

Overexpression of the Transcription Factor Msx1 Is Insufficient to Drive Complete Regeneration of Refractory Stage *Xenopus laevis* Hindlimbs

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Xenopus laevis tadpoles are capable of hindlimb regeneration, although this ability declines with age. Bmp signaling is one pathway known to be necessary for successful regeneration to occur. Using an inducible transgenic line containing an activated version of the Bmp target Msx1, we assessed the ability of this transcription factor to enhance regeneration in older limbs. Despite considerable evidence correlating *msx1* expression with regenerative success in vertebrate regeneration models, we show that induction of *msx1* during hindlimb regeneration fails to induce complete regeneration. However, we did observe some improvement in regenerative outcome, linked to morphological changes in the early wound epithelium and a corresponding increase in proliferation in the underlying distal mesenchyme, neither of which are maintained later. Additionally, we show that Msx1 is not able to rescue limb regeneration in a Bmp signalling-deficient background, indicating that additional Bmp targets are required for regeneration in anuran limbs. *Developmental Dynamics* 238:1366–1378, 2009. © 2009 Wiley-Liss, Inc.

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INTRODUCTION

The ability to regenerate an anatomically complex structure, such as a limb, is believed to be an ancestral feature lost in many vertebrates such as humans, but retained in others. Urodeles and larval anurans are relatively proficient at this, capable of regenerating their limbs, tails, jaws, and the lens of the eye (Sanchez Alvarado and Tsonis, 2006). However, while urodeles can regenerate their limbs proficiently as adults, in anurans, like the model organism *Xenopus laevis*, this ability declines during

metamorphosis, with hindlimb regeneration being lost altogether by Nieuwkoop and Faber developmental stage 57 (Dent, 1962; Overton, 1963; Nieuwkoop and Faber, 1967). Other vertebrates, such as chickens, cannot regenerate any part of their limbs (Summerbell, 1974). Mammals have a very limited regenerative capacity in that young mice and humans can regenerate just their extreme digit tips (Borgens, 1982; Reginelli et al., 1995; Allan et al., 2006).

Amphibian limbs regenerate by the process of epimorphosis, requiring the

formation of a proliferating blastema (Morgan, 1901). Successful epimorphic regeneration in amphibian limbs requires the formation of a wound epithelium (free from an underlying basement membrane and dermis), which covers the cut site almost immediately after amputation and aids in the development of the regenerative blastema (Tschumi, 1957; Neufeld et al., 1996). If this wound epithelium is replaced with mature skin in the form of a graft, regeneration fails (Mescher, 1976). This is thought to occur because the contin-

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ued development of the blastema, which underlies this distal epithelium, is driven by epithelial and blastemal cell interactions that would be inhibited by the presence of a basement membrane and dermis. The blastema itself is composed of dividing mesenchymal cells, already present in the developing limb (as in the case of *Xenopus laevis*), or alternatively formed from de-differentiated cells near the stump or by recruitment of reserve stem cell populations (as in the urodeles). Muscle satellite cells (stem cells) are recruited to the blastema in both urodele limb and anuran tail regeneration (Kumar et al., 2000; Chen et al., 2006; Morrison et al., 2006), but it is not known whether these cells are involved in *Xenopus* limb regeneration. Concurrent with blastema formation and development is the transition of the simple wound epithelium into the multilayered apical epithelial cap (AEC). This transition occurs within the first 2 to 3 days during *Xenopus* hindlimb regeneration (Pearl et al., 2008) and is important in ensuring the blastema reaches a critical mass capable of driving complete limb regeneration in amphibians (Christensen and Tassava, 2000).

Following a recent renaissance in regeneration research, fueled by the application of molecular techniques and the development of transgenic models, several pathways have been linked to regenerative success (reviewed in Stoick-Cooper et al., 2007; Yokoyama, 2008; Beck et al., 2009). One such pathway involves signaling by bone morphogenetic proteins (Bmps: Beck et al., 2003, 2006; Pearl et al., 2008), which form a subset of the large Tgf- β family of secreted molecules. Bmp signaling regulates a diverse array of processes including tissue specification and cellular proliferation, differentiation, and apoptosis (reviewed in Chen et al., 2004). Bmps 2, 4, and 7 are involved in the processes of tetrapod limb development, and are known to regulate apical epidermal ridge (AER) development, digit specification, apoptosis, and limb outgrowth (reviewed in Robert, 2007).

While several signaling pathways that are activated during regeneration have been identified in functional studies, the role of transcription factors that are known to act down-

stream of these pathways is less well understood. Signaling through either Bmp2 or Bmp4 can directly induce expression of the transcription factor Msx1 (Suzuki et al., 1997). During limb development, Msx1 has been proposed to regulate the role of the Bmp signaling pathway in induction of the AER (Pizette et al., 2001) in addition to maintaining the undifferentiated state of the limb bud mesenchyme (Carlson et al., 1998; Koshiba et al., 1998).

During regeneration, *msx1* is reexpressed in the regenerative blastema of urodele limbs (Crews et al., 1995; Simon et al., 1995; Koshiba et al., 1998), zebrafish fins (Poss et al., 2000; Murciano et al., 2002), mouse digit tips (Reginelli et al., 1995; Han et al., 2003), and *Xenopus* tails and hindlimbs (Beck et al., 2006), indicating a strong correlation of *msx1* re-expression with regenerative success. The loss of Bmp signaling through the overexpression of *noggin1* (which codes for an extracellular Bmp antagonist known to antagonize Bmps 2, 4, and 7) inhibits both *Xenopus* tail and limb bud regeneration characterized in part by the loss of *msx1* expression (Beck et al., 2006). Similarly, inhibition of Bmp signaling through ectopic expression of *chordin* during zebrafish fin regeneration results in the down-regulation of the *msx1* homolog *msxb* (Smith et al., 2006). Regenerative success in the mouse digit tip correlates with the expression zone of Msx1 and, furthermore, regeneration of the digit tip fails in the absence of *msx1* expression (Han et al., 2003). Moreover, induction of a hyperactive form of *msx1* through the use of a heat shock inducible transgene system restores regenerative ability of *Xenopus* tadpole tails during the refractory period in which tadpoles are transiently incapable of tail regeneration (Beck et al., 2003). Finally, Msx1 can induce dedifferentiation of cultured mouse myotubes into mononuclear dividing cells (Odelberg et al., 2000), and its loss prevents this dedifferentiation in cultured salamander myofibers (Kumar et al., 2004). Altogether this evidence strongly implicates Msx1 as a key mediator of Bmp signaling during regeneration and suggests that this gene may be a key player in determining regenerative success.

To investigate the potential of Msx1 to enhance regeneration in later stage amphibian hindlimbs, we have created a stable transgenic line of *Xenopus laevis* carrying a previously described inducible transgene containing a domain-switched, hyperactive version of *Xenopus* Msx1 under the control of the heat shock promoter *hsp70* (Beck et al., 2003). In the current study, we have utilized an *hsp70-eve-msx1-γ-crystallin-GFP* (henceforth M1) transgenic line to test the ability of this transcription factor to drive regenerative processes. The use of a stable line enables reduction of the natural variation in regenerative capability in the *Xenopus* population reported by others (Nye and Cameron, 2005) and observed by us. Using this system, we have demonstrated a role for Msx1 in the early stages of the regeneration process. However, we also show that Msx1 is not, by itself, sufficient to replace Bmp signaling during regeneration of the *Xenopus* hindlimb.

RESULTS

Msx1 Is Expressed in the Distal Mesenchyme and Interdigital Regions of the *Xenopus* Autopod

Xenopus msx1 has been previously shown to be expressed in the early limb bud progress zone mesenchyme (Christen and Slack, 1998), but its expression pattern during later limb development has not been described. Here, we show that *msx1* expression in *Xenopus laevis* limb development follows the expected pattern for tetrapod vertebrates, with expression in the distal mesenchyme from Nieuwkoop and Faber stage 52 and exclusion from the epithelium including the AER (Fig. 1A). Curiously, we also detect a patch of expression in the most proximal anterior mesenchyme, a region corresponding to the future stylopod, which was not previously reported (Fig. 1A, white arrowhead). Later, expression is found in the interdigital regions (Fig. 1B–D), a region corresponding to *Bmp4* expression (Beck et al., 2006). By stage 55, expression persists in interdigital regions but can also be seen in the most distal phalange of each forming toe (Fig. 1D).

