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FGF-10 Stimulates Limb Regeneration Ability in *Xenopus laevis*

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By reciprocal transplantation experiments with regenerative and nonregenerative *Xenopus* limbs, we recently demonstrated that the regenerative capacity of a *Xenopus* limb depends on mesenchymal tissue and we suggested that *fgf-10* is likely to be involved in this capacity (Yokoyama *et al.*, 2000, *Dev. Biol.* 219, 18–29). However, the data obtained in that study are not conclusive evidence that FGF-10 is responsible for the regenerative capacity. We therefore investigated the role of FGF-10 in regenerative capacity by directly introducing FGF-10 protein into nonregenerative *Xenopus* limb stumps. Exogenously applied FGF-10 successfully stimulated the regenerative capacity, resulting in the reinduction of all gene expressions (including *shh, msx-1,* and *fgf-10*) that we examined and the regeneration of well-patterned limb structures. We report here for the first time that a certain molecule activates the regenerative capacity of *Xenopus* limb, and this finding suggests that FGF-10 could be a key molecule in possible regeneration of nonregenerative limbs in higher vertebrates.

Key Words: **FGF (fibroblast growth factor)-10; limb; regeneration; *Xenopus laevis*; epidermal–mesenchymal interactions.**

INTRODUCTION

The basic research field of tissue regeneration is called regenerative biology, with major long-term goals of tissue and organ replacement therapies, which could be one of the most important clinical, medical, and biological fields in this century (Stocum, 1997). In most vertebrates, the capacity for regeneration is limited to a few tissues, such as liver, skin, bone, and skeletal muscle. Limb regeneration, including complete restoration of shape, pattern, and function of an organ, which is exclusively unique to amphibians, is a fascinating phenomenon in terms of whole organ regeneration. Among amphibians, anuran amphibians exhibit different degrees of capacity for limb regeneration at different stages of their life cycle, while urodele amphibians can regenerate their amputated limbs as adults. For example, *Xenopus* can completely regenetate developing hindlimb buds prior to the onset of metamorphosis, but the regenerative capacity declines gradually in a distal to proximal direction as metamorphosis proceeds (Dent, 1962; Muneoka *et al.*, 1986). Sessions and Bryant (1988) demonstrated that ontogenetic decline of regenerative capacity is due to intrinsic changes in the *Xenopus* limb bud itself. *Xenopus* limb regeneration therefore serves as an excellent model to investigate essential differences between regenerative limbs and nonregenerative ones. As yet, however, there has been no direct evidence that a certain molecule stimulates limb regenerative capacity, despite recent works suggesting several candidate molecules for regulation of limb regenerative ability in amphibians (Mullen *et al.*, 1996; Yokoyama *et al.*, 2000; Endo *et al.*, 2000).

Vertebrate limb buds are mainly composed of mesenchyme derived from the lateral plate mesoderm and epidermis derived from the ectoderm. It is well known that epidermal–mesenchymal interactions are necessary for limb regeneration (Polezhaev and Faworina, 1935; Goss, 1956; Stocum and Dearlove, 1972; Mesher, 1976) as well as for outgrowth of a developing limb bud (Saunders, 1948; Zwilling, 1956; Summerbell, 1974). With regard to a developing limb bud, recent studies have revealed that these interactions are mediated by FGF-10 in the mesenchyme and FGF-8 in the epidermis (see Martin, 1998, for review). Some FGF family members can substitute for the epidermal function (Niswander *et al.*, 1993; Fallon *et al.*, 1994), and, furthermore, amputation of a developing chick limb bud can be partially rescued by FGF-2 or -4 (Taylor *et al.*, 1994; Kostakopoulou *et al.*, 1996). However, even in these cases, FGFs reestablished distal structures including one or two unidentifiable digit-like structures only

