikinheimo et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995), *Fgf9* (Colvin et al., 1999) and *Fgf17* (Sun et al., 2000), only *Fgf17* RNA was detected in the VER. Its expression was restricted to the posterior two thirds of the VER (Fig. 1C,D). *Fgf17* RNA was also detected in the TBM (asterisk in Fig. 1C) and in midline structures, including the neural tube, the tail gut and the ventral mesoderm (Fig. 1D; Maruoka et al., 1998). *Fgf3*, *Fgf4*, *Fgf8*, *Fgf9*, *Fgf15*, *Fgf16* and *Fgf18* expression was detected in tail tissues other than the VER (Wilkinson et al., 1988; Niswander and Martin, 1992; Crossley and Martin, 1995; McWhirter et al., 1997; Maruoka et al., 1998; Colvin et al., 1999; Yamaguchi et al., 1999; and data not shown). *Fgf10* RNA was not detected in either the VER or other tail tissue (data not shown). We also assayed for expression of *Spry1*, *Spry2* and *Spry4* (Minowada et al., 1999), three members of the Sprouty gene family, which encodes antagonists of signaling via receptor tyrosine kinases, including FGF receptors (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). Expression of all three Sprouty genes was detected throughout much of the tail surface ectoderm, including the VER, as well as in other regions of the tail (data not shown).

We also assayed at ~E10.5 for the expression of BMP family members and several BMP antagonists (reviewed by Hogan, 1996; Smith, 1999). *Bmp2* RNA (Lyons et al., 1990) was detected specifically in the VER, along its entire length at that stage (Fig. 1E,F). Further analysis showed that *Bmp2* is expressed along the length of the VER, from ~E9.0 (17-18 somites) until ~E12 (50 somites). However, expression was not detected at ~E13 (60 somites), just before the VER regresses (data not shown). *Bmp4* RNA (Jones et al., 1991) was not detected in the VER, but it was detected in the tail gut and the mesoderm ventral to the gut (McMahon et al., 1998; Fig. 1G,H). We did not detect expression of the BMP antagonists noggin (*Nog*) (Zimmerman et al., 1996), chordin (*Chrd*) (Piccolo et al., 1996) or gremlin (Hsu et al., 1998) in the VER, although expression of *Nog* and *Chrd* was detected in the dorsal neural tube and notochord (McMahon et al., 1998; Fig. 1I,J, and data not shown). Of special interest was the observation that *Nog* RNA is localized in the mesoderm underlying the VER from ~E9.0 (17-18 somites) through ~E13 (60 somites) (Fig. 1I,J, and data not shown). This domain of expression appeared to overlap with the *Bmp4* expression domain in the ventral mesoderm. Together, these data indicate that the VER is a source of signaling molecules, such as FGF17 and BMP2, that might play a role in regulating tail outgrowth and patterning.

Fate mapping of the VER

We fate-mapped cells in three different regions of the VER (anterior, middle and posterior third) at ~E10.5, when the VER has attained its maximum size, by labeling cells in the ventral midline, which is the thickest and most prominent part of the VER, with DiI or DiO (Fig. 2A). After 25-28 hours in culture, dye-labeled tails were morphologically similar to unlabeled intact tails, in which the VER has lost its characteristic tall columnar morphology but continues to express *Bmp2*. Essentially the same number of somites formed in the dye-labeled tails (mean = 10.6 pairs; n=37) as in unlabeled controls (mean = 11.1 pairs; n=14), indicating that the labeling had no detrimental effect on tail development.

Descendants of cells labeled in the anterior third of the VER were all found in the ventral midline surface ectoderm of the tail, anterior to the VER (n=15; Fig. 2B). Labeled cells from

