Digit regeneration is regulated by *Msx1* and BMP4 in fetal mice

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Summary

The regeneration of digit tips in mammals, including humans and rodents, represents a model for organ regeneration in higher vertebrates. We had previously characterized digit tip regeneration during fetal and neonatal stages of digit formation in the mouse and found that regenerative capability correlated with the expression domain of the *Msx1* gene. Using the stage 11 (E14.5) digit, we now show that digit tip regeneration occurs in organ culture and that *Msx1*, but not *Msx2*, mutant mice display a regeneration defect. Associated with this phenotype, we find that *Bmp4* expression is downregulated in the *Msx1* mutant digit and that mutant digit regeneration can be rescued in a dose-dependent manner by treatment with exogenous BMP4. Studies with the BMP-binding protein noggin show that wild-type digit regeneration is inhibited without inhibiting the expression of *Msx1*, *Msx2* or *Bmp4*. These data identify a signaling pathway essential for digit regeneration, in which *Msx1* functions to regulate BMP4 production. We also provide evidence that endogenous *Bmp4* expression is regulated by the combined activity of *Msx1* and *Msx2* in the forming digit tip; however, we discovered a compensatory *Msx2* response that involves an expansion into the wild-type Msx1 domain. Thus, although both *Msx1* and *Msx2* function to regulate *Bmp4* expression in the digit tip, the data are not consistent with a model in which *Msx1* and *Msx2* serve completely redundant functions in the regeneration response. These studies provide the first functional analysis of mammalian fetal digit regeneration and identify a new function for *Msx1* and BMP4 as regulators of the regenerative response.

Key words: *Msx1*, BMP4, Regeneration, Digit, Mouse

Introduction

The amphibian limb has been the prototypical organ for the study of epimorphic regeneration both in adults and during development. These studies indicate a number of requirements for successful limb regeneration, including: (1) a wound healing response with the formation of an apical epithelial cap (AEC); (2) a cellular contribution, via the de-differentiation of mature tissues and/or stem cells, to form the blastemal mesenchyme; (3) a proliferative response regulated by multiple tissue interactions, including a neurotrophic effect, an AEC effect and a positional effect; and (4) morphogenesis and re-differentiation to restore the limb pattern (reviewed by Stocum, 1995; Tsonis, 1996). In general, the regeneration response can be separated into those events that are injury related and crucial for blastema formation, and those that represent a 're-development' response whereby regeneration recapitulates limb development (see Bryant et al., 2002). It is clear that the complexity of the regeneration response lends itself to multiple avenues whereby regeneration can be inhibited, and indeed numerous requirements for limb regeneration have been identified in this way. In parallel, there is a growing database of genes that are likely to play key roles, based on their regulated expression during regeneration, yet in most cases a functional understanding of how these genes act remains largely unexplored (see Gardiner et al., 2002).

The regeneration of amputated distal digit/finger tips has been reported in various mammals, including humans and rodents, and represents a mammalian system where successful epimorphic regeneration can occur (see Muller et al., 1999). In humans, regenerative potential is restricted to the distal tip of the finger in a region associated with the nail organ (Douglas, 1972; Illingworth, 1974). Experimental studies using rodent models demonstrate that regenerative capacity is level specific and, similarly, is associated with the proximal extent of the nail forming organ (Borgens, 1982; Zhao and Neufeld, 1995). A role for the nail organ in the regeneration response is supported by experiments showing that excluding the nail organ from the amputation wound results in no regenerative response; however, nail organ grafts result in ectopic bone formation and do not induce regeneration (Zhao and Neufeld, 1995; Mohammad et al., 1999). These experimental results, together with clinical studies in humans, provide evidence that the nail organ plays some role in the stimulation of adult digit tip regeneration. Genes specifically expressed in, or associated with, the nail organ are candidates for regulating this regenerative response. In previous studies, we demonstrated that the homeobox-containing genes *Msx1* and *Msx2* are expressed in association with the nail organ of neonatal digits, and at the apex of developing digits in the nail-forming region (Reginelli et al., 1995). Furthermore, mapping the regenerative ability of embryonic and fetal digit tips demonstrated that regenerative capacity correlated with amputation within the *Msx1*, but not the *Msx2*, expression domain in developing digits. These studies suggest a role for MSX genes in the digit regeneration response.

In all tetrapod vertebrates, *Msx1* and *Msx2* are co-expressed

in the apical mesenchyme during limb formation. In animals that regenerate their appendages, MSX genes are upregulated during the regeneration response and downregulated in association with re-differentiation (Crews et al., 1995; Simon et al., 1995; Yokoyama et al., 2001). In the regenerating urodele limb, *Msx2* is rapidly induced in the healing epidermis and subjacent tissues following amputation or simple wounding, whereas *Msx1* expression is restricted to blastemal cells (Koshiba et al., 1998; Carlson et al., 1998). During fetal digit tip regeneration in the mouse, both *Msx1* and *Msx2* are expressed in the regenerating digit mesenchyme, whereas neither is expressed following proximal amputations that fail to regenerate (Reginelli et al., 1995). Beyond these descriptive studies, the role that MSX genes play in regeneration of fish fins, amphibian limbs or mammalian digits is largely unexplored. However, the roles of MSX genes during limb development and in cultured cells have been extensively studied. Expression of MSX genes in the apical mesenchyme of the limb bud is dependent on signaling from the apical ectodermal ridge (AER), and also on interactions with neighboring mesenchymal cells (Davidson et al., 1991; Ros et al., 1992; Wang and Sassoon, 1995). A number of factors crucial for limb formation have been shown to regulate MSX gene expression, including members of the FGF (Watanabe and Ide, 1993; Fallon et al., 1994; Wang and Sassoon, 1995; Vogel et al., 1995), BMP (Wang and Sassoon, 1995; Ganan et al., 1996; Marazzi et al., 1997; Hofmann et al., 1996; Pizette and Niswander, 1999; Pizette et al., 2001) and TGFβ (Ganan et al., 1996) signaling families, and retinoic acid (Yokouchi et al., 1991; Wang and Sassoon, 1995). One function of *Msx1* in early limb development is to mediate a BMP signaling pathway that leads to the induction of the AER (Pizette et al., 2001). Nevertheless, mice carrying a targeted deletion of the *Msx1* gene form normal limbs, thus indicating that AER formation can occur in the absence of *Msx1* function (Satokata and Maas, 1994). Because *Msx1* and *Msx2* are co-expressed in the apical mesenchyme it remains possible that these genes function redundantly during limb formation.