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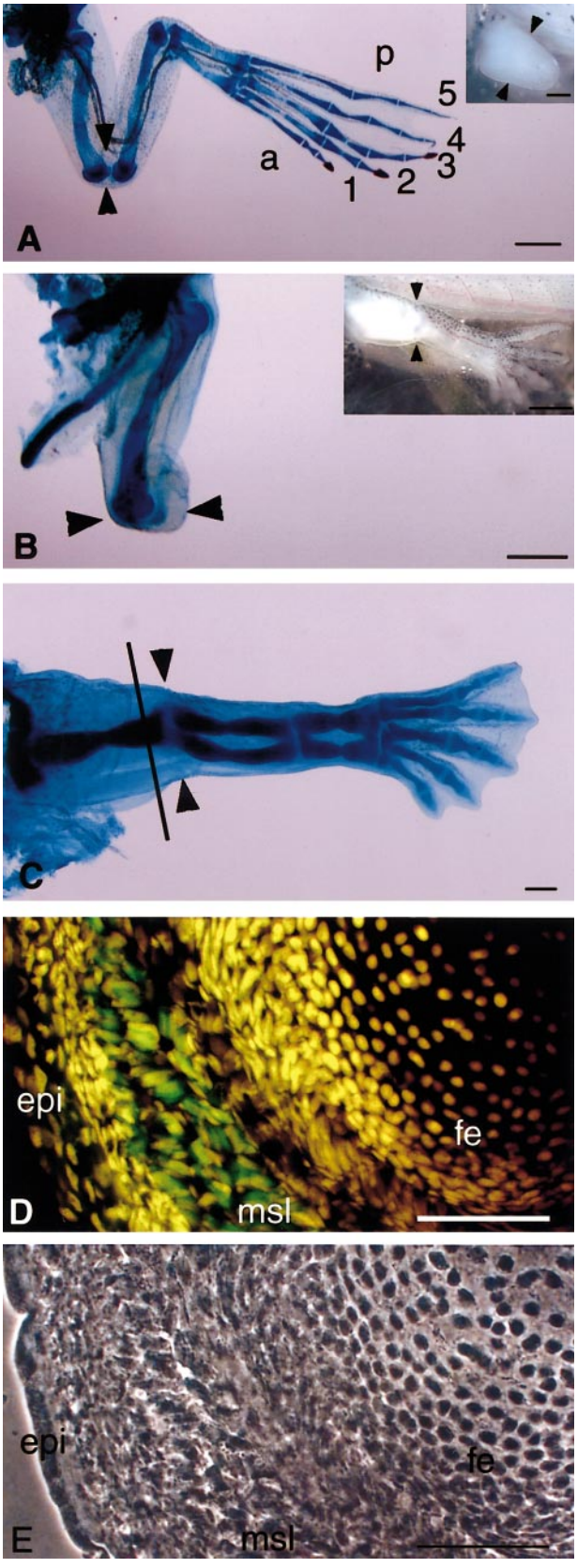

from undifferentiated-state cells in the early stages of developing limb buds. In *Xenopus* limb regeneration, we previously showed, by preparing recombinant limb buds composed of regenerative epidermis and nonregenerative mesenchyme or vice versa, that the mesenchyme, not the epidermis, controls regenerative capacity (Yokoyama *et al.*, 2000). Since *fgf-10* and *fgf-8* are synergistically reexpressed in regenerating blastemas, while neither *fgf-10* nor *fgf-8* is reexpressed after amputation of a nonregenerative limb, we proposed that FGF-10 may be a key mesenchymal molecule for controlling the regenerative capacity of vertebrate limbs (Yokoyama *et al.*, 2000). Based on this indirect evidence, we investigated the effect of ectopically applied FGF-10 on nonregenerative *Xenopus* limbs. Application of FGF-10-soaked beads induced reconstruction of gene expressions and produced well-patterned skeletal elements. These findings are the first direct evidence that FGF-10 stimulates vertebrate limb regeneration.