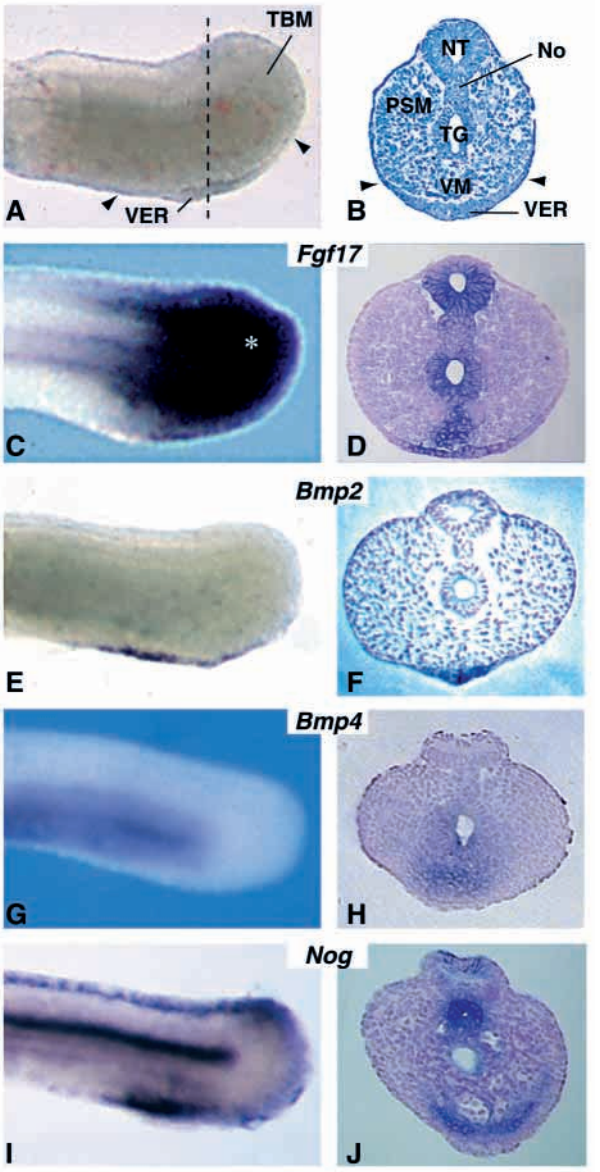

Fig. 1. Morphology and gene expression in the developing tail. (A,C,E,G,I) Whole-mount tails of mouse embryos with 35-41 somites. All tails are oriented with posterior on the right. (B,D,F,H,J) Transverse sections of the tails. The sections were cut at different positions along the AP axis of the tail, but all contain the VER. RNA in situ hybridization was performed in whole mount using probes for the genes indicated. The arrowheads in A,B indicate the boundaries of the VER. The dashed line in A indicates the approximate level of the section shown in B, which was stained with Toluidine blue. The asterisk in C indicates the domain of *Fgf17* expression in the TBM. Abbreviations: No, notochord; NT, neural tube; PSM, presomitic mesoderm; TBM, tail bud mesenchyme; TG, tail gut; VER, ventral ectodermal ridge; VM, ventral mesoderm.

the middle third of the VER were either contained in the VER or in the ventral surface ectoderm anterior to the VER (3/8 and 5/8 tails assayed, respectively). These cells were always found anterior to those derived from the posterior third of the VER (data not shown). In 27/29 tails that were labeled in the posterior third of the VER, labeled cells were mostly contained

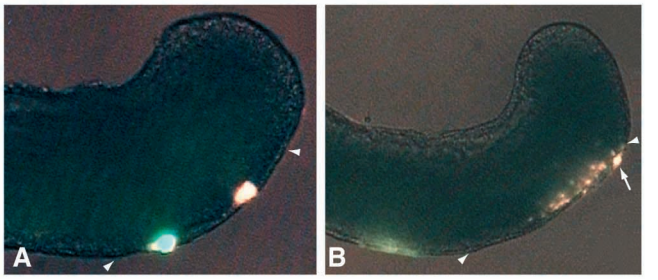

Fig. 2. Analysis of VER cell fate. (A) Cells in the anterior and posterior VER of an ~E10.5 tail were labeled with DiO (green) and DiI (red), respectively. (B) A different tail explant, showing the location of labeled cells after approximately 26 hours of culture in vitro. The arrowheads indicate the approximate boundaries of the VER. The arrow in B points to the region most intensely labeled with DiI and therefore the presumed site of injection in the posterior third of the VER. Note that DiI-labeled cells have spread anteriorly within the VER- and DiO-labeled cells have exited and are distributed in the ventral surface ectoderm anterior to the VER.

in the VER and were distributed along its full AP length (Fig. 2B). No mixing was observed between cells originating from different segments of the VER and the VER descendants did not colonize the lateral surface ectoderm (data not shown). These data show that cells in the posterior region of the VER at ~E10.5 and/or their descendants populate the whole VER and upon further development will exit the VER and contribute to the ventral surface ectoderm of the tail. This finding raises the possibility that cells in the posterior VER may serve as a stem cell population for the VER. One prediction of this model is that neighboring cell populations are not recruited into the VER. Consistent with this prediction, we have found that ectodermal cells posterior to the VER did not contribute to the VER or the ventral midline ectoderm (data not shown).

VER ablation and replacement

To determine the function of the VER in maintaining tail development, we have assessed the developmental consequences of complete and partial ablation of the VER (Fig. 3; Table 1). The formation of somites from TBM was used as a measure of the extent of tail bud development in vitro, because somite number is easily scored, and once formed, somites, unlike other TBM derivatives, do not undergo extensive apoptosis nor do they change their position within the tail. Furthermore, it has previously been established that a consistent number of somites develop in tail explants during a defined culture period (Tam, 1986). Thus, the number of new somite pairs that formed in tails isolated from ~E10.5 embryos and cultured for 25-28 hours was used as a quantitative measure of tail development.