MSX gene function is implicated in the control of cellular differentiation during embryogenesis (see Bendall and Abate-Shen, 2000). The expression pattern of MSX genes during limb development is consistent with a role in the control of cell proliferation and/or cell differentiation: *Msx1* and *Msx2* are expressed in the apical mesenchyme in association with undifferentiated proliferating cells, whereas proximal tissues where MSX genes are not expressed are associated with reduced growth and tissue differentiation. Forced expression implicates *Msx1* in the inhibition of myogenesis (Song et al., 1992; Woloshin et al., 1995), and there is evidence that this inhibitory activity can be generally extended to the differentiation of a number of mesenchymal (e.g. adipose, cartilage and bone) and epithelial (e.g. mammary) cell types (Hu et al., 2001). A similar conclusion can be drawn from the results of loss-of-function studies that indicate that both *Msx1* and *Msx2* mutants display defects in the formation of certain skeletal elements and ectodermally derived organs (Satokata and Maas, 1994; Satokata et al., 2000). An intriguing discovery that is potentially relevant to limb regeneration is the demonstration that regulated *Msx1* expression in differentiated C2C12 myotubes induces a de-differentiation response resulting in the establishment of multi-potent progenitor cells (Odelberg et al., 2000). In addition, an extracellular activity derived from newt blastema extract possesses a similar de-differentiation activity, thus suggesting that an intercellular signal, presumably acting through MSX1, is involved in de-differentiation during the early stages of limb regeneration (McGann et al., 2001).

The availability of mice carrying targeted deletions of the *Msx1* and *Msx2* genes, and the established regenerative response of fetal digit tips, allowed us to carry out a functional analysis of the MSX genes in regeneration. In this study, we have established a fetal digit tip regeneration model in cultured explanted autopods of E14.5 digits. Using this model, we discovered that *Msx1*, but not *Msx2*, mutant digits displayed a regeneration defect. Gene expression studies demonstrated that the *Msx2* expression domain was expanded into the *Msx1* domain in the *Msx1* mutant digit, thus showing that *Msx1* and *Msx2* are not functioning in a redundant manner in digit regeneration. By contrast, the *Bmp4* expression domain, which coincides with that of *Msx1* in wild-type digits, was apically restricted in the *Msx1* mutant digit, and *Bmp4* transcripts were not detected in the *Msx1*/*Msx2* double-mutant digit tip. Exogenous application of BMP4 was found to rescue the *Msx1* mutant-digit regeneration defect in a dose-dependent manner, and exogenous noggin application inhibited the regeneration response in wild-type digits. These studies provide functional evidence linking Msx1 function and BMP signaling to the control of digit tip regeneration in the mammalian fetus.

Materials and methods

Msx mutant mice

Mice carrying a targeted deletion of the *Msx1* gene (Satokata and Maas, 1994) or the *Msx2* gene (Satokata et al., 2000) were kindly provided by Dr Richard Maas. Homozygous *Msx1* or *Msx2* mutant embryos were obtained by heterozygote mating. *Msx1*/*Msx2* double-mutant embryos were obtained by double heterozygotes mating. At E14.5, *Msx1* and *Msx2* mutants cannot be distinguished from wild-type mice, so each experiment was carried out blind and tissues were collected for subsequent PCR genotyping as described (Satokata and Maas, 1994; Satokata et al., 2000). *Msx1*/*Msx2* double-mutant embryos are morphologically distinct at E14.5 and their genotype was verified by PCR. The limb buds of double mutants appear to be developmentally delayed; the morphology of E14.5 hindlimb (stage 11) is similar to the E13.5 wild-type hindlimb (stage 10). Limb stages are described by Wanek et al. (Wanek et al., 1989).

Digit amputation

For in vivo studies, stage 11 fetal digit tips were amputated at E14.5 using exo utero surgical techniques as described (Reginelli et al., 1995; Ngo-Muller and Muneoka, 2000a). Briefly, timed-pregnant mice were anesthetized with sodium pentobarbital (60 μg/g body weight), fentany (1.6 μg/animal) and droperidol (80 μg/animal). The abdomen was opened with a mid-ventral incision and fetuses were exposed by incision of anti-placental uterine wall. Access to the hindlimb was through an incision in the extraembryonic membranes and the hindlimb was teased out with a blunt probe. The three central hindlimb digits, digits 2, 3, and 4, were amputated at either a distal level, approximately 75 μm from the digit tip (see Fig. 1H), or a proximal level through the presumptive terminal interphalangeal joint. The uterus with fetuses attached was positioned within the abdominal cavity and the abdominal wall of female mouse was closed. Operated fetuses were allowed to develop for 2 to 4 days exo utero (Muneoka et al., 1986) after which the hindlimbs were collected for analysis of the digits.