MATERIALS AND METHODS

Experimental Manipulation

Xenopus tadpoles were staged after Nieuwkoop and Faber (1956). They were allowed to develop until they reached stage 56. For FGF application to limb stumps, the tadpoles were anesthetized in 1:5000 ethyl-3-aminobenzoate (ALDRICH) dissolved in Holtfreter's solution. Hindlimbs were amputated at the knee level [according to the outside view (see inset in Fig. 1B) and a fate map by Tschumi (1957)] with an ophthalmological scalpel. Two Affi-gel blue beads (Bio-Rad) soaked in 0.5 mg/ml, 1 mg/ml, or 2 mg/ml of each FGF, FGF-10 (recombinant human; Genzyme/Techne), FGF-8 (recombinant mouse; R & D Systems), and FGF-2 (bovine; R & D Systems), were immediately implanted in the amputated limb. Grafted beads were covered with wound epidermis soon after implantation. After metamorphosis, the limbs were fixed overnight in 10% formalin in Tyrode's solution, stained with 0.1% Alcian blue in 70% ethanol with 1% HCl at 37°C overnight, dehydrated, and cleared in methyl salicylate.

FIG. 1. Regenerative capacity and differentiation of *Xenopus* limb buds. (A) A complete regenerate from a stage 52 limb bud. Upper inset shows a stage 52 limb bud. Note that only anterior digits (1, 2, 3) have claws. (B) A sample after amputation of stage 56 limbs. No regenerates were formed. Upper inset shows a stage 56 limb. (C) Cartilage staining shows that the cartilage pattern is almost completely formed at stage 56. The line indicates the plane of a section of the stage 56 limb bud shown in (D). (D) Muscle differentiation at the amputation level of a stage 56 limb bud. MF20 staining (green) shows that many well-differentiated muscle cells have already migrated to the amputation site. (E) Phase-contrast photograph of (D). a, anterior; p, posterior; epi, epidermis; msl, muscle cells; fe, femur (cartilage). All arrowheads indicate amputation level (knee level). Bars, 1 mm for (A), (B), and upper inset in (B); 250 μm for upper inset in (A) and (C); 50 μm for (D) and (E).