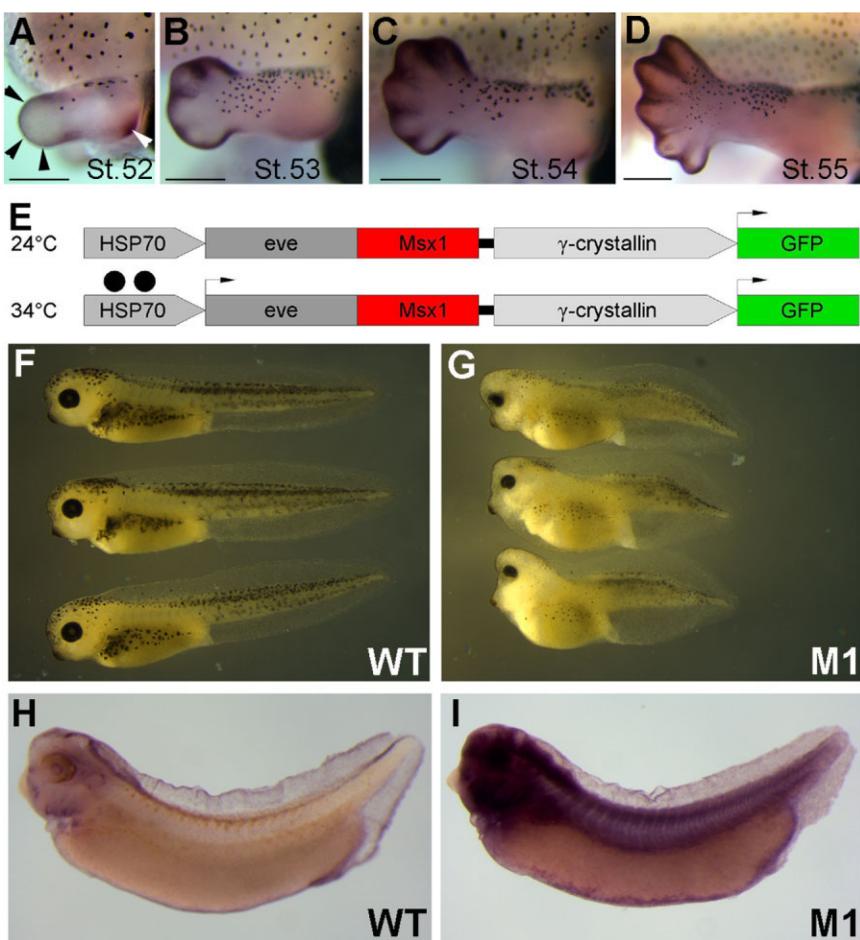


Fig. 1. Development of the M1 transgenic line of *Xenopus laevis*. **A–D:** Endogenous expression of *Msx1* in *Xenopus* hindlimbs from stage 52 to 55. Scale bars = 250 μ m, and limbs are oriented with distal to the left and posterior uppermost. Expression is restricted to the distal mesenchyme and excluded from the apical epidermal ridge (AER; black arrowheads in A). There is also an early proximal-anterior located expression (white arrowhead, A). Later expression is interdigital. **E:** Schematic representation of the eve*Msx1* transgene. At 24°C or below, the transgene is silent. At 34°C, however, heat shock factors (black circles) bind to the *hsp70* promoter sequence and activate transcription of eve*Msx1*. The construct is fused to a second transgene containing the γ -crystallin promoter driving green fluorescent protein (GFP). **F,G:** Phenotypes of tadpoles following heat shock at stage 14. WT embryos develop normally and are unaffected by the heat shock (F). Transgenic tadpoles have a flattened anterior head, reduced eye size, reduced number of melanocytes (pigment cells), and a shortened tail (G). **H,I:** In situ hybridization using an *Msx1* probe to show the normal expression of *Msx1* (H) is expanded and up-regulated in heat shocked transgenic animals (I).

The M1 Transgenic Line Is Activated Following Heat Shock, Enabling Ectopic *Msx1* Expression

A transgenic line of *Xenopus* (M1) was created using the previously described heat inducible *msx1* transgene (Beck et al., 2003; Fig. 1E–I). *Msx1* acts as a transcriptional repressor, and it has been previously shown that a swap of the repression domain of the *Xenopus laevis* protein with the strongly repressive domain from the *Drosophila* even-skipped protein (eve) creates a hyperactive form (Yamamoto et al.,

2000). Our transgene contains this hyperactive *Msx1* (eve*Msx1*) under the control of the inducible heat shock promoter *hsp70* linked to a second transgene in which green fluorescent protein (GFP) is under the control of the lens specific γ -crystallin promoter (Beck et al., 2003; Fig. 1E). Several founder animals containing this transgene have been raised, and all the experiments described here used F₁ or F₂ animals from the M1 transgenic line, which contains a single insertion site. These tadpoles develop normally under ambient temperatures, indicating no leakiness of ex-

pression from the *hsp70* promoter. During early development, *msx1* is expressed endogenously in the hindbrain, heart, branchial arches, and dorsal tail bud of embryos (Fig. 1 and Beck et al., 2003). The M1 tadpoles were tested for functional activity of the transgene by subjecting both wild-type (WT) and M1 animals to transient heat shock at the end of gastrulation (stage 14) and analyzing the resulting phenotypes at stage 40. At this stage, transgenic animals can be easily distinguished from their WT siblings by the presence of GFP in the lens of the eye. While WT animals developed normally to stage 40 (Fig. 1F), M1 sibling animals displayed a consistent phenotype with reduced forehead, small eyes, reduced number of pigment cells (melanophores) and a malformed craniofacial region, and were shorter in length, particularly in the tail (Fig. 1G). This phenotype is consistent with a role for *Msx1* in neural crest, eye, and tail development, and is similar to results obtained by Suzuki et al. (1997) following overexpression of *msx1* mRNA in embryos.

The *hsp70* promoter is thought to induce expression in all cells following heat shock (Wheeler et al., 2000; Beck et al., 2006). To test this for the M1 transgenic line, we heat shocked stage 35 embryos from a M1/WT cross; sorted them into transgenic and non-transgenic on the basis of GFP presence or absence, respectively, in the lens; and fixed them after 3 hr. The level of *msx1* transcript was then detected using an antisense RNA probe to *msx1* for whole-mount in situ hybridization. WT and M1 embryos were allowed to stain for the same amount of time, and the reaction stopped just after *msx1* staining became apparent in WT embryos. *Msx1* staining was much stronger in M1 embryos and was detected outside its normal expression zone (Fig. 1H,I). To further confirm that the transgene is being expressed at high levels as expected following heat shock, we used semi-quantitative polymerase chain reaction (qPCR) analysis to determine the relative expression levels of the transgene and the endogenous *msx1* gene. Three day postampputation limb blastemas/pseudoblastemas were harvested from bilaterally operated WT stage 52, Wt stage 54 or heat shocked

M1 stage 54 tadpoles, and RNA extracted for each tadpole. Large amounts of transgenic *eveMsx1* transcript were present in the M1 pseudoblastemas (Critical threshold or Ct = 22.02 ± 0.22), almost equivalent to the levels of cytoskeletal actin (Ct = 19.94 ± 0.06). Surprisingly, high levels of the endogenous gene were also expressed in M1 pseudoblastemas (Ct = 24.52 ± 0.17), suggesting auto-regulation of the endogenous gene by the transgene. In contrast, endogenous *msx1* was present in much lower levels in either stage 52 WT blastemas (Ct = 30.72 ± 0.83) or stage 54 WT pseudoblastemas (Ct = 31.92 ± 0.34). Amplified cDNA from regeneration competent stage 52 blastemas, therefore, reached the Ct approximately 31 cycles and that from regeneration incompetent stage 54 pseudoblastemas approximately 32 cycles. In comparison, the amount of transgenic expressed *eveMsx1* only requires 22 cycles, and, therefore, the amount of transgenic transcript massively exceeds that of the endogenous gene.