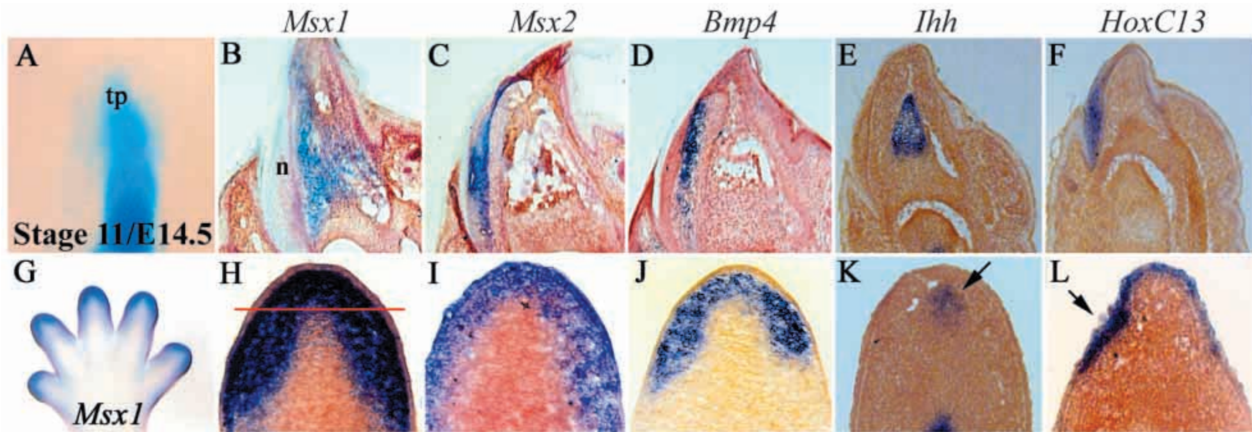

Fig. 1. Fetal digit tip formation. (A) An Alcian Blue stained stage 11 digit (E14.5), showing the chondrogenic structure of the digit at the time of amputation. tp, terminal phalanx. (B-F, K-L) In situ hybridization of sagittal sections of neonatal digits. Left side of each image is dorsal; top is distal. (H-J) In situ hybridization of frontal sections of the E14.5 digits. The top of each image is distal. (B) *Msx1* transcripts are localized to the loose connective tissue subjacent to the nail organ (n) and surrounding the dorsal region of the terminal phalanx in the P7 digit. (C) *Msx2* transcripts are localized in the nail bed and nail matrix of the P7 digit. (D) *Bmp4* transcripts are expressed in dorsal loose connective tissue cells beneath the nail bed in the P2 digit. (E) *Ihh* is expressed at the distal tip of terminal phalanx in the newborn digit. (F) *Hoxc13* is expressed in the nail bed and nail matrix of the newborn digit. (G) Whole-mount in situ hybridization of *Msx1* in the stage 11 autopod. (H) *Msx1* is expressed in the apical mesenchymal cells surrounding the forming terminal phalanx. The line indicates the amputation level that elicits a regeneration response. Digit amputation at a level proximal to this line does not elicit a regeneration response. (I) *Msx2* is expressed in the apical epidermis and in mesenchymal cells subjacent to the epidermis. (J) *Bmp4* is expressed in apical mesenchymal cells in a domain similar to that of *Msx1*. (K) *Ihh* is expressed in digit tip cells, initiating endochondral ossification of the terminal phalanx (arrow). (L) The nail organ marker, *Hoxc13*, is expressed in the distal epidermis associated with presumptive nail tissue (arrow).

Organ culture

For organ culture, stage 11 hindlimbs were collected from E14.5 fetuses and transferred to a dish containing lactated Ringer's solution, where digits were amputated as described above. Hindlimbs were trimmed proximally at the level of the ankle and were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum on filters (Millipore, 0.45 μm pore size, 13 mm diameter) supported by a metal grid (Zhang et al., 2000). Hindlimbs with amputated digits were cultured either with or without added growth factors for 2 or 3 days with daily changes of the medium. Growth factors used in these studies included recombinant human BMP2 and BMP4 (kindly provided by Genetics Institute), and noggin (R&D). BMP2 activity was tested independently, based on the induction of *Nodal* during left-right axis formation following BMP2 bead implantation into the chick embryo (Schlange et al., 2002).

In situ hybridization

For in situ hybridization, digoxigenin-labeled riboprobes complementary to *Msx1* (Ngo-Muller and Muneoka, 2000b), *Msx2* (Ngo-Muller and Muneoka, 2000b), *Bmp4* (Jones et al., 1991), *Hoxc13* (Godwin and Capecchi, 1998) and *Ihh* (St-Jacques et al., 1999) were used in whole-mount or paraffin-sectioned preparations as described (Schaller and Muneoka, 2001; Omi et al., 2002). Fetal digit tissues were fixed by immersion in 4% paraformaldehyde and post-natal digits were fixed by injection of fixative into the digits followed by immersion in 4% paraformaldehyde. For tissues processed in parallel, all aspects of the in situ hybridization staining protocol were carried out in synchrony and imaged identically. For *Msx1/Msx2* double-mutant tissue that is negative for *Bmp4* expression, we used the expression of *Bmp2* in limb tissues as a positive control for tissue viability.

Histology and cell proliferation

For differentiating skeletal analysis in the developing digits, tissues were stained with Alcian Blue/Alizarin Red S according to methods described by McLeod (McLeod, 1980). For BrdU incorporation studies, BrdU was added to the culture 1 hour prior to tissue fixation following a protocol recommended by the manufacturer (Roche). Paraffin-sectioned tissue samples were incubated with anti-BrdU and differentiated with anti-mouse Ig-alkaline phosphatase. Incorporated BrdU was detected using NBT and X-phosphate as substrates for alkaline phosphatase.

Results

Formation of the fetal digit tip

In a previous study we established a correlation between the expression domains of *Msx1* and *Msx2* and the level-specific regenerative capacity of mature mouse digits (Reginelli et al., 1995). In situ hybridization studies of neonatal digits indicates that *Msx1* is expressed in loose connective tissue that is sandwiched between the nail bed and the terminal phalangeal skeletal element (Fig. 1B). We observed that *Msx2* expression is restricted to the cells of the nail bed (Fig. 1C), so that, in the mature digit, *Msx1* and *Msx2* are both expressed in a similar region of the digit tip but their expression domains are non-overlapping. Our earlier studies showed that various stages of fetal digit tip development have the capacity to regenerate in a level-specific manner (Reginelli et al., 1995). We have since focused on the regeneration of the stage 11 digit tip because at this stage the digit tip has initiated differentiation, and it retains the ability to undergo a rapid regeneration response. Overt differentiation of the digit tip is initiated by this stage as the condensing terminal phalangeal element is apparent in whole-mount Alcian Blue stained digits (Fig. 1A). At this stage, *Msx1* and *Msx2* are expressed in distinct domains that overlap in the

distal mesenchyme. *Msx1* is expressed in all mesenchymal cells at the digit tip and forms a domain that is complementary to the central chondrofying terminal phalangeal element (Ngo-Muller and Muneoka, 2000b) (Fig. 1G,H). *Msx2* is expressed in the apical ectoderm and in distal mesenchymal cells subjacent to the ectoderm (Fig. 1I), thus the region of *Msx1* and *Msx2* co-expression is restricted to the apical mesenchyme. During the maturation of the digit, the *Msx1* expression domain becomes restricted to the dorsal loose connective tissue that lies between the terminal phalanx and the nail organ. During that same timeframe, *Msx2* expression is downregulated in the distal mesenchyme and the ectodermal domain shifts to the dorsally located nail organ.

In addition to *Msx1* and *Msx2* expression, *Bmp4* is expressed in the stage 11 distal digit mesenchyme in a pattern similar to that of *Msx1* (Fig. 1J). At this stage, the onset of endochondral differentiation of the terminal phalanx is indicated by the expression of *Ihh* at the distal tip of the digit (Fig. 1K), and the initiation of nail organ formation is indicated by the expression of *Hoxc13* on the dorsal surface of the digit (Fig. 1L). In neonatal digits (E18.5 and postnatal), the anatomy of the terminal digit region is complete. In the dorsal mesenchyme separating the nail organ and the terminal phalanx, *Msx1* is expressed prominently, both at birth (not shown) and postnatally (Fig. 1B), whereas *Bmp4* is expressed weakly at birth (data not shown) but is expressed strongly postnatally (Fig. 1D). *Msx2* expression is downregulated in the dorsal mesenchyme and its expression in the epidermis is restricted to the nail bed (Fig. 1C). *Hoxc13* expression remains associated with the forming nail and is prominent in the neonatal nail bed (Fig. 1F). The formation of the terminal phalanx itself is characterized by the distal expression of *Ihh* (Fig. 1E).