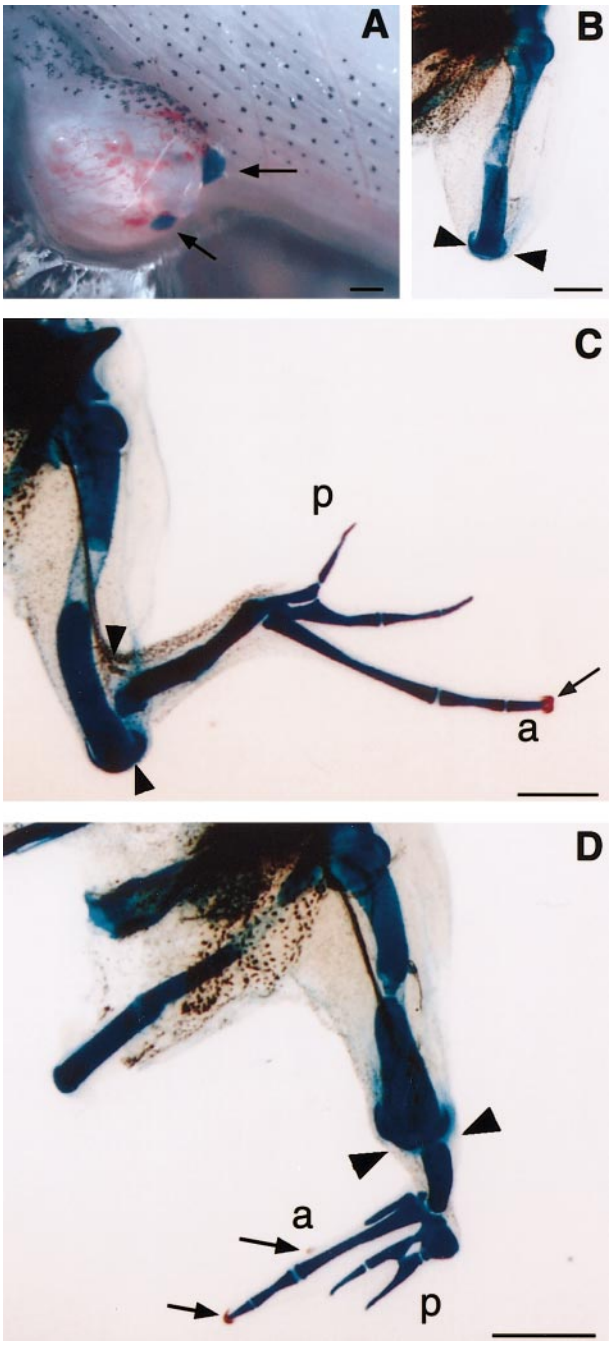

FIG. 2. Effect of FGF-10 application on *Xenopus* limb regeneration. (A) A beads-implanted stump of a stage 56 limb amputated at knee level. Two affi-gel blue beads (arrows) were implanted near anterior and posterior sides of the amputated plane at the distal edge of the femur. (B) A sample after amputation and PBS-soaked beads implantation. Nothing is regenerated. (C and D) Regenerates from FGF-10-applied stage 56 limbs. Note that only anterior digits have claws (arrows). a, anterior; p, posterior. All arrowheads indicate amputation level (knee level). Bars, 1 mm for (B–D); 250 μm for (A).

Immunohistochemistry and in Situ Hybridization

To examine the extent of muscle cell differentiation in stage 56 limb buds, immunohistochemical staining was carried out using MF20 [a monoclonal antibody against the myosin heavy chain, Development Studies Hybridoma Bank (Iowa); Bader *et al.,* 1982] that recognizes differentiated muscle cells also in amphibians (Neff *et al.,* 1989). Stage 56 limb buds were fixed in 4% paraformaldehyde/PBS at 4°C overnight, washed with PBS several times, and immersed in 10% and 20% sucrose/PBS overnight. Fixed limbs were embedded in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and sectioned at 10 μm using a cryostat. After treatment with 0.5% skim milk/PBS for blocking, they were incubated with 40 μg/ml MF20 at 4°C overnight. After three washes with PBS, sections were incubated with goat-anti-mouse IgGs conjugated with fluorescein isothiocyanate (Chemicon) and then observed under a fluorescence microscope (Olympus).

Preparation of a DIG-labeled RNA probe and whole-mount *in situ* hybridization in the *Xenopus* limb bud were performed as described previously (Endo *et al.,* 1997; Yokoyama *et al.,* 2000; Endo *et al.,* 2000). For making serial cryosections, regenerating limb buds were fixed in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde), embedded in OCT compound (Miles), and serially sectioned at 10 μm. RNA was detected by non-RI *in situ* hybridization on frozen sections using the procedures described by Yoshida *et al.* (1996).

RESULTS AND DISCUSSION

Regenerative Capacity and Cell Differentiation in Xenopus Limb Buds

Xenopus can completely regenerate early-stage hindlimb buds (stage 52) after amputation at the presumptive knee level (Fig. 1A; see also Yokoyama *et al.,* 2000), but they cannot regenerate late-stage limb buds amputated at the presumptive knee level (Fig. 1B, stage 56, a stage that is preceded by stages of gradual regenerative decline, and note that stage 56 limbs still have a little regenerative capacity at more distal level; Muneoka *et al.,* 1986; Wolfe *et al.,* 2000). To estimate the extent of cartilage differentiation of stage 56 limb buds, limb buds were stained with Alcian blue to visualize their cartilage patterns. This staining showed that the hindlimb cartilage pattern had already been almost completely formed at this stage and that the anterior–posterior (AP) polarity of the limb had also been established at this stage (Fig. 1C, compare with Fig. 1A). We also examined muscle cell differentiation in stage 56 limb buds by immunohistochemical staining with MF20, a monoclonal antibody against the myosin heavy chain. MF20 staining revealed that the stage 56 limb bud stump is composed of well-differentiated muscle cells (Fig. 1D), as well as chondrocytes shown in Fig. 1C. Based on these observations, we judged stage 56 limb buds amputated at the knee level to be in a nonregenerative condition in which limb tissues are well-differentiated, and such limb buds were used for the following experiments.