Ectopic Expression of *Msx1* Can Increase the Success of Anuran Hindlimb Regeneration

We wanted to see if our M1 transgenic animals would be able to regenerate more successfully than their WT siblings. Regenerative capability in *Xenopus* hindlimbs declines rapidly as development proceeds, correlating with ossification of the bones (Dent, 1962; Wolfe et al., 2000). Following partial amputation at stage 54, when all the limb elements are formed, the outcome is generally poor for WT hindlimbs, as either none or very limited regeneration occurs. We, therefore, decided to compare the regeneration of WT stage 54 limbs with transgenic M1 limbs, to see if ectopic expression of *eveMsx1* could improve regenerative ability. Several tadpoles arising from a WT/M1 mating were grown to stage 54, heat shocked, and the distal limb amputated at knee level, after 2–3 hr. This ensures that the transgene, if present, will be expressed strongly at the time of amputation. Heat shocks were then applied again after 24, 48, and 72 hr; thereafter, tadpoles were grown at 25°C until

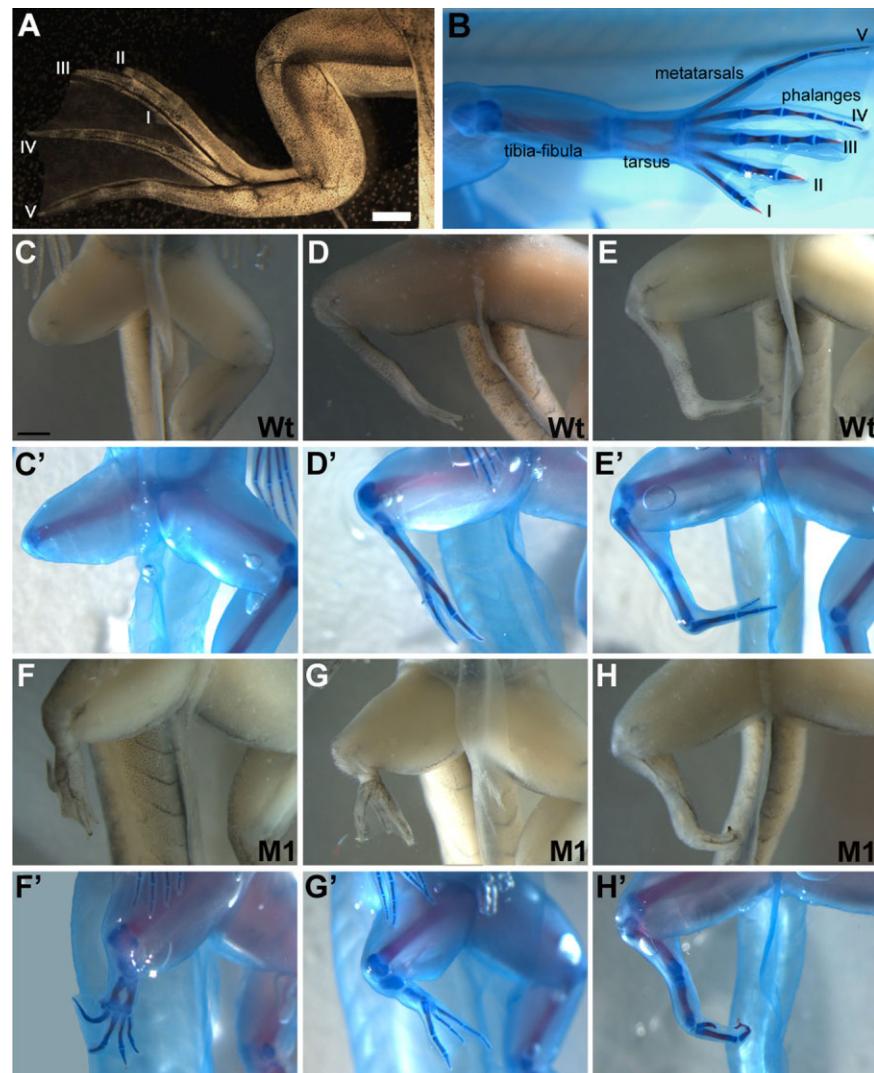


Fig. 2. Regeneration phenotypes in stage 54 amputated *Xenopus* wild-type (WT) or M1 limbs. **A,B:** M1 unoperated (left) contralateral limbs. **A:** Dorsal view of unoperated limb of stage 57 M1 tadpole and **B** ventral view of skeletal preparation showing the limb elements present at stage 57. **C–H:** ventral view of operated (right) limb following amputation at knee level at stage 54, heat shocked and fixed at stage 57. **C–C'**: Skeletal preparation of the corresponding WT tadpole in which no regeneration has taken place and a stump is formed ending at the knee. **D,D'**: WT limb that has regenerated two toes and has no tibia-fibula and an abnormal metatarsal. **E,E'**: WT limb that has regenerated a tibia-fibula, abnormal metatarsal, one normal toe, and a vestigial distal phalange (counted as two toes). **F,F'**: M1 limb that regenerated four toes but lacks a tibia-fibula. **G,G'**: M1 limb that regenerated an abnormal metatarsal and tibia-fibula as well as three toes. **H,H'**: M1 limb with regenerated tibia-fibula, abnormal metatarsal and a single toe. A vestigial proximal phalange is also present (counted as 1 toe).

they reached stage 57, at which point they were genotyped for the presence of the transgene (by scoring GFP in the lens) and the number of toes recovered on the operated limb were counted. Skeletal preparations were then made from the tadpoles to confirm the extent of skeletal regeneration (Fig. 2).

A normal *Xenopus* hindlimb autopod contains five toes, with the poste-

rior three toes (digits III, IV, and V) having three phalanges and a claw and the anterior two (digits I and II) having a fourth phalanx in place of the claw (Fig. 2A,B). Following knee level amputation at stage 54, the most frequently observed outcome for WT tadpoles was no regeneration, with the limb healing over to form a stump ending at the knee (Table 1, Fig. 2C,C'). In some cases, the tibia-fibula,

TABLE 1. Comparison of Regenerative Ability Between M1 and WT Tadpoles Following Amputation of the Limb at Knee Level at Stage 54^a

Genotype	Number of toes regenerated						n	score/n	% reg
	0	1	2	3	4	5			
WT	6	0	2	0	0	0	8	0.5	25%
M1	3	2	2	3	1	0	11	1.73	73%

^aLimbs were scored for the number of toes regenerated by stage 57 in either wild-type (WT) or M1 tadpoles following heat shocks at -3, +24, +48, and +72 hr relative to amputation. Each regenerated toe results in a score of 1; n = number of tadpoles; score/n is, therefore, the average number of toes per hindlimb. M1 tadpoles regenerated significantly more toes than WT tadpoles (*t*-test, $P = 0.048$). The percentage of animals showing any level of regeneration (in the form of toes) is recorded as % reg.

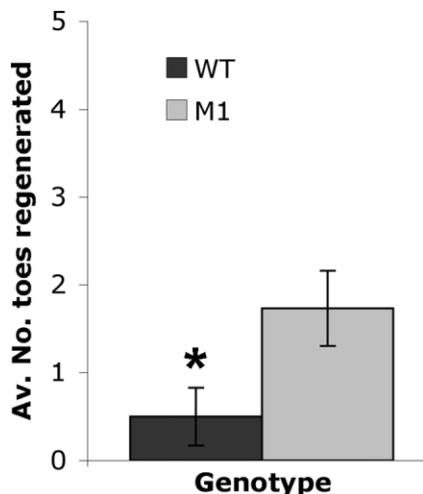


Fig. 3. M1 tadpoles regenerate more toes following amputation at knee level at stage 54. Graph showing the average number of toes regenerated following amputation at knee level at stage 54 in wild-type (WT) or M1 tadpoles. Tadpoles were heat shocked to induce the transgene, if present, 2–3 hr before amputation and again after 24, 48, and 72 hr to maintain transgene expression. A fully regenerated limb would have 5 toes, and no regeneration at all results in a stump (0 toes). Toes were counted once the animals had reached stage 57 (forelimbs emerged). Error bars show standard error; n = 8 for WT animals and 11 for M1 animals. M1 tadpoles regenerated significantly more toes than WT ($P < 0.05$) but never achieved perfect regeneration.

abnormal metatarsals, and digits were regenerated (Table 1; Fig. 2D–E'), with the maximum number of regenerated toes being two. The M1 animals fared a little better, with most tadpoles having more than one toe regenerated in addition to a tibia–fibula and abnormal metatarsals (Table 1; Fig. 2F–H,F'–H'). M1 tadpoles recovered more toes than WT animals ($P =$

0.048, unpaired *t*-test), and more of them regenerated some structures (73% vs. 25% in WTs; Fig. 3). However, perfect limb regeneration was never observed and these animals sometimes appeared to lack a tibia–fibula (zeugopod), giving the hindlimbs a distinctive shortened appearance (Fig. 2F,G').