Digit regeneration in vivo

In vivo amputation of the stage 11 digit tip of E14.5 fetuses results in a wound healing response that is followed by the regeneration of the digit tip blastema and the eventual formation of an anatomically complete digit. This process is completed in a 4 day period so that at birth (E18.5) the digit tip has a relatively normal appearance, albeit somewhat shorter by comparison to non-amputated control digits (compare the regenerated central digits with the non-amputated peripheral digit in Fig. 2A). Gene expression studies of the 4-day-regenerated digit tip corroborate anatomical observations and indicate that the regenerate is normal. *Msx1* is expressed in the dorsal mesenchyme between the nail organ and the terminal phalanx (Fig. 2B). *Msx2* is downregulated in the dorsal mesenchyme, but is expressed in the epidermis associated with the nail organ (Fig. 2C). *Bmp4* is weakly expressed in the dorsal mesenchyme (Fig. 2D). The differentiation markers, *Ihh* and *Hoxc13*, are expressed in the terminal phalanx and the forming nailbed, respectively (Fig. 2E,F). Analysis of the regenerating digit tip 2 days post-amputation shows that the response involves the reformation of a digit blastema distal to, and surrounding, a central cartilaginous element. Within this digit blastema marker genes are expressed largely in a manner similar to those of the developing digit tip (Fig. 2H-L). Thus, *Msx1*, *Msx2* and *Bmp4* are all expressed in the regenerating digit blastema mesenchyme, *Msx2* and *Hoxc13* are expressed in the apical epidermis, and *Ihh* is expressed in the central cartilaginous element. However, one difference is that the *Msx2* expression domain in the distal mesenchyme is expanded so as to coincide with that of *Msx1* (Fig. 2I). Amputation of the digit tip at a more proximal level does not result in a regenerative response, and distal digit marker genes (*Msx1*, *Msx2* and *Bmp4*) are not expressed at the site of amputation injury (Reginelli et al., 1995) (Fig. 2G).

Digit regeneration in vitro

We used techniques for organ culture to determine whether the regeneration of stage 11 digits can occur in vitro. Stage 11 autopods were isolated from E14.5 fetuses, and the tips of 2-3 central digits were amputated and cultured for up to 4 days. After 2 days of culture a clear regeneration response is evident based on the reformation of the digit blastema, and after 3-4 days of culture the digit blastema elongates distally (Fig. 3A). Based on external anatomy, the in vitro regeneration response

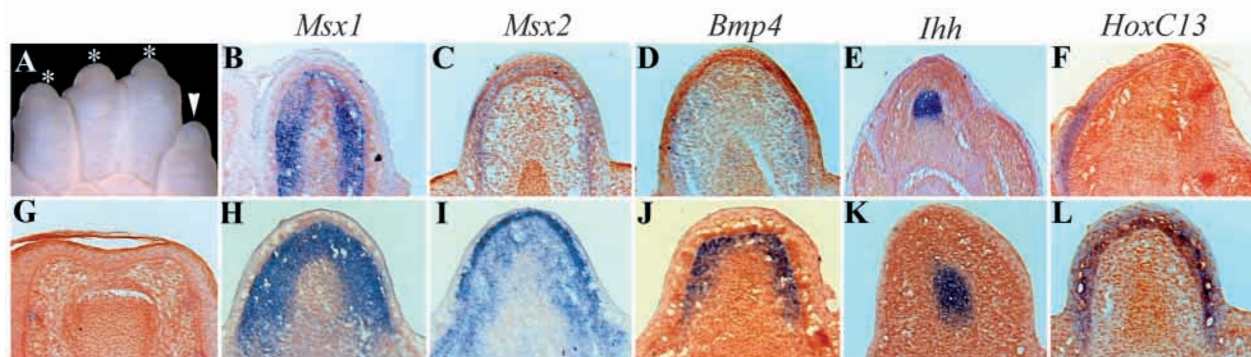

Fig. 2. Digit regeneration in vivo. (A) E17.5 autopod (stage 13), ventral view. The central 3 digits, digits 2, 3 and 4, were amputated at E14.5 and analyzed 3 days later. Note the regenerated digit tips (asterisk) are shorter than a non-amputated control digit tip (arrowhead). (B-F,H-L) In situ hybridization of regenerated digit tips 4 days (B-F) and 2 days (H-L) after amputation. (B-D,H-L) Frontal sections with distal toward the top of the image. (E-F) Sagittal sections with distal toward the top and dorsal to the left of the image. Sections in B-F show expression of *Msx1* in the mesenchyme surrounding terminal phalanx (B), *Msx2* in the apical epidermis (C), low levels of *Bmp4* in the apical mesenchyme (D), *Ihh* in the forming terminal phalanx (E), and *Hoxc13* in the nail organ (F). (G) Proximally amputated digits fail to mount a regeneration response and are negative for the expression of *Bmp4*. (H-L) In situ hybridization of 2 day regenerates showing expression of *Msx1* (H), *Msx2* (I), *Bmp4* (J), *Ihh* (K) and *Hoxc13* (L). The expression patterns of these marker genes in regenerating digits are largely similar to those of developing digits with the exception of *Msx2*, which displays an expanded mesenchymal domain.