Although, in whole mount, the VER appears to be localized to the ventral midline of the tail, histological analysis showed that the VER actually extends more laterally and may occupy a larger than expected area of the tail ectoderm (Fig. 1B). To ensure that the VER was ablated completely, we always removed the entire ectoderm extending from the posterior border of the last formed somite to the tip of the tail and spanning the ventral third of the circumference of the tail. Following culture, we examined the tail explants histologically, carried out an in situ hybridization analysis for

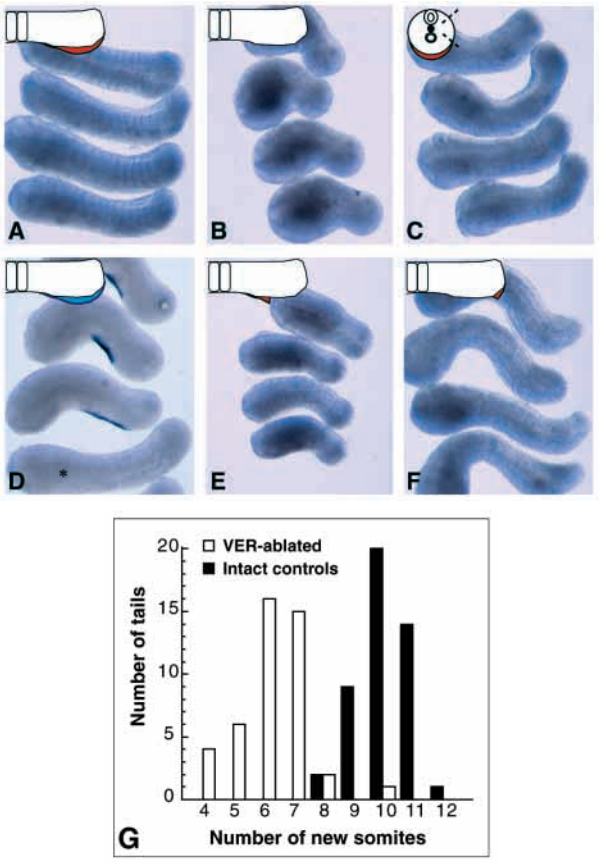

Fig. 3. VER removal inhibits somitogenesis. (A-F) Each panel shows tails cultured for 25-28 hours after the experimental manipulation illustrated in the upper left was performed. (A) Intact control tails, with the VER shown in red. Tails from which (B) the entire VER or (C) the lateral ectoderm was removed. (D) Tails that received grafts of *lacZ*-expressing VER fragments (illustrated in blue) after removal of the entire VER. Asterisk indicates an intact control tail. The grafted cells can be recognized on the ventral surface of the tails because they display β-galactosidase actvity. Tails in which only (E) a posterior fragment or (F) an anterior fragment of the VER was left intact (illustrated in red). (G) Histogram showing the number of new somites formed during a 25-28 hour culture period in intact control tails and following removal of the entire VER.

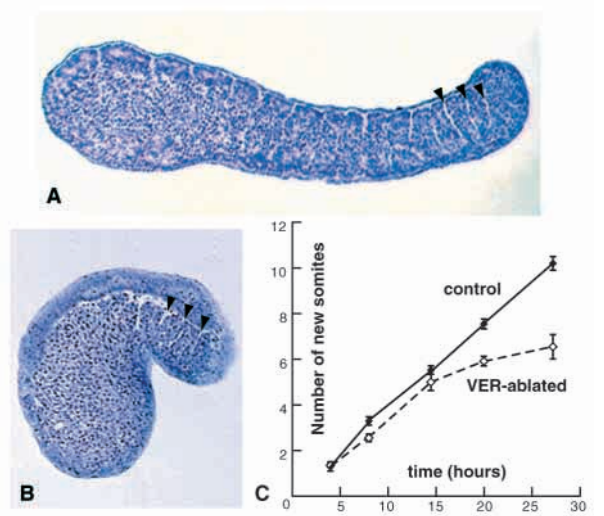

Fig. 4. Effects of VER ablation on the rate of somitogenesis. (A,B) Parasagittal sections of (A) control and (B) VER-ablated tails cultured for 25-28 hours. Arrowheads point to boundaries between somites at the caudal end of the tail. (C) The data illustrate the progressive change in somite number in 9 VER-ablated and 8 intact control tail explants that were cultured for up to 27 hours. The bars indicate standard error of the mean.

Bmp2 expression (a marker for the VER) and counted the number of epithelialized somites (as visualized in whole mount). We found that, although the ectoderm healed over the region ablated, the VER was not reconstituted, as revealed by the lack of *Bmp2* expression (*n*=5, data not shown). VER-ablated explants were much shorter and thicker than intact tail explants (Fig. 3A,B), and a mean of 6.2 pairs of somites formed (*n*=45), whereas in the intact tail explants, a mean of 10.1 somite pairs formed (*n*=48; significantly different between ablated and intact tails, Table 1). The somites in the VER-ablated tails appeared histologically normal (Fig. 4A,B) and displayed AP patterning as assessed by expression of *Meox1* (formerly *Mox1*) (Hrabe de Angelis et al., 1997) (data not shown). The data from this study, which are summarized in Fig. 3G, clearly show that ablation of the ~E10.5 VER compromises tail explant development.

We also assessed the consequences of removing the ectoderm covering the lateral walls on one (*n*=6) or both (*n*=15) sides of the tail (Fig. 3C; Table 1). Ablation of the lateral ectoderm had little effect on tail development. On average, 9.9 somite pairs were formed in tails lacking lateral ectoderm (*n*=21) compared with 10.2 somite pairs formed in intact controls (*n*=13). The somites in the lateral ectoderm-ablated tails were histologically normal (data not shown). These results indicate that, in contrast to the VER, the lateral ectoderm is dispensable for somite formation.