Histological Analyses of M1 and WT Tadpoles Reveal an Early Difference in Postamputation Morphology

We have previously demonstrated a requirement for the Bmp signaling pathway in the successful development of regeneration specific structures. Inhibition of the Bmp pathway through activation of noggin1 results in a loss of regenerative capacity reflected in the absence of a proliferative blastema and functional, multilayered AEC (Beck et al., 2006; Pearl et al., 2008) resulting in ultimately failed regeneration. Similar events have been reported to precede the failure of older WT hindlimbs to regenerate (Wolfe et al., 2000). We, therefore, investigated whether overexpression of the Bmp-activated target transcription factor Msx1 could result in the formation of an improved AEC from the wound epithelium.

Within the first 24 hr, a wound epithelium forms over the cut site priming the underlying stump tissue for regeneration (Tschumi, 1957). The layered morphology of the AEC, which develops from the wound epithelium at its apex, has been previously described in urodeles (Christensen and Tassava, 2000). In *Xenopus* hindlimbs

that have been partially amputated before the ability to regenerate declines, a similar multilayered AEC is formed (Pearl et al., 2008). Following amputation at stage 54 at the level of the forming knee, M1 or WT hindlimbs were assessed histologically for signs of regenerative potential. All tadpoles were heat shocked 3 hr before amputation and every 24 hr afterward to induce the transgene if present. Individuals were fixed after 1, 2, 3, or 5 days, sectioned, and stained with hematoxylin and eosin. An organized, multilayered epithelium was evident in both the WT and M1 regenerating limbs; however, in the M1 limbs, the wound epithelium appeared thicker (six to seven cell layers), and, therefore, more reminiscent of an AEC than the WT equivalents (three to four cell layers; Fig. 4A,A',E,E'; Table 2). In both cases, the basal layer of epithelial cells, which directly contact the underlying mesenchyme, appeared cuboidal in shape, rather than columnar. Transition to columnar basal cell morphology is strongly associated with a functionally active AEC (Christensen and Tassava, 2000). Additionally, accumulation of eosin-stained connective tissue was almost always evident within the M1 blastema (six of seven cases examined, Fig. 4E',E"; Table 2). In approximately half of these, extensive deposition of connective tissue resulted in separation of the AEC from the underlying cells, and was accompanied by infiltration of strongly eosin stained cells with large nuclei, resembling eosinophils (three of seven cases examined, Fig. 4E"; Table 2). Connective tissue was noted less frequently and in smaller quantities in WT samples (Fig. 4A'), and was never accompanied by eosinophils or separation of the AEC from the mesenchyme.

By 48 hr after limb amputation, most (four of six) M1 amputated limbs and a few (two of seven) WT limbs had morphologically distinct multilayered AECs (Fig. 4B,B',F,F'; Table 2). The WT AEC, when present, had slightly fewer cell layers (six to seven) than the M1s (seven to eight). The basal epithelial cells of the AEC had developed a somewhat columnar morphology, associated with successful regeneration (Fig. 4B',F' insets). Additionally, in both WT and M1 limb sections, a large number of

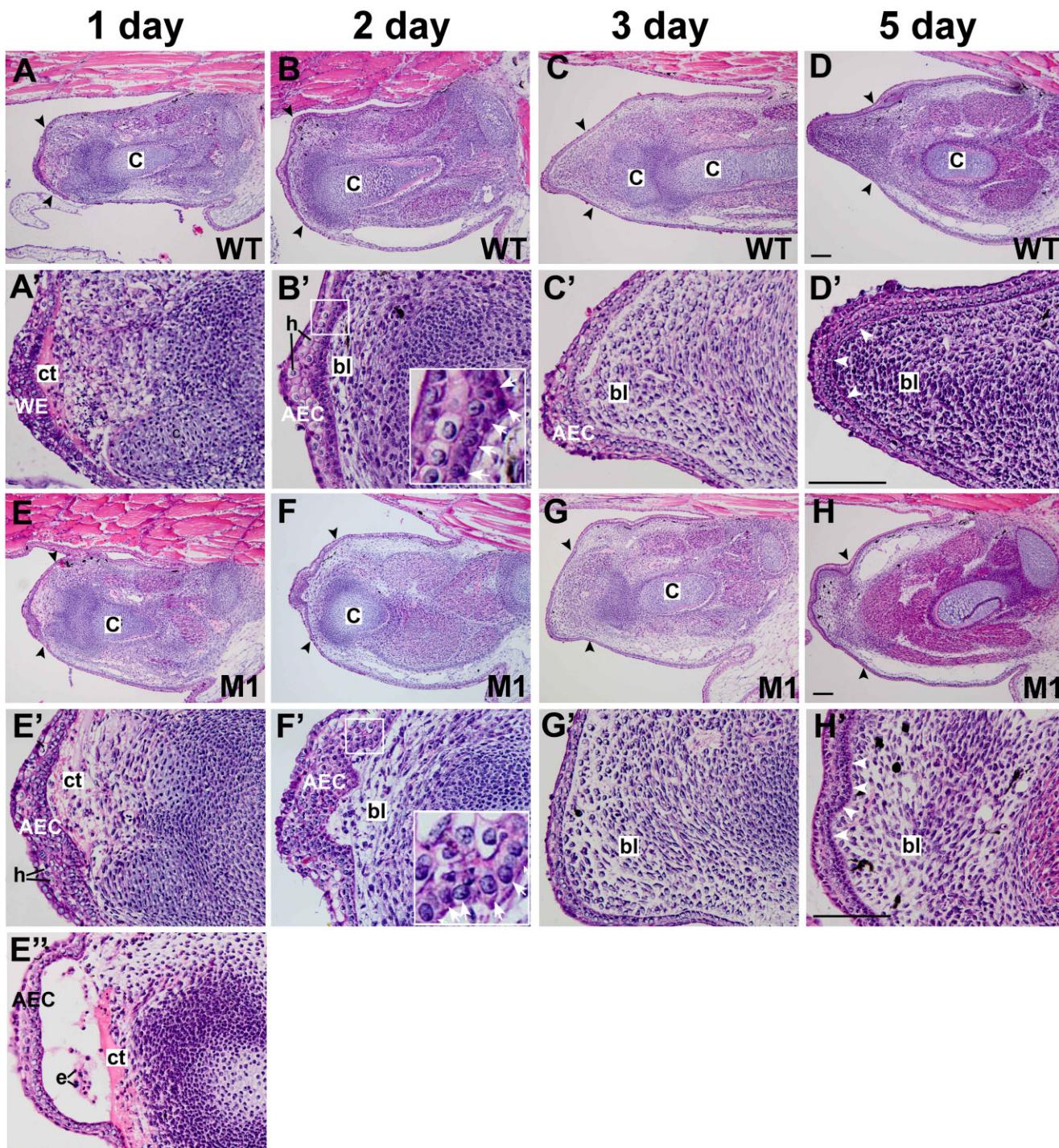


Fig. 4. Histological analysis of M1 and wild-type (WT) partially amputated hindlimbs. **A–D:** Sections of WT limbs showing the whole limb after 1–5 days of regeneration following stage 54 knee level amputation. **A'–D':** Higher magnification of the distal tip in sections A–D, respectively. **E–H:** Sections of M1 limbs showing the whole limb after 1–5 days of regeneration. **E'–H':** Higher magnification of the distal tip of sections E–H, respectively. **E''**: Shows a 1 day postamputation M1 limb distal tip in which the apical epithelial cap (AEC) has detached from the underlying mesenchyme creating a gap containing connective tissue and presumptive eosinophils. **c**, cartilage; **ct**, connective tissue; **bl**, blastema; **e**, eosinophils; **WE**, wound epithelium; **h**, hypertrophic cells. Black arrowheads in A–H indicate the approximate level of amputation. White arrowheads in D', H' indicate the eosin stained basement membrane. Insets in B', F' correspond to white box and arrows mark columnar-like cells. Tadpoles are oriented with dorsal side uppermost, and anterior to the right. Limbs are, therefore, distal to the left, posterior uppermost. Scale bars = 100 μm in D,H (applies to A–H), 100 μm in D',H' (applies to A'–H',E'').