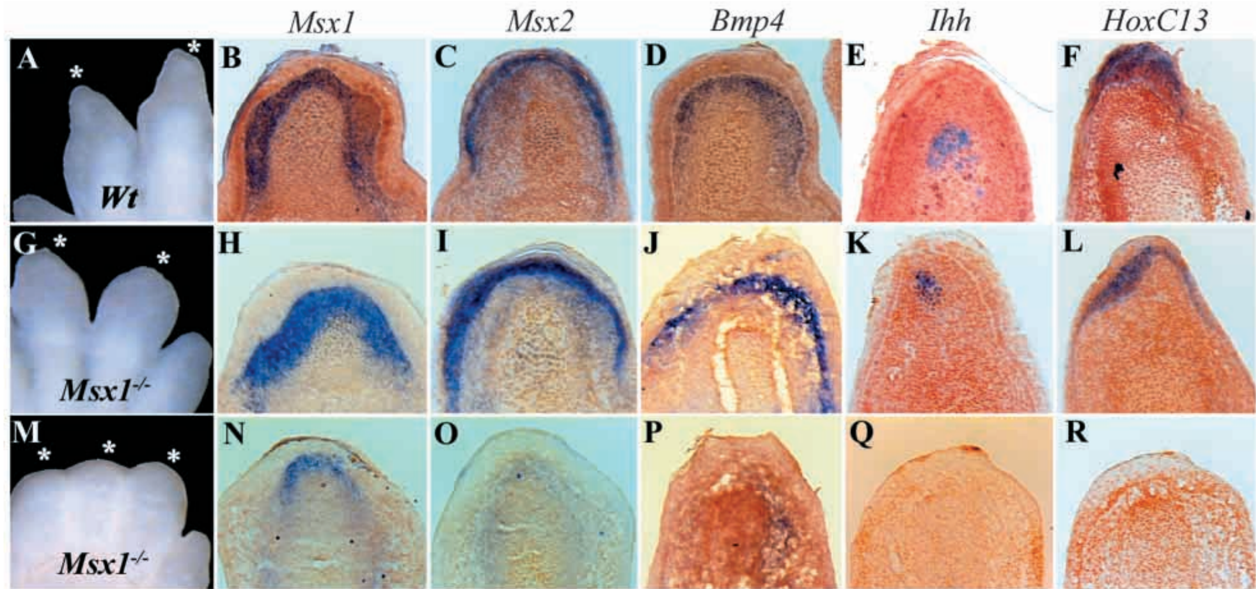

Fig. 3. *Msx1* mutant digits display a regeneration defect. (A) Stage 11 wild-type digit tips regenerate in 3-day organ culture. Note the distal outgrowth (asterisk) associated with the regeneration response. (B-F,H-L,N-R) The expression patterns of marker genes in 3-4 day cultures (B-F) or 2-day cultures (H-L,N-R) of amputated wild-type (B-F,H-L) or *Msx1* mutant digits (N-R). (B-D,H-J,N-P) Frontal sections with distal toward the top of the image. (E-F,K-L,Q-R) Sagittal sections with distal toward the top and dorsal to the left of the image. (B) *Msx1* is expressed in the regenerating mesenchymal cells of wild-type digit blastemas. (C) *Msx2* is expressed in the basal layer of the apical epidermis and is weakly expressed in the distal digit mesenchyme of wild-type regenerates. (D) *Bmp4* is expressed in the distal mesenchyme of regenerating wild-type digits. (E) *Ihh* is expressed in the differentiating terminal phalanx of regenerating wild-type digits. (F) *Hoxc13* expression in the apical epidermis is associated with the forming nail in regenerating wild-type digits. (G) Digit amputation fails to elicit a regeneration response (asterisk) from *Msx1* mutant digits after 3 days of culture. (H-L) Regenerating wild-type digits cultured for 2 days show the reformation of the digit blastema. *Msx1* (H) and *Bmp4* (J) are expressed distal mesenchymal cells, and *Msx2* (I) is expressed in the distal mesenchyme and apical epidermis. *Ihh* (K) is expressed in the differentiating terminal phalanx. *Hoxc13* (L) expression is associated with the distal epidermis associated with the presumptive nail organ. (M) *Msx1* mutant digits amputated in vivo and analyzed 3 days post-amputation fail to mount a regeneration response. Asterisks indicate non-regenerating digit tips. (N-R) Non-regenerating *Msx1* mutant digits fail to express regeneration marker genes 2 days after amputation. (N) Expression of non-functional *Msx1* transcripts is detected in the distal mesenchyme. *Msx2* (O), *Bmp4* (P), *Ihh* (Q) and *Hoxc13* (R) are not expressed in the mutant digit following amputation.

is comparable to the in vivo response. The cultured autopods appear morphologically different than in vivo limbs, primarily because the re-fusion of digits, which occurs late in limb development (Maconnachie, 1979), does not occur in cultured autopods. In addition to the anatomical response, we characterized the expression of genes associated with digit regeneration in vivo. In 4-day-cultured regenerates, we observed robust expression of *Msx1* and *Bmp4* in mesenchymal cells surrounding the terminal phalanx, and *Msx2* expression restricted largely to the apical epidermis (Fig. 3B-D). Expression of *Ihh* in 4-day-cultured regenerates is associated with skeletogenesis of the terminal phalanx (Fig. 3E), whereas *Hoxc13* expression is associated with the apical epidermis, and is spatially more variable than in in vivo regenerates (Fig. 3F). In 2-day-cultured regenerates, an apical blastema of mesenchymal cells is present and *Msx1* is expressed strongly throughout the regenerating digit tip (Fig. 3H). *Msx2* is expressed strongly in the basal layer of the apical epidermis and is expressed weakly in the most distal mesenchyme (Fig. 3I). Like *Msx1*, *Bmp4* is expressed in the distal mesenchyme (Fig. 3J). We also investigated the expression of the differentiation marker genes *Ihh* and *Hoxc13*, associated with the terminal phalanx and the forming nailbed, respectively (Fig. 3K,L). Overall, we observed an anatomical regeneration response in more than 90% of cultured wild-type digits (Table 1), and we found perfect correlation between a regeneration response and the expression of *Msx1*, *Msx2* and *Bmp4*.

Msx1 mutant digits display a regeneration defect

We used the regeneration of cultured digits as a screen to test the hypothesis that MSX genes play a role in the regeneration response. E14.5 *Msx1* and *Msx2* mutants were generated by heterozygote cross, and all resulting fetuses were collected and tested for digit tip regeneration. To eliminate bias, anatomical scoring for a regeneration response was carried out without knowledge of genotype. Thus, in each experiment the regenerative ability of wild-type, heterozygote and mutant digits was tested. Our studies show that *Msx2* mutants regenerate in a manner that is indistinguishable from wild-type or $Msx2^{+/-}$ digits, thus demonstrating that *Msx2* function is not required for the regeneration response (Table 1). However, regeneration studies with *Msx1* mutant digits indicate a regeneration defective phenotype, in which mutant digits regenerated at a frequency (28%) that was much lower than their heterozygote and wild-type counterparts (Table 1, Fig. 3G). This finding was verified in vivo, where *Msx1* mutant digits were found to regenerate at a similarly low frequency (37%; Table 1, Fig. 3M). An analysis of gene expression in in

Table 1. Regeneration response of fetal digit tips

Genotype	Experiment	Total amputated	Regenerates	% Regeneration
Wild type and *Msx1*$^{+/-}$	Organ culture	77	70	90.9
Msx1$^{-/-}$	Organ culture	36	10	27.8
Msx2$^{-/-}$	Organ culture	21	17	81.0
Wild type and *Msx2*$^{+/-}$	Organ culture	50	43	86.0
Wild type and *Msx1*$^{+/-}$	exo utero (in vivo)	33	28	88.9
Msx1$^{-/-}$	exo utero (in vivo)	8	3	37.5
Msx1$^{-/-}$	BMP4 (200 ng/ml)	15	11	73.0
Msx1$^{-/-}$	BMP4 (1000 ng/ml)	21	18	86.0
Msx1$^{-/-}$	BMP2 (200 ng/ml)	18	5	27.8
Msx1$^{-/-}$	Noggin (200 ng/ml)	23	2	8.7
Wild type and *Msx1*$^{+/-}$	Noggin (200 ng/ml)	82	15	18.3

vivo and in vitro mutant digits revealed that expression of non-functional *Msx1* transcripts was detected at the amputation wound of 2-day regenerates (Fig. 3N), whereas *Msx2* and *Bmp4* transcripts were not detected (Fig. 3O,P). We were also unable to detect expression of either *Ihh* or *Hoxc13* in 2-day non-regenerating *Msx1* mutant digits (Fig. 3Q,R). In the few *Msx1* mutant digits that did regenerate, we found expression of *Msx2* and *Bmp4* associated with the regeneration response, and we were unable to distinguish this mutant regeneration response from regenerating wild-type digits (data not shown).