To determine whether the negative effect on tail development was caused by damage to the underlying mesoderm during VER ablation, we tested if somitogenesis could be restored by replacing fragments of the VER. Pieces of the posterior 2/3 of the VER, which included the midline but spanned only about 1/3 the total width of the VER, were isolated from *lacZ*-expressing donors (Tam and Tan, 1992) and were orthotopically grafted to VER-ablated tails. After 25-28 hours in culture, the tails were stained for β-galactosidase activity to determine if the graft was retained, and the number of epithelial somites was counted. A total of 26 tails retained the VER grafts. These tails displayed morphologies ranging from that of VER-ablated tails to that of intact tail explants (Fig. 3D, and data not shown). A mean of 8.5 pairs of new somites formed in the VER-replaced tails but the number of somites formed was much more variable than in other experiments (Table 1). This mean number was significantly higher than in VER-ablated tails (P<0.01 by Kolmogorov-Smirnov 2-sample test). This reflects the fact that, in many (11/26; 42%) of the orthotopically grafted tails, 10 or more somite pairs formed, whereas the formation of 10 or more pairs of somites was rarely observed (1/22; 5%) in VER-ablated tails without an orthotopic graft. These findings indicate that the negative effects of VER ablation on somitogenesis are unlikely to be due to mechanical damage to the underlying mesoderm.

Table 1. Summary of effects on somitogenesis of VER removal and other experimental manipulations

	Number of tails analyzed	Mean no. of somite pairs	Standard deviation	Statistical analysis*
Entire VER				
Ablated	45	6.2	1.17	$P<0.01$
Intact control	48	10.1	0.86	
Non-VER ectoderm				
Lateral ectoderm ablated	21	9.9	0.91	n.s.
Intact control	13	10.2	0.73	
Regions of VER left intact				
Anterior only	23	7.3	1.11	$P<0.001$
Posterior only	15	9.8	0.86	n.s.
Anterior and middle	12	9.8	0.97	n.s.
Intact control	18	10.5	0.92	
VER replacement				
None	22	7.1	1.29	$P<0.001$
VER graft	26	8.5	1.79	$P<0.01$
Tails with 10 or more somites	11	10.1		
Tails with 9 or fewer somites	15	7.4		
AER or lateral ectoderm graft	10	6.6	1.84	$P<0.001$
Intact control	24	10.5	0.93	

*Kolmogorov-Smirnov test of experimental data versus control.
n.s., not significant.

We therefore conclude that the VER is necessary to maintain somite formation and tail elongation.

We next sought to determine whether there is any functional difference between different regions of the VER. Several different experiments were performed in which only part of the VER was left intact (Table 1). When only the anteriormost region was present (approximately 20% of VER length), an average of 7.3 somite pairs formed and the tail failed to elongate (n=23; Fig. 3E), suggesting that the anterior VER alone could not support somite formation and tail development. In contrast, when only the posteriormost region was present (approximately 20% of VER length), the average number of somite pairs formed was 9.8 (n=15; Fig. 3F), similar to that in VER-intact controls (mean = 10.5 somite pairs; n=18). These data indicate the posterior region of the VER contains cells that can provide the signals necessary to support tail development. However, we also found that, when a longer anterior fragment of VER was left in place (60-70% of VER length, encompassing both anterior and middle regions), tail development was relatively normal (9.8 somite pairs, n=12), suggesting that cells capable of producing these signals are also present in the middle region of the VER.

Finally, we sought to determine whether the ability to rescue tail development is specific to the VER. Pieces of non-VER ectoderm, such as ectoderm from the lateral regions of the tail (n=3) or fragments of the AER (n=7), were grafted to tails from which the VER was removed. In all cases, the heterotopically grafted tails morphologically resembled tails lacking the VER and formed approximately the same average number (6.6) of somite pairs (Table 1). Consistent with the finding that the AER did not rescue somitogenesis in VER-ablated tails, we found that beads soaked in FGF4 protein, which can rescue limb development following AER removal (Niswander et al., 1993), did not rescue tail development when implanted in VER-ablated tail mesoderm (data not shown). We also found that beads soaked in BMP2 protein did not rescue the development of VER-ablated tails. Indeed, in most cases, the beads compromised tail development. In particular, the BMP2 beads caused extensive cell death when implanted in either VER-ablated or unoperated tails, whereas control beads soaked in PBS had no effect (data not shown). This result is surprising given that *Bmp2* is normally expressed in the VER. However, excess BMP signaling is known to cause apoptosis in a variety of developmental settings (Graham et al., 1994; Ganan et al., 1996; Schmidt et al., 1998) and it is possible that the local concentration of BMP2 supplied by the beads was higher than that normally produced by the VER, thus causing cell death.