TABLE 1
Regenerative Capacity of Stage 56 FGF-Treated Limbs

Type of treatment	Total no. of limbs	Wound healing (without regeneration)	Pattern formed: Incomplete ← spike	1 digit	2 digits	3 digits	→ Complete: 4 digits	5 digits
PBS (control)	12	12	0	0	0	0	0	0
FGF-10 (0.5 mg/ml)	9	4	1	3	1	0	0	0
FGF-10 (1 mg/ml)	11	4	0	2	2	1	2	0
FGF-10 (2 mg/ml)	8	5	0	3	0	0	0	0
FGF-8 (1 mg/ml)	11	8	1	1	1	0	0	0
FGF-2 (1 mg/ml)	8	8	0	0	0	0	0	0

FGF-10 Provides Regenerative Capacity in Nonregenerative Xenopus Limb Buds

FGF-10-soaked affi-gel blue beads were implanted in non-regenerative limb bud (stage 56) stumps amputated at the knee level (Fig. 2A). PBS-soaked beads were grafted onto stage 56 limb bud stumps as controls. Since stage 56 limb buds with PBS beads did not regenerated any structures (12/12; Fig. 2B; Table 1), we concluded that the bead-implantation itself has no effect on nonregenerative limb buds. Treatment with FGF-10 just after amputation resulted in a significant enhancement of regeneration in stage 56 limb buds. In about two-thirds of all cases (7/11), stage 56 limb buds with 1 mg/ml FGF-10 regenerated distal structures, including digits with segmented cartilage (Figs. 2C and 2D; Table 1). In the best cases, three or four digits were regenerated (See Figs. 2C and 2D; note that only the anterior-sided digits have claws.). Since only three anterior digits in a normal *Xenopus* hindlimb have claws (see Fig. 1A), these regenerates appear to have an anterior–posterior (AP) polarity in their digits. This speculation was supported by data of *shh* reexpression (see below and Fig. 3B.). More proximal structures, such as the tarsus and tibia/fibula, were often incomplete or reduced in FGF-10-treated limbs. In urodele amphibians, expression analysis of *HoxA* cluster genes suggests that the first step in limb regeneration is formation of the most-distal identity and that the intercalation of the proximal-distal (PD) axis secondarily produces more proximal structures to fill the gap between the stump and the distal structure (Gardiner *et al.*, 1995). Deletion of proximal structures in FGF-10-treated limbs suggests that our method of FGF-10 application is not sufficient for the intercalation step of the PD axis. We speculate that distal structures induced by FGF-10 application are formed, not as a result of a prolonged developmental process that proceeds from proximal to distal, but by a regenerative response as in urodele limb regeneration.

We considered the possibility that a higher (or lower) concentration of FGF-10 would enhance its function, resulting in more complete proximal units. To examine the optimal concentration of FGF-10 to induce a regeneration response, we implanted beads soaked in solutions of lower (0.5 mg/ml) or higher (2 mg/ml) FGF-10 concentration into stage 56 limb buds. However, only one or two digits at best were regenerated in a very few cases (Table 1). Since these concentrations proved to be less effective, 1 mg/ml FGF-10 seems to be the optimal concentration for inducing a maximal regenerative response in stage 56 limb stumps.