Msx2 compensation in *Msx1* mutant digits

The variability of the *Msx1* mutant digit regeneration phenotype suggests the existence of a redundant activity that might replace Msx1 function in the regenerative response. An obvious candidate in the forming digit is Msx2 because: (1) its expression is associated with digit regeneration, (2) Msx1 and Msx2 display similar biochemical characteristics (Bendall and Abate-Shen, 2000), and (3) the *Msx2* expression domain overlaps with *Msx1* in the distal digit mesenchyme. To explore this possibility, we carried out an analysis comparing *Msx2* expression in wild-type and *Msx1* mutant digits. In initial studies, we processed wild-type and *Msx1* mutant digits side by side for whole-mount in situ hybridization for *Msx2* expression, and discovered that *Msx2* expression is visibly enhanced in the *Msx1* mutant digit as compared with wild-type (Fig. 4A,B), thus suggesting that *Msx2* expression was compensating for the absence of *Msx1*. When the spatial pattern of *Msx2* expression was analyzed in the *Msx1* mutant digit, we discovered that the mesenchymal *Msx2* expression domain was expanded to encompass the wild-type *Msx1* expression domain (Fig. 4C). These observations suggest that *Msx1* functions to restrict the *Msx2* expression domain during digit formation, although this inhibition must involve additional activities, as both genes are co-expressed in the distal mesenchyme. These data are consistent with a redundancy in *Msx1* and *Msx2* function during digit development; however, because the *Msx1* mutant digit displays a regeneration defect, the evidence suggests that *Msx1* and *Msx2* are functioning in a partially redundant way in regeneration.

BMP4 rescues the *Msx1* mutant regeneration defect

To explore the defective regeneration response in *Msx1* mutant digits, we analyzed the expression of *Bmp4*. By processing *Msx1* mutant and wild-type digits for whole-mount in situ hybridization in parallel, we found that *Bmp4* expression was visibly reduced in the *Msx1* mutant (Fig. 4D,E). Analyzing for changes in spatial expression of *Bmp4* indicated that the *Bmp4* expression domain changed from being coincident with the *Msx1* expression domain to being coincident with the wild-type *Msx2* expression domain in *Msx1* mutant digits (Fig. 4F). We next analyzed *Bmp4* expression in the tip of *Msx1*/*Msx2* double-mutant digits and found no detectable transcripts by in situ hybridization (data not shown). These data are consistent with the conclusion that *Bmp4* is regulated by both *Msx1* and *Msx2*, and raises the possibility that BMP4 may be a limiting factor in *Msx1* mutant digit regeneration.

To test this hypothesis, we carried out rescue experiments with exogenous BMP4. Amputated hindlimb digits obtained from *Msx1*$^{+/-}$ crosses were cultured in medium containing recombinant human BMP4 at two different concentrations, 200 ng/ml and 1000 ng/ml. Treatment at the lower BMP4 concentration resulted in a partial rescue of the regeneration response (73%, Table 1), whereas treatment at the higher concentration enhanced the regeneration response to a level comparable to that seen in wild-type controls (86%, Table 1). In parallel studies, application of BMP2 (200 ng/ml) had no effect on *Msx1* mutant digit regeneration (27.8%, Table 1), indicating that the rescue effect is specific to BMP4. The

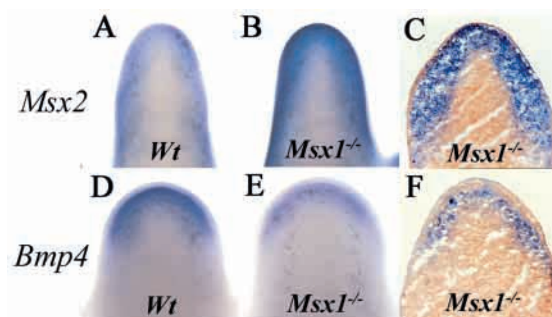

Fig. 4. Expression patterns of *Msx2* and *Bmp4* in *Msx1* mutant digits. (A,B) *Msx2* whole-mount in situ hybridization of stage 11 wild-type (A) and *Msx1* mutant (B) digits that were processed and imaged in parallel, showing an upregulation of *Msx2* expression in the *Msx1* mutant. (C) Frontal section of an *Msx1* mutant digit showing the expansion of the *Msx2* expression domain (compare with Fig. 1I). (D,E) *Bmp4* whole-mount in situ hybridization of stage 11 wild-type (D) and *Msx1* mutant (E) digits that were processed and imaged in parallel. *Bmp4* expression is downregulated in the *Msx1* mutant digit. (F) Frontal section of an *Msx1* mutant digit showing compression of the *Bmp4* expression domain to the apical mesenchyme (compare with Fig. 1J).

BMP4 rescue of the Msx1 regeneration defect is associated with a striking upregulation of *Msx2* expression in the apical epidermis (Fig. 5A), and of *Bmp4* expression in the induced digit blastemal mesenchyme (Fig. 5B). *Hoxc13* is also expressed in the dorsal epidermis in the BMP4-induced regenerate (Fig. 5C), and *Ihh* is expressed in the forming terminal skeletal element (data not shown). In summary, these results show that BMP4 functions in a dose-dependent manner downstream of *Msx1*, and identifies BMP4 as an essential regulator of the regeneration response.

As an alternative approach to investigate the role of BMP4 in digit tip regeneration, we treated cultures of amputated wild-type and mutant digits with the BMP-binding protein noggin. As *Bmp2* is not expressed during later stages of mouse digit formation (M.H., unpublished), and as exogenous BMP2 does not rescue the *Msx1* mutant regeneration defect, noggin treatment is likely to act as a specific inhibitor of BMP4 in our digit regeneration studies. The influence of noggin (200 ng/ml) on regeneration was tested in wild-type digits, and in *Msx1* heterozygote and *Msx1* mutant digits resulting from $Msx1^{+/-}$ crosses. Overall, noggin treatment caused a reduction of regeneration frequency in all three groups. *Msx1* mutant digit regeneration was reduced to 8.7% (Table 1), a 3-fold reduction compared with the 27.8% regeneration observed without noggin treatment. This finding provides support for the conclusion that reduced levels of BMP4, as indicated by in situ hybridization analyses, is responsible for the low level regeneration response in *Msx1* mutant digits. This observation also implicates *Bmp4* as the downstream target gene responsible for the partial redundancy displayed by *Msx1* and *Msx2* in digit regeneration. *Msx2* or *Bmp4* transcripts were not detected by in situ hybridization in noggin-inhibited *Msx1* mutant digits (data not shown). Noggin treatment of *Msx1* heterozygote and wild-type digits reduced the regeneration frequency 5-fold, from 90.9% to 18.3% (Table 1). Unlike the *Msx1* mutant digits, noggin-inhibited amputated wild-type digits do express *Msx1*, *Msx2* and *Bmp4* transcripts at the wound site (Fig. 5D-F). This data suggests that *Msx2* and *Bmp4* are functionally downstream of *Msx1*, and that BMP4 signaling is not a prerequisite for the maintenance of either *Msx1* or *Msx2* expression. In summary, these data provide additional support for the conclusion that BMP4 signaling is essential for the fetal digit tip regeneration response.