hypertrophic epithelial cells were evident in the central region of the AEC (Fig. 4B',F',E'; Table 2). A small num-

ber of these hypertrophic cells were also evident in 24-hr regenerate sections. We have previously observed these cells

in the AECs of regenerating WT stage 52 limbs, beginning at 3 days after amputation and suggest that these are dy-

TABLE 2. Histological Assessment of Regenerative Quality in Early M1 and WT Limb Regenerates^a

Regeneration feature	Average score			
	+24 hr		+48 hr	
	WT	M1	WT	M1
Blastema/mesenchyme accumulation*	+	+	+	++
Conical blastema outgrowth*	+	++	+	++
AEC > 4 cell layers*	-	+	+	++
Columnar basal epithelial cells*	-	-	+	+
Connective tissue	+	++	-	+
Hypertrophic epithelial cells	+	+	++	+++
Eosinophil invasion	-	+	-	-

^aHistological features in sections of stage 54 right hindlimbs 24 or 48 hr after amputation at knee level were scored for absence (-), or presence to strong presence (+ to +++) for each regeneration feature. Features indicative of good regeneration are marked with an *, those indicative of poor regeneration are not (Wolfe et al., 2000).

ing cells within the AEC, indicative of AEC regression (Pearl et al., 2008). Indeed, by 3 days after amputation, the AEC of stage 54 limbs had regressed to an average thickness of only 2–3 cells (Fig. 4C',G') indicating a declining regenerative potential. Additionally, a basement membrane, visible as a thin eosin stained line running between the basal AEC cells and underlying mesenchyme, was visible in most sections by day 5 (Fig. 4D',H'). Despite this, a cone-shaped blastema was often apparent in both WT and M1 samples at 3 and 5 days after amputation, suggesting at least a partial regenerative environment may be established before the decline of the AEC (Fig. 4C–D',G–H'). However, the relatively low number of regenerates (partial or complete) suggests that the formation of a cone-shaped blastema by 3–5 days after amputation may not be enough to ensure eventual regenerative success, especially of the autopod, as previously suggested by Wolfe and colleagues (Wolfe et al., 2000).

Induction of eveMsx1 Following Limb Amputation Results in an Early Increase in Proliferation in the Blastema That Is Not Maintained Later

Regenerative success is dependent on the development of a proliferative

blastema capable of driving the regeneration of all the required tissues. Failure to achieve this critical mass generates a decline in the regenerative success of the limb, resulting in either poor regeneration (observed as reduced numbers of toes) or in the complete absence of regeneration (reviewed in Yokoyama, 2008). We have previously shown that the proliferation of the blastemal cells is dependent on correct signaling through the Bmp signaling pathway (Beck et al., 2006). We therefore used M1 tadpoles to assess the capacity of eveMsx1 to stimulate increased proliferation of both the blastema and AEC. M1 and WT sibling tadpoles were heat shocked 3 hr before and every 24 hr after amputation until they were fixed for analysis. Tadpoles were injected with bromodeoxyuridine (BrdU) either 28 or 52 hr after amputation of the right hindlimb, and the tissue fixed for analysis of dividing cells 20 hr later (i.e., 48 or 72 hr after amputation). Immunohistochemistry of sectioned limbs was used to label dividing cells. Labeled cells in the distal blastema and epithelium regions of representative M1 and WT sections were counted, and average counts were compared between M1 and WT siblings. A significant increase in proliferation in the blastemal cell population was observed in the M1 animals labeled between 28 and 48 hr after amputation (Fig. 5A; $P < 0.05$, ran-

domization test). In contrast, the later labeling times showed that by 52–72 hr after amputation, the level of proliferation had reduced in M1 blastemas, and was no different to that seen in WT samples (Fig. 5A). The increased proliferation in M1 blastemas was not due to a global increase in mitosis, as shown by the similar level of counts seen in proximal limb mesenchyme (Fig. 5B). Consistent with previous results, the number of dividing cells in the AEC was seen to be much lower than in the blastema (Beck et al., 2006). Counts of dividing cells in the AEC showed no significant difference in WT or M1 limb sections at either time point (Fig. 5C).

Induced Expression of eveMsx1 Is Not Sufficient to Compensate for the Loss of Bmp Signaling During Limb Regeneration

Regenerative ability of stage 52 *Xenopus* hindlimbs is inhibited by means of the induction of the Bmp antagonist noggin during the first 48 hr of regeneration (Beck et al., 2006). This failure in regenerative ability in the N1 line coincides with a decline in the expression of msx1, suggesting that the absence of Msx1 protein may be the cause of regenerative failure in these tadpoles. To evaluate this possibility, an N1 × M1 cross was performed and the resulting tadpoles were evaluated for the ability to regenerate the right hindlimb at a regeneration permissive stage. Stage 52 tadpoles carrying one or both transgenes (assessed by the presence of GFP in the lens) were heat shocked 3 hr before limb amputation and 24, 48, and 72 hr afterward. Tadpoles were allowed to recover to stage 57 when they were killed and their tails used for genotyping. The number of recovered toes on the amputated limb and contralateral limb was recorded (Table 3). Regenerative ability of the N1 line was generally poor, with only 44% of tadpoles regenerating, and most of those only having a tibiafibula and a single toe ($n = 16$; Fig. 6; Table 3), as previously described (Beck et al., 2006). In contrast, 100% of M1 tadpoles regenerated at least one toe ($n = 5$, Fig. 6). Furthermore, the average number of toes regener-