Effect of FGF-10 on Gene Expression

The reduction in limb regenerative activity is reflected in the reductions of gene expressions. Several gene expressions that are thought to be regulatory for limb regeneration are reduced or diminished in nonregenerative *Xenopus* limbs (Endo *et al.*, 1997; Christen and Slack, 1997; Yokoyama *et al.*, 2000; Endo *et al.*, 2000; Matsuda *et al.*, 2000; see also Figs. 3G–3J). We examined the expressions of those molecules in order to determine whether FGF10 application can rescue those gene expressions that are destroyed in nonregenerative limbs. Previous works have shown that *fgf-8* is reexpressed in the distal epidermis of regenerating *Xenopus* limb buds but not reexpressed in nonregenerative limbs after amputation (Christen and Slack, 1997; Yokoyama *et al.*, 2000; see Fig. 3H). In our experiments, *fgf-8* expression was detectable after 3 days, in response to amputation and concomitant treatment with FGF-10 (Fig. 3A), while *fgf-8* was not expressed in limbs with PBS beads (data not shown). Since other genes that we examined (including *fgf-10*, *shh*, *msx-1*, and *hoxa-13*) were not detectable in the blastema after 3 days (not shown), the rapid response of *fgf-8* expression could be a relatively early step of the reaction to application of FGF-10, possibly loading the epidermal–mesenchymal interaction, which is essential for the establishment of regenerating blastemas. It is possible that the stimulation of this early step in limb regeneration will lead to successful regeneration of other non-regenerative limbs. Within 8 days after amputation and FGF-10 application, cone-shaped blastemas were formed (compare the shapes of blastemas in Figs. 3C–3F with those in Figs. 3G–3J.). *fgf-10* (Fig. 3C), as well as *fgf-8* (Fig. 3D), was reexpressed in the distal blastema, suggesting that FGF-10 application stimu-