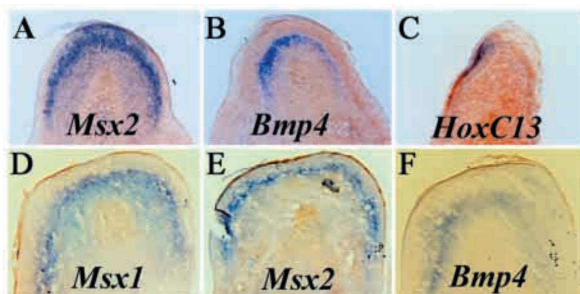

Fig. 5. Effect of BMP4 and noggin on digit regeneration. (A-C) Exogenous treatment with BMP4 rescues the *Msx1* regeneration defect. (A,B) Frontal section in situ hybridization with distal toward the top of the image. (C) Sagittal section in situ hybridization with distal to the top and dorsal to the left of the image. BMP4-induced mutant digit regeneration displays normal expression of *Msx2* (A), *Bmp4* (B) and *Hoxc13* (C). (D-F) Exogenous treatment with the BMP-binding protein noggin inhibits regeneration in wild-type digits. (D-F) Frontal section in situ hybridization with distal toward the top of the image. Despite the absence of a regeneration response, stump tissues maintain expression of *Msx1* (D), *Msx2* (E) and *Bmp4* (F).

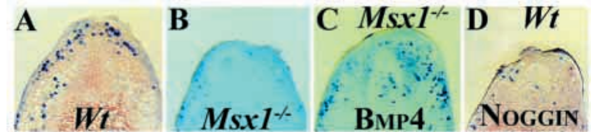

Fig. 6. Cell proliferation and digit regeneration. BrdU incorporation was studied in 2-day regenerating digits. (A-D) Frontal sections with distal toward the top of the image. (A) BrdU incorporation identifies a population of proliferating cells at the apex of a regenerating wild-type blastema. (B) The absence of a regeneration response in *Msx1* mutant digits is associated with very little BrdU incorporation. (C) BrdU-labeled cells are shown in the regenerating blastema of *Msx1* mutant digits treated with BMP4. (D) Noggin treatment inhibits cell proliferation in amputated wild-type digits.

Cell proliferation correlates with the regeneration response

In tetrapod vertebrates, epimorphic regeneration of adult and developing limb tissues is associated with a localized growth response at the site of injury. To analyze cell proliferation during the fetal digit tip regeneration response, we carried out BrdU incorporation studies of amputated wild-type and *Msx1* mutant digits after treatments to modulate the regeneration response. After 2 days in culture, ectodermal wound closure is complete and a morphological response is evident. In wild-type digits, we found localized incorporation of BrdU associated with outgrowth of the regenerating digit blastema (Fig. 6A). By contrast, we observed little BrdU incorporation at the injury site in *Msx1* mutant digits that fail to mount a regeneration response (Fig. 6B). BMP4-treated *Msx1* mutant digits displayed BrdU-labeled cells associated with the rescued regeneration response (Fig. 6C), providing evidence that BMP4 induces a proliferative response in regenerating digit blastema cells. Consistent with this conclusion, BrdU incorporation was reduced in noggin-treated wild-type digits in which regeneration is inhibited (Fig. 6D), which indicates that BMP signaling is required for proliferation. These studies provide evidence that fetal digit tip regeneration is an epimorphic response associated with an apical growth zone, and suggests a role for *Msx1* and BMP4 in the control of cell proliferation following amputation injury.

Discussion

Amputation of the murine fetal digit tip results in an epimorphic regenerative response that restores the forming digit blastema, which subsequently undergoes differentiation to form a normal digit tip. This regenerative response occurs both in vivo and in vitro, and we have used this model to provide functional evidence that both MSX1 and BMP4 are required for successful regeneration. Regeneration rescue experiments show that regeneration-defective *Msx1* mutant digits are induced to regenerate by the exogenous application of BMP4, and that this response is dose-dependent. Gene

expression studies suggest that regeneration may be regulated by both MSX1 and MSX2, via their combined involvement in controlling *Bmp4* expression during digit development. Experiments on wild-type digits showing that regeneration is inhibited by application of the BMP-binding protein noggin confirm a role for BMP signaling in the control digit regeneration. These studies provide both loss- and gain-of-function evidence that MSX1 and BMP signaling are required for the successful regeneration of the fetal mouse digit tip.

Regeneration in vitro

The capacity of embryonic/fetal tissues to undergo enhanced regenerative repair identify developing tissues as relatively simple models for investigating regenerative responses (see Muller et al., 1999). The regenerating mammalian digit tip is no exception to this rule. In mice, mature digit tip regeneration is level-specific, associated with the nail organ, and proceeds very slowly, requiring weeks to months to complete. Fetal digit tip regeneration is also level-specific and associated with the nail anlagen, but is completed within a 4 day period. Because the regeneration response occurs so rapidly in fetal digits, we have been able culture this regenerating structure in vitro, where the regeneration environment can be manipulated. The fact that a regeneration response can be elicited in explanted limb tissue indicates that components that are likely to play crucial roles in mature digit regeneration, such as functional innervation, vascularization and the availability of circulating hormones, are not required for fetal regeneration. Thus, the simplicity of the regeneration response provides a means to experimentally dissect underlying regulatory mechanisms that might otherwise be inaccessible in the mature digit.