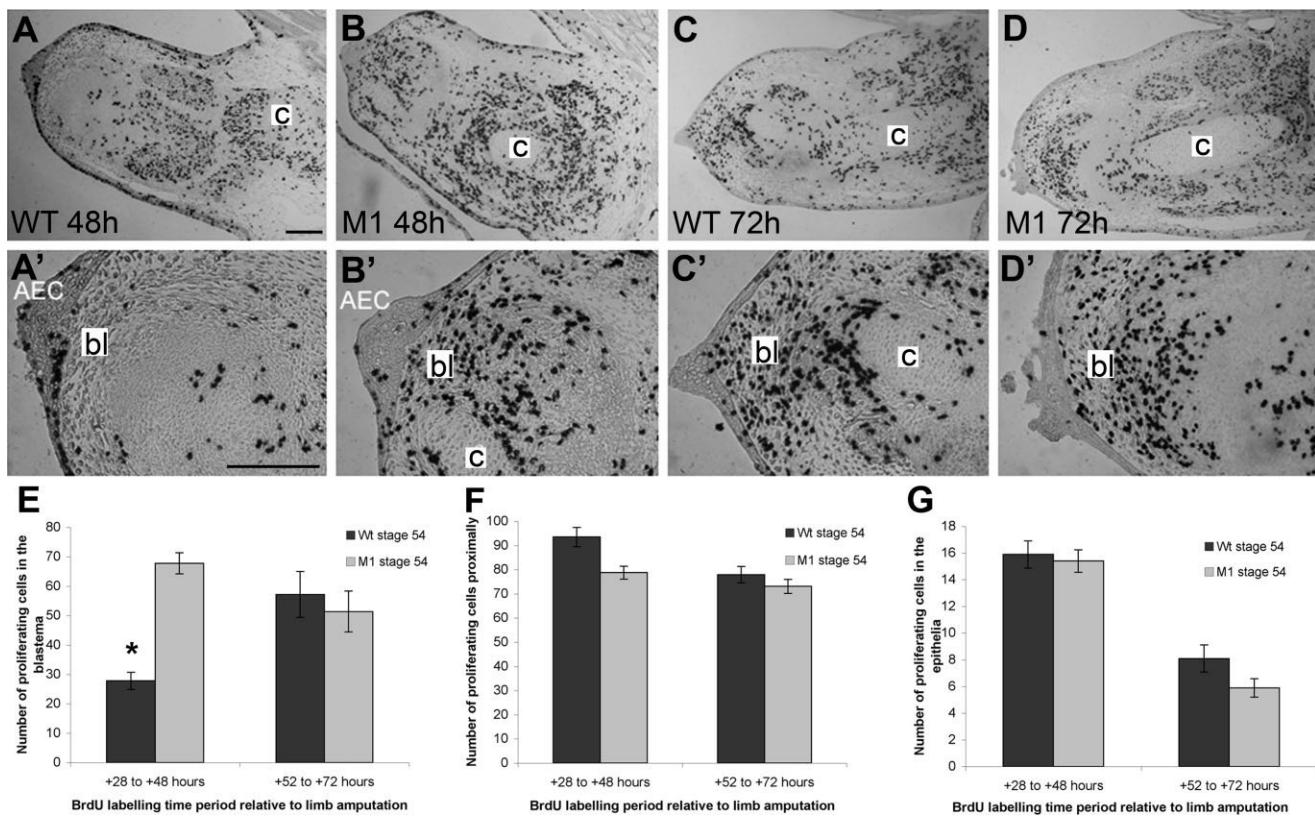


Fig. 5. Cell proliferation analysis in M1 and wild-type (WT) hindlimbs after amputation. Tadpoles were injected with 5'-bromo-2'-deoxyuridine (BrdU) either 28 or 52 hr after amputation of the right hindlimb at stage 54, and fixed 20 hr later. Cells that have incorporated BrdU during cell divisions have darkly stained nuclei. **A–D:** Representative sections showing the pattern of BrdU accumulation along the whole limb. **A'–D':** Higher magnification of the distal tip of the limb showing the apical epithelial cap (AEC) and blastema (bl). **E–G:** Bar graphs showing the number of BrdU-labeled cells in a 200 × 200 μm area for mesenchyme or a 200 μm length along the distal tip. Bars show the average of 3 counts for each of 3 representative limbs (except $n = 2$ limbs for M1 72 h) and error bars are standard error. **E:** Proliferating cells in the blastema/distal limb; an asterisk indicates a significant difference ($P = 0.047$) between the number of proliferating cells in WT and M1 limbs at the earlier time point. **F:** Proliferating cells in the proximal limb. **G:** Proliferating cells in the epithelia/AEC. Scale bar = 200 μm in A (applies to A–D), 200 μm in A' (applies to A'–D').

TABLE 3. Regenerative Capability of N1, M1, and N1/M1 Transgenic Animals Following Hindlimb Amputation at Knee Level at Stage 52^a

Genotype	Number of toes regenerated						n	score/n	% reg
	0	1	2	3	4	5			
M1	0	1	1	0	1	2	5	3.4	100%
N1	9	6	0	0	1	0	16	0.6	44%
M1/N1	5	5	1	0	0	0	11	0.6	55%
WT, no HS	0	2	0	0	4	4	10	3.8	100%

^aLimbs were scored for the number of toes regenerated by stage 57 in either M1, N1, N1/M1 following heat shocks (HS) at -3, +24, +48, and +72 hr relative to amputation or wild-type (WT) tadpoles. Each regenerated toe results in a score of 1; n = number of tadpoles; score/n is, therefore, the average number of toes per hindlimb. The percentage of animals showing any level of regeneration (in the form of toes) is recorded as % reg.

ated per limb (3.4) was significantly higher in M1 individuals (unpaired *t*-test; $P < 0.05$). Comparison with previous results on WT limbs (Beck et al., 2006; 100% of stage 52 WT limbs undergo regeneration), as well as those

shown here for non-heat-shocked tadpoles (Table 3; Fig. 6) suggests that M1 tadpoles regenerate appropriately for their stage. There is no significant difference in the number of toes regenerated by either data set compared

with M1s (unpaired *t*-test; $P > 0.05$). When both transgenes were present, however, the N1 phenotype dominated, and only 55% of limbs regenerated, with the majority having a single toe (Fig. 6; Table 3). There was no significant difference between the average number of toes regenerated by N1 or N1/M1 animals (unpaired *t*-test; $P > 0.05$).

Contralateral, unoperated left limbs were also examined, to determine any effects of the transgenes on normal limb development from stage 52. The number of digits was unaffected in M1 contralateral limbs (five toes in all five individuals). Digit number was significantly reduced when the N1 transgene was present, with an average of 3.8 toes in N1 individuals (unpaired *t*-test; $P < 0.05$ compared with M1), and 4.0 in N1/M1 individuals (unpaired *t*-test; $P < 0.05$ compared with M1). The presence of the M1 trans-

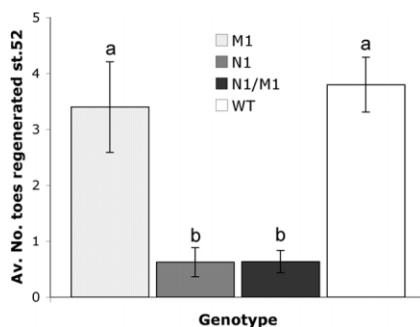


Fig. 6. Overexpression of Msx1 cannot rescue regeneration in limbs with dysfunctional Bmp signaling. Bar graph showing the average number of toes regenerated after knee level amputation of the right side hindlimb of stage 52 transgenic animals. Tadpoles resulting from a cross between an N1 female and M1 male were heat shocked to induce expression of the transgene and allowed to grow to stage 57 before counting the number of regenerated digits. The animals were then genotyped, and the average number of toes regenerated for each genotype is shown ($n = 17$ for M1, 10 for N1 and 7 for N1/M1). Similarly operated, non-heat-shocked animals are also shown for comparison (wild-type, WT). Error bars represent standard error and different lower case letters above columns indicate a statistically significant difference between two data sets. There was a significant difference between regenerative success of M1 and N1 (unpaired *t*-test; $P = 0.0003$) and M1 and N1/M1 double transgenics (unpaired *t*-test; $P = 0.0005$). WT animals did not differ significantly from M1 (unpaired *t*-test; $P = 0.67$).

gene in the double transgenic animals did not significantly alter the number of toes formed by the unoperated hindlimbs (unpaired *t*-test; $P > 0.05$, N1 compared with N1/M1). These results suggest that *noggin* expression at stage 52 impairs development of the autopod and that Msx1 is not able to rescue this phenotype.

DISCUSSION

Although larval *Xenopus* are capable of regenerating a complete hindlimb, this ability decreases in a proximal to distal manner as metamorphosis progresses (Dent, 1962; Overton, 1963; Wolfe et al., 2000). Consequently, late stage 54 tadpoles are relatively inept at this process, and stage 57 tadpoles are only capable of simple wound healing and stump formation. Here, we show that activation of a transgene resulting in ectopic expression of a hyperactive version of the transcription factor Msx1 can somewhat improve the outcome of late stage, poorly regenerating hindlimbs.

Although the difference was only just significant, three quarters of M1 limbs regenerated at least one toe, whereas only one quarter of WT achieved this. However, the timing of *evemsx1* expression may be critical, because our attempts to further boost regenerative success by extending the period of time that the transgene is activated after amputation were unsuccessful, and in fact resulted in regenerative outcomes not significantly better than those of WT siblings (D.B., unpublished observations). Perfect regeneration of five digits was never obtained, and defects were also apparent in the zeugopod. However, we have not assessed the effect of earlier *evemsx1* expression, beginning at stage 52, to see if cells can be maintained in a regeneration competent state.

Msx1 Is Unable to Rescue Regeneration in Refractory Stage Hindlimbs

Previously, it has been shown that founder transgenic tadpoles expressing the *hsp70-evemsx1-γ-crystallin-GFP* transgene were able to regenerate tails following amputation of the distal portion during the refractory stage, when their WT siblings fail to do so (Beck et al., 2003). The current experiments were done using a stable transgenic line, whereas the tail experiments used founders, each of which harbor a unique insertion of the transgene. Many founder tadpoles do not survive to adulthood, suggesting that perhaps the survivors' transgene insertion sites may naturally result in lower expression. This seems unlikely because we have shown that the transgene is expressed at very high levels in M1 tadpoles and limb blastemas following heat shock, so availability of Msx1 is unlikely to be the issue. Of interest, a study of axolotl tail regeneration showed no effect of Msx1 morpholino knockdown or over-expression on muscle cell dedifferentiation *in vivo*, suggesting that in axolotls, Msx1 is not, after all, required for this aspect of the regenerative process (Schnapp and Tanaka, 2005). Knockdown of Msx1 has yet to be tried in *Xenopus*, and so its absolute requirement in regeneration is still in question.