Effects of VER ablation on somite patterning and development of the notochord, neural tube and tail gut

The data described above demonstrate that removal of the VER adversely affects somitogenesis. To determine whether the decrease in somite number could be accounted for by a decrease in the overall rate of somitogenesis, we counted the number of epithelialized somite pairs in control (n=8) and VER-ablated tails (n=9) at various times during the culture period. The rate of somitogenesis was found to be nearly identical in both groups up to the time at which the sixth pair of new somites formed (Fig. 4C). However, after that, somitogenesis largely ceased in the VER-ablated tails, whereas new somite pairs continued to form at a constant rate in the unoperated control tails. At any given time, the presomitic mesoderm contains the precursors of six pairs of somites (i.e., 6 pairs of somitomeres; Tam, 1986). The observation that somitogenesis virtually ceased after the sixth pair of somites formed suggests that the somites that do form in the VER-ablated tails develop from somitomeres already within the PSM at the time of VER ablation. This raises the possibility that no new somitomeres are formed in the absence of the VER, perhaps because of a paucity of paraxial mesoderm. However, it is also possible that somitomeres are formed, but are unable to segment into epithelialized somites.

We made an effort to determine what aspect of somitogenesis is affected by VER removal by assaying for the expression of genes known to be involved in somite formation. These assays were performed at the end of the culture period when the effect of VER removal was most pronounced. *Tbx6* and *Wnt3a* are normally expressed in a broad domain in the TBM and in the mesoderm anterior to it and are required for somite specification in the developing tail (Takada et al., 1994; Chapman et al., 1996; Greco et al., 1996; Chapman and Papaioannou, 1998). *T*, *Fgf3* and *Wnt5a* are expressed in a similar domain (Wilkinson et al., 1988, 1990; Takada et al., 1994) and are required for tail outgrowth (Chesley, 1935; Mansour et al., 1993; Yamaguchi et al., 1999). Expression of all these genes was detected at the caudal end of the VER-ablated tails, presumably in the TBM (Fig. 5A,B, and data not shown). *Evx1* and *Sax1* are normally expressed in a dorsal domain in the TBM, presumably marking neural precursors (Schubert et al., 1995; Gofflot et al., 1997). The expression of both genes was detected in the dorsal-caudal region of VER-ablated tails (data not shown). Thus, we detected no obvious

abnormalities in the expression patterns of several genes that normally mark the TBM, suggesting that the TBM is not grossly abnormal in tails lacking a VER.

We also assayed for the expression of two genes in the Notch/Delta signaling pathway, which is required for segmentation of the PSM and epithelialization of somites (reviewed by Gossler and Hrabe de Angelis, 1998; McGrew et al., 1998; Tam et al., 2000). *Notch1* is normally strongly expressed in a somite-sized stripe in the anterior PSM, just caudal to the last somite and is weakly expressed throughout the PSM and TBM (Fig. 5C; Reaume et al., 1992). In VER-ablated tails, *Notch1* RNA was detected in a stripe near the caudal end of the abnormally shaped tails as well as in cells caudal to it (Fig. 5D). *Dll1*, which is normally expressed throughout the PSM and TBM (Bettenhausen et al., 1995), was detected in the caudal region of the VER-ablated tails (data not shown). In addition, *Meox1* expression, which is normally detected in a domain that overlaps with that of *Notch1* in the anterior PSM (Candia et al., 1992), was detected near the caudal end of the VER-ablated tails (data not shown). These data suggest that the anterior PSM is present and is patterned in the VER-ablated tails. Markers specific for the posterior PSM are not presently available.

Since overexpression of BMP family members can adversely affect somitogenesis (Tonegawa et al., 1997), we also assayed for expression of *Bmp2* and *Bmp4* following VER removal. *Bmp2* RNA, which is normally restricted to the VER, was not detected, but *Bmp4* RNA was detected in its normal domains (see Fig. 1G,H) in VER-ablated tails (data not shown). Thus, VER removal does not result in ectopic expression of either *Bmp2* or *Bmp4*.

We next sought to determine whether the formation of other tissues in the tail bud might be adversely affected by VER removal. Histological analysis revealed gross morphological changes in the VER-ablated tails (Fig. 5E,F). There appeared to be a dramatic increase in the number of cells underlying the tail gut and a concomitant decrease in the number of cells lateral to it, thus displacing the gut to an abnormally dorsal position within the tail. The notochord was also shifted dorsally, was often displaced laterally and occasionally it was forked. In many cases, the neural tube was disorganized and appeared to have collapsed. Although abnormally localized in the VER-ablated tails, these tissues displayed relatively normal patterns of gene expression. For example, *Nog* RNA was detected in the tail gut, notochord and neural tube (Fig. 5E,F). Likewise, *Foxa2* (formerly *Hnf3β*) expression, which is normally detected in the gut, notochord and floor plate of the neural tube (Ang et al., 1993; Sasaki and Hogan, 1993), was also detected in those tissues in the VER-ablated tails (Fig. 5G,H). *Shh* RNA is normally detected in the notochord and the anteriormost portion of the tail gut, near the prospective base of tail (Gofflot et al., 1997; and data not shown). It was also detected in the notochord of VER-ablated tails (Fig. 5A,B). Taken together, our data suggest that, although removal of the VER dramatically affects the morphology of the tail, the processes of neural tube, notochord and tail gut development occur, at least to some extent, and that these structures are correctly patterned.