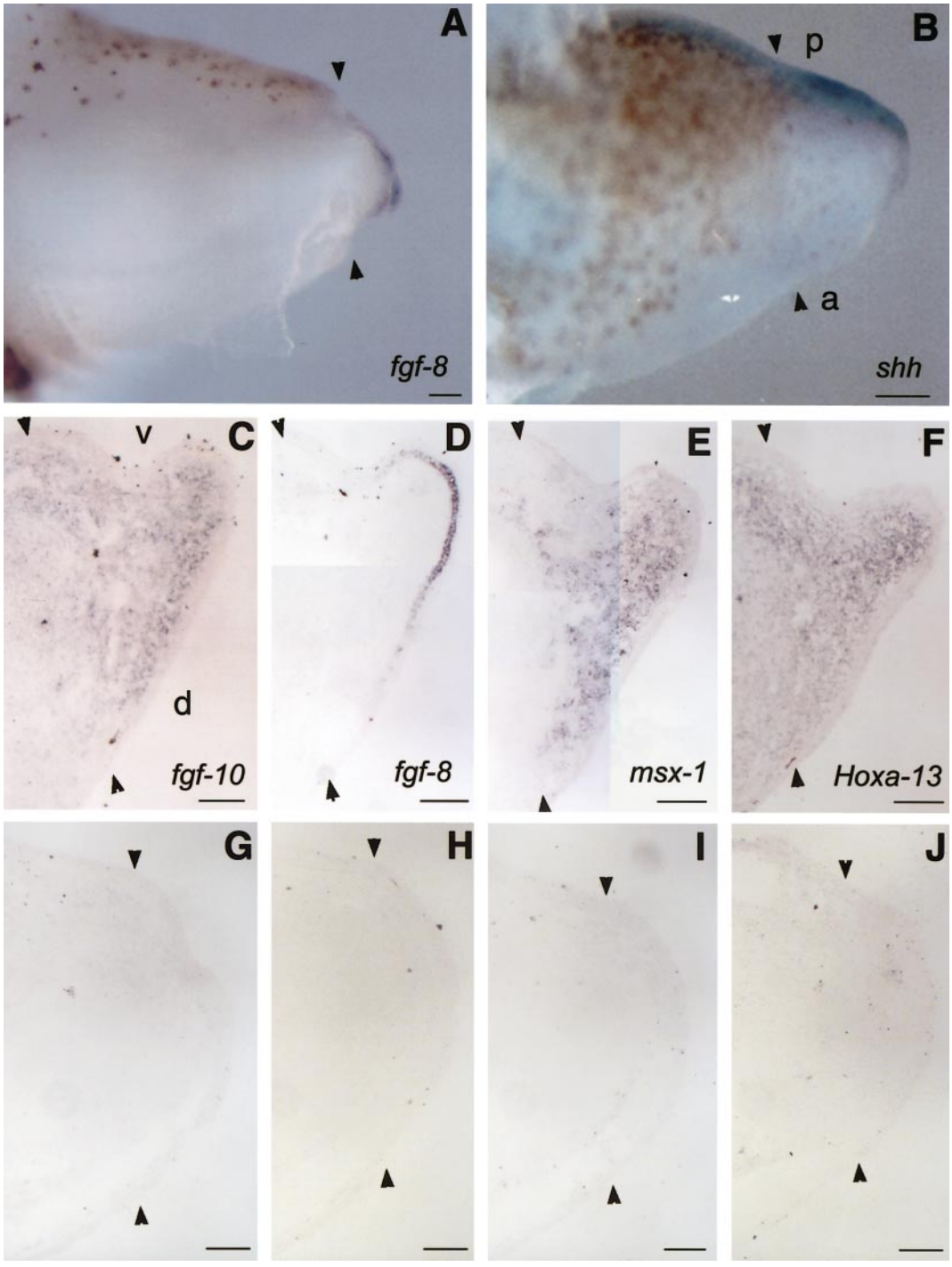

FIG. 3. Gene expressions in FGF-10-treated blastemas. (A) *fgf-8* expression in an FGF-10-applied stage 56 limb 3 days after amputation. (B) *shh* expression in an FGF-10-applied stage 56 limb 8 days after amputation. (C–F) Gene expressions in an FGF-10-applied stage 56 limb 8 days after amputation. *fgf-10* (C), *fgf-8* (D), *msx-1* (E), and *Hoxa-13* (F) expressions were examined by *in situ* hybridization in serial sections. (G–J) Gene expressions in a PBS-applied stage 56 limb 8 days after amputation. *fgf-10* (G), *fgf-8* (H), *msx-1* (I), and *Hoxa-13* (J) expressions were not induced by the PBS-soaked beads. a, anterior; p, posterior; d, dorsal; v, ventral. All arrowheads indicate amputation level (knee level). Bars, 100 μm.