Despite these conflicting results,

there is still much evidence for the involvement of Msx in regeneration, as discussed earlier. Because tail regeneration is hard to score qualitatively, we decided to focus on limb regeneration where the number and quality of regenerated skeletal structures can give a clearer picture of regenerative success. Using this system, we show that Msx1 can enhance regeneration, but is not sufficient to completely rescue regeneration of the limb in older tadpoles when the natural ability to regenerate is declining, at least in this transgenic line.

Our data indicate that, at least in the M1 line, Msx1 is insufficient to rescue regeneration of limbs, but there are several possible limitations to this work. First, the transgene system we use is inducible but results in expression throughout the tadpole, hence Msx1 would be active in the AEC, blastema, and proximal limb. Normally during regeneration *Msx1* is re-expressed only in the distal mesenchyme blastema (Beck et al., 2006), and this restricted distribution may be necessary for complete regeneration. Second, we are using an active, strongly repressing fusion protein version of Msx1, eveMsx1, which may repress genes that are not normally targets of this transcription factor. Third, Msx1 itself may not be the limiting factor in regeneration and may require a cofactor, which is not present in older limbs. In support of this, we have shown here using qPCR that endogenous *msx1* levels are only slightly lower in nonregenerating limbs compared with regeneration competent limbs. Fourth, in mammals, the *msx1* gene produces a natural antisense transcript, which is expressed in a tissue specific manner and thought to be important in regulating the level of Msx1 during early development (Blin-Wakkach et al., 2001). While this antisense transcript appears to play an important regulatory role during development of the craniofacial regions and teeth of mammals, its role in limb development is less clear (Coudert et al., 2005). In early mouse limb bud stages, the antisense transcript is expressed weakly in the proximal tissues, in a pattern complementary to that of the sense transcript. In later stages, it is found just proximal to, and abutting, the distal region occu-

plied by the sense transcript (Coudert et al., 2005). This transcript has been tentatively identified in other vertebrates (Blin-Wakkach et al., 2001), although it has not been reported in *Xenopus* or other amphibians. Overexpression of the sense transcript may disrupt the normal sense/antisense balance of transcripts and hence patterning of the regenerate resulting in poorer regeneration. This seems unlikely, however, given that the nonoperated limbs develop normally after induction of the transgene at either stage 52 or 54.

While there is much evidence that *Msx1* expression correlates with regenerative success in several vertebrate species, a functional role for this gene has only been demonstrated in cell culture and neonatal mice. In urodeles, *Msx1* appears to cause the fragmentation of myotubes into mononucleate, proliferating cells (Kumar et al., 2004). However, this is not the case in vivo (Schnapp and Tanaka, 2005). This dedifferentiation does not occur in *Xenopus* tail regeneration (Gargioli and Slack, 2004) and is, therefore, unlikely to occur during the regeneration of the hindlimbs. In *msx1*–/– mice, limbs develop normally but digit tip regeneration is impaired, suggesting the gene is needed in regenerating limbs but not developing ones (Han et al., 2003). We have shown here that overexpression of *evemsx1* in *Xenopus* does not affect limb development. *Msx1* may in fact be functionally redundant in *Xenopus* limb development, most likely with its orthologue *msx2*, which has overlapping expression. This is indeed the case in mice, because double knockout *msx1*–/–*msx2*–/– mice have severe limb defects (Lallemand et al., 2005) and mice lacking either gene alone do not (Satokata and Maas, 1994; Satokata et al., 2000).

Msx1 Induces an Early Enhancement of the Blastema and AEC but This Is Not Maintained Later

Ectopic expression of *msx1* enhances the cell proliferation rate of the blastema within the first 24 hr of amputation, which coincided with an improved morphology of both the blastema and AEC. However, it ap-

pears that during anuran hindlimb regeneration, *Msx1* alone is insufficient to drive complete regeneration, as observed in the samples fixed for histology after 3 days after amputation, in which no difference could be seen between WT and M1 tadpole limb stumps.

Msx1 is expressed in the distal mesenchyme during *Xenopus* limb regeneration but is excluded from the epithelia including the AEC (Beck et al., 2006). Similarly, *msx1* expression is restricted to the distal limb bud mesenchyme during limb development and is excluded from the analogous epithelial signaling center, the AER. Moreover, it has been shown that maintenance of *msx1* expression in the limb bud mesenchyme depends on the presence of an AER and reciprocal signaling between the AER and mesenchyme appears to be mediated through transduction of the Bmp signal by means of *msx1* (Pizette et al., 2001). The AEC is thought to be functionally similar to the AER, suggesting that *Msx1* might also be involved in AEC development. This was evident to some extent in the M1 stage 54 hindlimbs after amputation, especially in the first 2 days, when the M1 wound epithelium was notably thicker and more similar to an AEC than in corresponding WT animals. The enhanced AEC, however, was short lived, declining by three days post amputation as evidenced by the appearance of a basement membrane between the AEC and underlying blastemal cells. This may be due to increased apoptosis (as evidenced by the presence of large numbers of hypertrophic cells in the AEC), combined with a demonstrated drop off in proliferation rates in the distal limb mesenchyme between 52 and 72 hr. Supporting this, Bmps are known to regulate the destruction of the interdigital regions during limb development in vertebrates, sculpting the autopod (Ganan et al., 1998). *Msx1* could be modulating Bmp expression, driving cells in the central AEC into hypertrophic states. Transgenic mice expressing *noggin* under the *keratin 14* promoter, however, display apoptosis defects such as syndactyly without any apparent change in expression of either *msx1* or 2, suggesting that *msx* genes are not involved in regulating

apoptosis of the interdigital regions during limb development in mammals (Guha et al., 2002). It is also likely that the AEC requires other signals, independent of the Bmp/*Msx* pathway, which are deficient in both WT and M1 stage 54 limbs and, therefore, it regresses rapidly after initial formation.

Analysis of the proliferative status of the amputated transgenic limbs between 28 and 48 hr showed a clear and significant increase in cell division in the distal mesenchyme of transgenic M1 tadpoles compared with WT siblings. Curiously, this is not maintained by 52–72 hr, suggesting that *msx1* expression can only drive blastema proliferation in the very early stages of regeneration. The reduction in distal mesenchyme cell division correlates with our observations of the AEC, which declines at approximately the same stage suggesting that a breakdown in reciprocal signaling between the mesenchyme and overlying AEC has occurred. Despite this, the M1 tadpoles regenerated better than WT animals, with a significantly larger number of toes being reformed. This finding suggests that the initially higher rate of proliferation results in a “better” blastema, capable of developing into distal limb elements. Alternatively, because the zeugopod was often poorly formed in these regenerating limbs, the blastema might be biased toward the production of distal limb elements (autopod) rather than the more proximal tibia–fibula.

The AEC itself appeared thicker in M1 limbs 24 hr after amputation but this was not reflected in the number of dividing cells in this tissue between 28 and 48 hr, which was not significantly different from that of WT AECs. This finding suggests that an earlier proliferative burst in M1 tadpoles may cause the transient enhancement of the AEC. Alternatively, the thickened AEC may result from aberrant migration of stump epithelia, as has been shown following amputation of the fin in the zebrafish *fgf20* mutant (Whitehead et al., 2005). However, aberrant migration would not be expected to cause the observed increase in cell proliferation in the underlying mesenchyme. Of interest, the effects of *Msx1* appear to be localized to the distal region of the amputated limb, as prolif-

eration in the proximal stump region (at both time points) remained similar to WT controls.