To determine whether the abnormal morphology of the VER-ablated tails might be due to changes in the rate of cell proliferation and/or survival in the mesoderm, we assayed for cell proliferation and apoptosis 16-18 hours after VER removal (see Materials and Methods). No statistically significant differences were found between the control and experimental tails (data not shown), although it should be noted that a substantial amount of cell death was detected in both control and experimental tails at the end of the culture period. Thus, removal of the VER does not appear to have an obvious effect on cell proliferation or survival in the tail mesoderm.

Effect of VER ablation on noggin expression in the ventral mesoderm

The observation that the number of cells located between the tail gut and the ventral surface is greatly increased in VER-ablated tails (Fig. 5E-H) raised the possibility that cells that would normally be destined for the paraxial mesoderm lineage might have changed fate and formed ventral mesoderm in the absence of the VER. We therefore analyzed expression of *Nog* and *Bmp4*, which are normally detected specifically in the ventral mesoderm of the tail (Figs 1G-J, 5I). As noted above, we found no evidence that VER removal affected *Bmp4* expression, but changes were observed in the pattern of *Nog* expression. Although *Nog* RNA was detected in its normal domain immediately after VER removal (data not shown), 16 hours later, *Nog* RNA was no longer detected in the ventral tail mesoderm (Fig. 5J). In contrast, *Nog* continued to be expressed in other regions of the VER-ablated tails (Fig. 5E,F). Although these results do not resolve the question of whether the ventral mesoderm population is expanded following VER removal, they indicate that the *Nog* gene is regulated, directly or indirectly, by signals from the VER.

These results raise the possibility that loss of *Nog* expression in the ventral mesoderm is responsible, directly or indirectly, for the observed effects of VER removal on tail development. This hypothesis is consistent with the finding that tail development fails in $Nog^{-/-}$ mice (McMahon et al., 1998). We attempted to restore tail development following VER removal by implanting a bead soaked in 0.25-0.5 μg/μl of NOGGIN protein. However, there was no obvious morphogenetic effect of the NOGGIN bead (data not shown), perhaps because the local concentration of NOGGIN was inappropriate or the bead was not correctly positioned.

DISCUSSION

Since the VER in the mouse embryonic tail shares some morphological similarities with the AER of the limb bud, it has been speculated that the VER may be functionally analogous to the AER and produce molecules required for outgrowth and patterning of the tail (Grüneberg, 1956). Here we present an analysis of the fate, function and molecular characteristics of the VER in the mouse embryonic tail. Our fate-mapping data indicate that there is extensive spreading of cells within the VER from posterior to anterior, and that over time cells exit the VER and populate the surface ectoderm in the ventral midline of the tail. We have investigated the function of the VER by testing the effect on tail development following the ablation of this tissue and showed that the anterior VER is dispensable, but the posterior VER is required for somitogenesis and tail elongation. Interestingly, somite precursors already present in the presomitic mesoderm at the

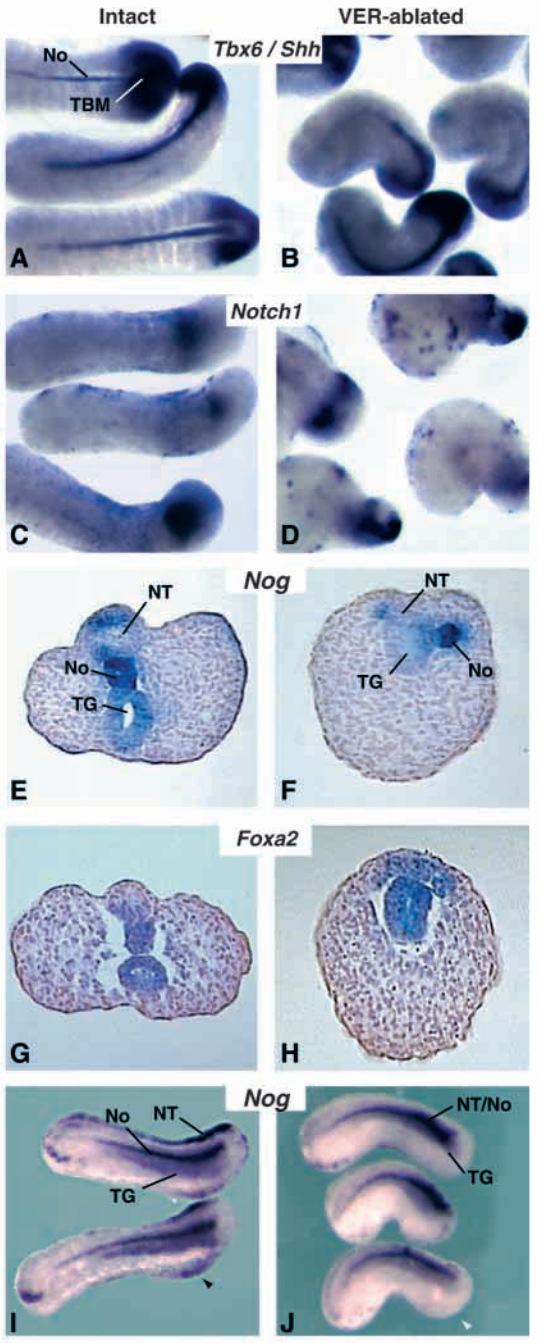

Fig. 5. Morphology and gene expression following VER ablation. RNA in situ hybridization in whole mount of tail explants cultured in vitro for (A-H) 25-28 hours or (I,J) 16-18 hours using probes for the genes indicated. The transverse sections shown in E-H illustrate the abnormal morphology of the VER-ablated tails. The black arrowhead in I points to the *Nog* expression domain in the ventral mesoderm. Note the absence of *Nog* expression in this domain in the VER-ablated tails (white arrowhead in J). Abbreviations as in the legend to Fig. 1.