lates endogenous *Xenopus fgf-10* expression itself (perhaps via early induction of *fgf-8* expression). These results also suggest that the blastema of this stage could have a positive loop of interaction between *fgf-10* and *fgf-8*, as do developing and regenerating limbs (Ohuchi *et al.*, 1997; Yokoyama *et al.*, 2000). *Sonic hedgehog* (*shh*), which specifies the AP axis of both developing and regenerating limbs (Riddle *et al.*, 1993; Roy *et al.*, 2000), is known to be expressed in the posterior border of regenerating *Xenopus* limb buds amputated at early stages (Endo *et al.*, 1997). In the FGF-10-treated stage 56 limbs, *shh* transcripts were detectable in the posterior border of a blastema 8 days after amputation (Fig. 3B). This result is consistent with polarized digit patterns in resultant regenerates (Figs. 2C and 2D). *msx-1*, which is a good marker of the progress zone of the developing chick limb bud (Ros *et al.*, 1992; Suzuki *et al.*, 1991), is also expressed in the regenerating blastema of urodele (Koshiba *et al.*, 1998) and anuran (Christen and Slack, 1998; Endo *et al.*, 2000) limbs, and the *msx-1* expression is thought to correlate with mouse limb bud regeneration (Reginelli *et al.*, 1995). *msx-1* was expressed in the blastema of a stage 56 limb bud with FGF-10 (Fig. 3E). Since a stage 56 limb stump is mostly composed of well-differentiated tissues (Figs. 1C and 1D), it is possible that *msx-1*-positive undifferentiated blastemal cells could be derived from differentiated cells through a dedifferentiation process. However, this does not exclude the possibility that these undifferentiated cells might be derived from some stem cell-like reserved cells that are stored in the differentiated tissues. Wherever the blastema cells are derived from, what is important is that these results suggest that FGF-10 can induce the accumulation of undifferentiated *msx-1*-positive cells in the *Xenopus* limb stump. We used *hoxa-13* as a marker of the most-distal identity (presumptive autopod), as described in developing and regenerating limbs (Yokouchi *et al.*, 1991; Gardiner *et al.*, 1995; Endo *et al.*, 2000). *hoxa-13* expression was found in the blastema (Fig. 3F). These results demonstrate that FGF-10-treated blastemal cells have an adequate positional identity along the proximal–distal axis. None of these genes was expressed in control limbs with PBS beads (Figs. 3G–3J).

Can Other FGFs Stimulate Xenopus Limb Regeneration?

Each FGF appears to have a distinct expression and function in limb development (Martin, 1998). To ensure that the induced regeneration in stage 56 limbs is a specific effect of FGF-10, we applied FGF-8- or FGF-2-soaked beads to stage 56 limb stumps. FGF-8-applied limbs regenerated one or two digits at best in only a few cases (2/11; Table 1), suggesting that FGF-8 is much less effective than FGF-10 (1 mg/ml). These results also suggest that FGF-10 induces significant regenerative responses not only through the induction of *fgf-8* expression but also through an *fgf-8*-independent pathway. In the blastema of FGF-8-applied limbs, *msx-1* expression is detectable 8 days after amputation (data not shown), while the *msx-1* expression induced by exogenous FGF-8 seems not to be sufficient for the significant enhancement of limb regeneration. Recent studies using gene targeting have revealed that *fgf-10*-deficient mice can express neither *fgf-8* nor *shh*, resulting in limb defects (Min *et al.*, 1998; Sekine *et al.*, 1999), while *fgf-8*-deficient mice can form normal limbs (Meyers *et al.*, 1998). Taken together with our results, it appears that FGF-10 could be an endogenous initiator of not only limb development but also limb regeneration. FGF-2 could not induce any regeneration in stage 56 limb stumps (8/8; Table 1). Therefore, FGF-10-induced *Xenopus* limb regeneration should be distinct from chick limb bud "regeneration" induced by FGF-2 application to distal stumps of early limb buds (Tayor *et al.*, 1994). Based on the fact that FGF-2 can rescue limb regeneration in denervated axolotl limbs which cannot regenerate without the application of a neurotrophic factor (Mullen *et al.*, 1996), FGF-10 seems to act in a different manner from the rescue of nerve-dependent limbs.

CONCLUSION

In this study, we showed that FGF-10 can induce gene reexpressions and produce a well-patterned regenerate, and we indicated that it induces a strong regenerative response in the proximal stump of nonregenerative *Xenopus* limb buds. This report is the first to describe enhancement of reduced ability of limb regeneration. As in nonregenerative *Xenopus* limb buds, normal limb regeneration ability in higher tetrapod vertebrate groups (mammals, birds, and reptiles) is either entirely lacking or limited to the distal tip of digits (Muller *et al.*, 1999). The findings in the present study that *fgf-10* expression and the molecular interactions evoked by FGF-10 must be rescued for regeneration provide a clue for the possible realization of regeneration of nonregenerative limbs.

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