***Msx1* Overexpression Generates Poor as Well as Good Indicators of Regeneration and Cannot Substitute for Active Bmp Signaling**

In approximately half the M1 limbs sectioned 24 hr after amputation, large amounts of connective tissue were evident, effectively severing the connection between the wound epithelium and the underlying mesenchyme. This tissue appeared to be invaded by cells resembling eosinophils, a phagocytic blood cell, presumably originating from severed blood vessels in the vicinity of the stump. The eosinophils appear to be degrading the connective tissue, because both are less apparent in later stage M1 blastemas. Our results suggest that *msx1* overexpression induces the production of inappropriate amounts connective tissue from dermal fibroblasts, which is then degraded by the eosinophils as previously described in mammals (Hibbs et al., 1982). Although it is not possible to determine whether these limbs would have regenerated successfully or not, given the separation of the wound epithelium and mesenchyme at a critical time for reciprocal signaling, it is tempting to speculate that M1 tadpoles that failed to regenerate any toes (27%; n = 11) did so because of this early connective tissue phenotype. Whatever the reason, it is clear from our experiments that *Msx1* overexpression is not enough to compensate for lack of Bmp signaling in limb regeneration. Crossing N1 (transgenic animals with inducible noggin, a Bmp inhibitor) and M1 frogs led to the production of M1, N1, and N1/M1 offspring. N1 tadpoles do not regenerate limbs even at normally permissive stages, and coexpression of *msx1* was not able to alleviate this block to regeneration, suggesting that other Bmp targets are also critical for the regenerative process in stage 52 limb buds. Because tadpoles expressing a heat inducible constitutive receptor Bmpr1A were able to regenerate their tails effectively during the refractory

period (Beck et al., 2003), it would be interesting to see if an activated Bmp receptor could enhance regeneration, because this would be expected to activate all the normal targets of Bmp signaling.

EXPERIMENTAL PROCEDURES

Culture of *Xenopus laevis* Tadpoles

Xenopus embryos were generated as described previously (Godsave and Slack, 1988) and cultured in 0.1 × MMR without antibiotics until stage 48. At stage 48, they were transferred to 10-liter tanks containing 0.1 × MMR and fed a small amount of slurry made from spirulina powder and salmon starter food. Each day, 10% of the water was exchanged for filtered tap water. After approximately 2 weeks, they were transferred into 4-liter tanks in a Marine Biotech XR1 aquarium at low density (25 tadpoles per liter) with automatic water recirculation, and grown to limb stages. They were staged according to Nieuwkoop and Faber (1967).

All animal experiments were subject to New Zealand's animal welfare standards for vertebrates and were reviewed by the University of Otago Animal Ethics Committee. The Animal Ethics Committee approved all experiments under protocols AEC57/03 and AEC78/06.

In Situ Hybridization

Whole-mount *in situ* hybridization of embryos and tadpole limbs was performed as previously described in (Harland, 1991) with modifications as in (Pownall et al., 1996). An *msx1* probe was generated by digestion of *Bluescript msx1* plasmid (Suzuki et al., 1997) with *Eco*RI and transcription with T3 polymerase.

Transgenic Lines

The N1 stable line of transgenic *Xenopus laevis* has been previously described (Beck et al., 2006; Pearl et al., 2008). The M1 stable line of transgenic *Xenopus* was developed from outcrossing a male founder frog containing a single insertion of the *hsp70-eveMSX1-γ-crystallin-GFP* transgene,

described previously (Beck et al., 2003; Fig. 1A). Briefly, the animals contain a transgene comprised of two linked parts, the first containing *X. laevis noggin1* (N1) or *eveMSX1* (M1) coding sequence under the control of the *hsp70* promoter, and the second the GFP coding sequence under the control of the lens specific promoter *γ-crystallin*. In the case of the M1 line, the repressor domain of Msx1 was replaced with that of the *Drosophila melanogaster* even-skipped (eve) creating a hyperactive form of Msx1 (Yamamoto et al., 2000). Both lines are derived from a single insertion founder made by sperm nuclear injection using the method of Kroll and Amaya (Kroll and Amaya, 1996) modified as in (Beck et al., 2003).

Double Transgenic Animals and Genotyping

A cross between N1 and M1 individuals was performed to generate *Xenopus* containing both inducible genes at a 1:1:1:1 ratio with WT, N1, and M1 tadpoles. As expected, 75% of the offspring expressed GFP in the lens of the eye, indicating the presence of one or more transgene. Tail samples (posterior 2/3) were taken from transgenic tadpoles for genomic DNA extraction. DNA was extracted by incubating tails in 300–500 μl 5% suspension of Chelex 100 chelating resin (Sigma) and 2 μl of 25 mg/ml Proteinase K (Sigma) at 65°C for 4 hr to overnight (Walsh et al., 1991). Samples were then briefly heat inactivated at 95°C and the resin removed by centrifugation (5 min 14K rpm). One microliter of the resulting DNA was used for genotyping PCR with the following oligonucleotide primer pairs for the N1 transgene: T3 promoter primer (AATTAACCTCACTAAAGGGGA) and *noggin* R2 primer (TGGTTATC-CACGCACACTTC), or for the M1 transgene SP6 promoter primer (CAT-ACGATTAGGTGACACTATAG) and *msx1* primer (CTGCCCTCTCTGC-TATGGAC). These primer pairs amplify only the transgene and not the endogenous gene, each giving a single band. For each primer set, 35 PCR cycles were run with annealing temperature at 50°C.

Hindlimb Transections and Transgene Activation

All hindlimb amputations were at knee level on the right side limb and were performed using iridectomy scissors while the tadpoles were anesthetized in 1/4,000 (w/v) tricaine (MS222, Sigma). Transgene activation (heat shock) was initiated by immersion of tadpoles in water at 34°C for 30 min followed by return to normal aquarium temperature (25°C).

Semiquantitative qPCR

Total RNA was extracted from pairs of blastemas or pseudoblastemas taken from bilaterally operated tadpoles 3 days after amputation of the hindlimbs at knee level. Samples were placed in *RNAlater* (Sigma) immediately after dissection and RNA was isolated and DNase treated using the Ambion RNAqueous Micro kit (Applied Biosystems). A total of 100 ng of total RNA was used to make cDNA using the Invitrogen VILO SuperScript kit. cDNA was diluted 1 in 5 and 4 µl was used in 20 µl qPCR reactions with SYBR GreenER (Invitrogen) and 200 nM of each primer using a Stratagene Mx3000p PCR machine, with annealing temperature set to 58°C. Ct refers to the number of cycles required to reach a critical threshold. Primers used to detect only the endogenous *msx1* transcript were AGAG-CACGATCAAGGTGTGG (forward) and TCGGAGTAGACGGCTGCGTA (reverse). Primers used to detect only the transgenic *evensx1* transcript were ACGACAACGTGATTGCGGA (forward) and GATACTGCTTCTGCCT-GAAC (reverse). Actin control primers were TTTCCTTGATGGAAATCCTG (forward) and AACAGTATTGGCATAGAGGTCC (reverse).

Histology

Tadpoles were fixed overnight in ethanol/glycine fixative (70% ethanol, 15 mM glycine pH 2.0) at -20°C and stored in 100% methanol until required. Fixed samples were dehydrated through ethanol and xylene and embedded in paraffin wax. Transverse 5-µm sections were cut using a Leica microtome and stained with hematoxylin and eosin.

BrdU Labeling

Stage 54 tadpoles were heat shocked 3 hr before hindlimb amputation and again 24 hr after amputation or at both 24 and 48 hr after amputation depending on the labeling period. Four hours after the final heat shock, tadpoles were anesthetized and injected intraperitoneally with 3 µl of 5'-bromo-2'-deoxyuridine (BrdU) from the Roche cell proliferation kit. After 20 hr of labeling, the tadpoles were fixed overnight in cold ethanol/glycine fixative, and then dehydrated through ethanol and xylene and embedded in paraffin wax. Transections (5 µm thick) were taken using a Leica microtome from 3 tadpoles in each experimental condition with the exception of the M1 tadpoles labeled from 52–72 hr after amputation where n = 2. The central most ribbon of limb sections (encompassing the AEC) was chosen for analysis and stained using the Roche cell proliferation kit, which stains S-phase cells a deep blue. Sections were counterstained with nuclear fast red. A randomization test was performed in Microsoft Excel due to the small sample set.

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