time of VER ablation continue to segment, but no new somites are formed after all the presomitic mesoderm is converted into epithelial somites and the tail fails to elongate. These results indicate that the VER functions to maintain a somite progenitor population in the TBM and is required for tail morphogenesis. We have identified two genes encoding secreted signaling molecules, *Fgf17* and *Bmp2*, that are expressed in the VER and therefore might contribute to VER function. In addition, we identified a previously unreported expression domain of the gene encoding the BMP antagonist NOGGIN in the mesoderm beneath the VER and abutting the presomitic mesoderm, and found that *Nog* expression is extinguished in this domain when the VER is absent. These observations suggest a model in which the normal function of the VER is to maintain this mesenchymal expression of *Nog*, which is required to modulate BMP signaling that, if unchecked, would disrupt somitogenesis and terminate tail development.

The VER is a source of midline ventral ectoderm

The VER appears to be derived from epiblast cells in the primitive streak at late stages of gastrulation. This conclusion is based on data from fate-mapping studies, which showed that the epiblast overlying the primitive streak in both mouse (~E8.5; 7-10 somites) and chick (stages 11-13; 12-17 somites) contributes to the VER, as well as to the surface ectoderm anterior to the mouse VER (Schoenwolf, 1981; Tam and Beddington, 1987; Catala et al., 1995; Wilson and Beddington, 1996). Because epiblast cells were still ingressing into the primitive streak at the stages when the ectoderm was labeled, it is unclear whether the VER is made of cells that have ingressed through the streak or is derived from non-ingressed epiblast that spread ventrally during formation of the tail fold.

Our ~E10.5 VER fate-mapping data suggest that, as the tail extends, cells labeled in the posterior third of the VER are found at successively more anterior positions, initially within the middle, then at the anterior end of the VER, and finally exit the VER to colonize the ventral surface ectoderm. The observed anterior spread may be achieved, at least in part, by cell rearrangement, since labeled cells were found intermingling with non-labeled cells, indicating that cell mixing occurs over time. Moreover, cell proliferation is likely to play a role in this process since we (data not shown) and others (Gofflot et al., 1997) have observed that cells in the VER are mitotically active. These results raise the possibility that cells from the epiblast that populate the VER may form a self-contained progenitor cell population that is localized near the posterior end of the VER, which serves as a source of tail midline ventral ectoderm as well as a signaling center for tail morphogenesis.

The VER is a source of signaling molecules required for tail development

The progressive segmentation of somites from the paraxial mesoderm, a derivative of the TBM, is the most obvious manifestation of tail outgrowth. We found that VER ablation does not inhibit somitogenesis of tissue already allocated to the presomitic mesoderm, but it blocks either the formation of new somitomeres or their subsequent differentiation. VER removal also causes abnormalities in the tail explants, including a dorsal shift in the position of the tail gut, a lateral shift in the position of the notochord and disorganization of the neural tube. It is unclear whether these abnormalities contribute to the inhibition of somitogenesis or if they reflect parallel disruptions in tail morphogenesis.

Our gene expression analysis identified FGF17 and BMP2

as candidate signaling molecules responsible for VER function in maintaining somitogenesis. To date, the only other signaling molecule gene reported to be specifically expressed in the VER is *Wnt5a* (Gofflot et al., 1997). Given the importance of FGFs produced in the surface ectoderm for outgrowth of the limb (reviewed by Martin, 1998) and the first branchial arch (Trumpp et al., 1999), we were surprised to find that only one of the many FGF genes that we assayed is expressed in the VER. The expression domain of *Fgf17* is restricted to the posterior two-thirds of the VER, the region that is necessary for maintaining normal tail outgrowth. It is therefore tempting to speculate that FGF17 plays a role in VER function during tail development. However, loss of *Fgf17* function has no obvious effect on tail morphogenesis (Xu et al., 2000). Furthermore, implanting a bead soaked in FGF4 did not rescue development of VER-ablated tails (data not shown). Thus the available evidence argues against the hypothesis that FGF signaling contributes to VER function. Nevertheless, it remains possible that other FGF family members that we did not assay, or that are as yet unidentified, are expressed in the VER and are essential for its function.