Regeneration studies on the mature mammalian digit tip have focused almost exclusively on the role of the nail organ and the distal growth of the terminal phalanx (see Zhao and Neufeld, 1995). This association is derived from both clinical studies in humans and experimental studies on rodents (Douglas, 1972; Illingworth, 1974; Borgens, 1982). By studying both developing and mature digits, we found that regenerative potential is associated with the expression domain of *Msx1* (Reginelli et al., 1995), but that *Msx1* expression in mature digits is restricted to loose connective tissue fibroblasts subjacent to the nail, and is not found in the nail organ itself. The demonstration that *Msx1* mutant digits display a regeneration defect indicates that *Msx1*-expressing cells play a crucial role in the response. These findings suggest that digit tip regeneration may not be dependent on the nail organ, but rather on the connective tissue cells underlying the nail organ. Because this population of cells is closely associated with the nail organ it would be difficult to distinguish between a nail organ effect and the influence of the underlying connective tissues. The importance of this cell population in mature digit regeneration is also supported by histological observations noting a streaming of fibroblastic cells toward the regenerating digit tip (Revardel and Chebouki, 1987; Muller et al., 1999), and by the observation that a regeneration response itself need not include the nail plate (Reginelli et al., 1995).

MSX genes in digit formation and regeneration

The expression patterns of the MSX genes at the digit apex represent a simple nested relationship that partitions the digit apex into a distal domain, in which both MSX genes are expressed, and a sub-distal domain, in which only *Msx1* is expressed. Digit formation occurs normally in *Msx1* and *Msx2* mutant mice, indicating that individual MSX gene function is not essential during outgrowth (Satokata and Maas 1994; Satokata et al., 2000). The co-expression of *Msx1* and *Msx2* in apical mesenchymal cells raises the possibility that these two genes function redundantly during digit development. The expansion of the *Msx2* expression domain in *Msx1* mutant digit tips suggests a compensatory response by *Msx2* in the absence of *Msx1*, and is consistent with a redundancy hypothesis. This response also suggests that *Msx1* plays an inhibitory role in *Msx2* expression within the sub-distal cells where only *Msx1* is expressed in wild-type digits. However, if MSX1 inhibits *Msx2* expression, its activity must be repressed in the distal zone where both genes are co-expressed. *Dlx5* is also expressed in the distal digit mesenchyme (Robledo et al., 2002) (M.H., unpublished), and has been shown to compete with and/or repress the activity of MSX genes (see Bendall and Abate-Shen, 2000), thus it represents a candidate for modulating MSX activity in the digit tip.

Our studies indicate that MSX1 functions in a regeneration-specific manner. In the absence of MSX1, digit formation is normal yet digit regeneration is defective, thus MSX1 function is necessary for regeneration but not for development. In addition, *Msx2* compensation in the *Msx1* mutant suggests an incomplete or partial redundancy of function that is restricted to digit regeneration. Studies on cultured cells indicate that one activity of MSX1 involves the control of cell differentiation (see Hu et al., 2001). Based on amphibian limb regeneration studies, a significant regeneration-specific event is the de-differentiation of cells at the wound site to form the blastema. Cellular de-differentiation has been best documented for multinucleated amphibian myotubes that are induced to form individual cells in vitro and, after grafting, in vivo (Lo et al., 1993). Studies using differentiated C2C12 myotubes and regulated *Msx1* expression provide evidence that MSX1 induces de-differentiation in vitro, and that subsequent suppression of *Msx1* expression can lead to transdifferentiation to multiple cell types, including cartilage, bone, adipose and muscle (Odelberg et al., 2000). Although these de-differentiation studies are specific to muscle tissue, forced *Msx1* expression studies demonstrate that multiple mesenchymal and epithelial cell types are inhibited from differentiation in culture, and that differentiation of mammary epithelial tissue is impaired in vivo (Hu et al., 2001). Thus, the available evidence suggests that MSX1 functions in multiple cell types to control differentiation and, in the context of regeneration, de-differentiation. As regenerative potential is restricted to domains of *Msx1* expression in the mature and fetal digit tip, we hypothesize that MSX1 functions to maintain a population of undifferentiated mesenchymal cells that can participate in a regeneration response. One significant difference between amphibian limb regeneration and regeneration of the mammalian digit tip is that *Msx1* expression is induced in response to amputation in amphibian limbs, whereas, in mammals, regenerative potential is linked to regions where *Msx1* expression is maintained in the mature digit.

BMP signaling and regeneration

BMP signaling is crucial for fetal digit regeneration. A number

of lines of evidence support this conclusion. First, BMP4 rescues the *Msx1* mutant regeneration defect in a dose-dependent manner. Second, *Bmp4* expression is downregulated in the *Msx1* mutant digit, which is consistent with the idea that residual regenerative capability is associated with this reduced level of BMP availability. Third, noggin-treated *Msx1* mutant digits display a more severe phenotype associated with the absence of *Bmp4* expression. Fourth, noggin treatment of wild-type digits results in a more than 5-fold reduction in regenerative capability. *Noggin* is not expressed within the forming digit tip, but is expressed in chondrofying skeletal elements at proximal digit levels that lack regenerative potential (Brunet et al., 1998; Capdevila and Johnson, 1998; Merino et al., 1998). Other BMP signaling antagonists, such as *follistatin, gremlin* (*Cktsf1b1* – Mouse Genome Informatics) and *chordin*, are also expressed in developing limbs (D'Souza and Patel, 1999; Merino et al., 1999; Zhang et al., 2002). The expression of multiple BMP signaling antagonists associated with digit formation suggests that the inability of proximal digit amputations to regenerate may be a consequence of regulated BMP activity associated with skeletal differentiation.

The sole function of MSX1 in digit regeneration lies in the regulation of *Bmp4* expression. BMP4 rescue of *Msx1* mutant digits indicates that MSX1 function is not required downstream of BMP signaling. Successful regeneration of a low percentage of *Msx1* mutant digits correlates with a reduced level of *Bmp4* expression and provides definitive evidence that successful digit regeneration can occur in the absence of MSX1 function. During digit formation, we have identified *Bmp4* as functionally downstream of both *Msx1* and *Msx2*. The shift of the *Bmp4* expression domain to coincide with the wild-type *Msx2* expression domain is consistent with the hypothesis that *Bmp4* is regulated by both *Msx1* and *Msx2* in the distal digit domain, but by only *Msx1* in the sub-distal domain. However, the compensatory response of *Msx2* in the *Msx1* mutant complicates this interpretation and indicates the presence of an *Msx2*-dependent *Bmp4* regulatory component that is co-expressed in the distal digit compartment. It is interesting that in vitro studies of the transcriptional regulator *Runx2* (previously known as *Cbfa1*) indicate that it is regulated by MSX2 (Shirakabe et al., 2001) and that it regulates *Bmp4* (Helvering et al., 2000), and that we find it co-expressed with *Bmp4* in the developing digit tip (M.H., unpublished). Thus, *Runx2* represents a candidate for mediating the regulation of *Bmp4* expression by MSX genes. Unravelling the details underlying the regulation of BMP4 signaling in the digit tip should provide key insights into the control of fetal digit regeneration.

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