Bmp2 expression is detected in the VER from the earliest stages of its development, but becomes undetectable just before the VER regresses and tail outgrowth ceases. In the chick, BMPs appear to be necessary for tail outgrowth, since expression of a dominant-negative form of BMP receptor IB, which presumably blocks BMP2 signaling, leads to tail truncations (Zou and Niswander, 1996). One possible function of BMP2 produced in the VER may be to negatively regulate the size of the VER. This hypothesis is based on recent studies showing that, in the developing limb bud, the size of the AER, which is critical for normal limb morphogenesis, is controlled, at least in part, by BMP signaling. Thus it has been found that overexpression of *Nog* in the limb bud, which presumably antagonizes the activity of the BMPs produced in the AER and limb bud mesenchyme, causes an increase in both the length of the AER and the amount of time that it persists (Pizette and Niswander, 1999). As in the AER, there is extensive cell death in the VER at all stages (~E10.5-~E13 [34-60 somites]; Gajovic and Kostovic-Knezevic, 1995; and data not shown) suggesting that its size is also regulated by a mechanism that controls the rate of cell death. It seems likely that BMP2 produced in the VER plays some role in this process. The precise role BMP2 plays in tail development has yet to be determined by genetic analysis. Although *Bmp2*$^{-/-}$ mice have been produced, they die at around E9, before definitive tail outgrowth stages (Zhang and Bradley, 1996). Thus, it will be necessary to perform VER-specific gene inactivation studies to further explore BMP2 function in tail outgrowth.

The VER is required for expression of noggin in the ventral mesoderm of the tail

The gene encoding the BMP antagonist NOGGIN is normally expressed in several domains of the developing tail including the dorsal neural tube, the notochord and the ventral mesoderm underlying the VER at nearly all stages of tail outgrowth. The absence of *Nog* expression in the ventral mesoderm was the only molecular abnormality that we detected following removal of the VER, identifying NOGGIN as a candidate for a molecule required for somitogenesis in the tail. Little is known at present about the molecules that regulate *Nog* expression in vivo, but our data raise the possibility that BMP2 produced in the VER might be responsible for inducing and/or maintaining *Nog* gene activity in the tail ventral mesoderm. Such an inductive relationship between BMPs and noggin has been reported in cultured fetal osteoblasts and fibroblasts (Gazzerro et al., 1998).

Loss-of-function studies in the mouse have demonstrated that *Nog* is necessary for tail formation after ~E10.5. Interestingly, genes known to be required for TBM maintenance and paraxial mesoderm specification are expressed in both *Nog*$^{-/-}$ embryos (McMahon et al., 1998) and VER-ablated tails (our data), suggesting that *Nog* is required only after the paraxial mesoderm has been specified. However, the specific domain in which *Nog* function is required for the continuation of tail development is not known. Our data point to the ventral mesoderm as a possible source of the NOGGIN protein essential for tail somitogenesis and perhaps other aspects of tail development. Experiments in which *Nog* is inactivated specifically in the ventral mesoderm will be needed to test this hypothesis.

How might the loss of *Nog* expression in the tail affect somitogenesis and tail outgrowth? One possibility is that NOGGIN protein antagonizes BMP signaling in the ventral mesoderm and thereby affects somitogenesis. This hypothesis is based on the finding that ectopic expression of *Nog* in the chick lateral plate mesoderm induces ectopic somite formation (Tonegawa and Takahashi, 1998). Conversely, ectopic BMP expression in the PSM can inhibit the formation of somites and promote differentiation of lateral plate mesoderm (Tonegawa et al., 1997). However, under some conditions, excess BMP4 can lead to extensive cell death in the PSM (Schmidt et al., 1998). Presumably it is the local concentration of BMP that determines whether cell differentiation or survival is affected. In the developing tail, the ventral mesoderm, which abuts the PSM, expresses *Bmp4* and *Bmp7* (Lyons et al., 1995; McMahon et al., 1998) and it seems likely that NOGGIN produced in the ventral mesoderm plays some role in regulating their activity. We therefore propose that the loss of *Nog* expression in the ventral mesoderm of the tail, either as a consequence of VER removal or in *Nog*$^{-/-}$ mice, may effectively increase the amount of BMP signaling to the paraxial mesoderm domain, disrupt somitogenesis and simultaneously lead to a cessation of tail development.

Concluding remarks

In summary, our data support the hypothesis that the VER is required for tail somitogenesis and outgrowth. They also point to some similarities, in addition to morphological characteristics, between the tail VER and the limb bud AER. In particular, the fates of cells in the VER (our data) and the AER (Vargesson et al., 1997) appear to be remarkably similar. In both types of ridge, labeled cells move from posterior to anterior within the ridge over time, and labeled cells from the anterior or middle thirds exit the anterior ridge and contribute to surface ectoderm. However, our data do not lend support to the speculation that the VER and AER are strictly analogous structures with equivalent functions. The AER does not appear to be able to substitute for the VER in tail explants. Moreover, unlike the AER, the VER does not appear to influence cell proliferation or cell survival in the underlying mesoderm. This might be because the production of FGFs, which is a primary

function of the AER (Niswander et al., 1993; Fallon et al., 1994), does not appear to be an essential feature of VER function. Based on our gene expression studies, we propose that one of the major functions of the VER is to provide signals that regulate BMP activity, which in turn controls somitogenesis.

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