Mesenchyme with fgf-10 Expression Is Responsible for Regenerative Capacity in Xenopus Limb Buds

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A young tadpole of an anuran amphibian can completely regenerate an amputated limb, and it exhibits an ontogenetic decline in the ability to regenerate its limbs. However, whether mesenchymal or epidermal tissue is responsible for this decrease of the capacity remains unclear. Moreover, little is known about the molecular interactions between these two tissues during regeneration. The results of this study showed that fgf-10 expression in the limb mesenchymal cells clearly corresponds to the regenerative capacity and that fgf-10 and fgf-8 are synergistically reexpressed in regenerating blastemas. However, neither fgf-10 nor fgf-8 is reexpressed after amputation of a nonregenerative limb. Nevertheless, nonregenerative epidermal tissue can reexpress fgf-8 under the influence of regenerative mesenchyme, as was demonstrated by experiments using a recombinant limb composed of regenerative limb mesenchyme and nonregenerative limb epidermis. Taken together, our data demonstrate that the regenerative capacity depends on mesenchymal tissue and suggest that fgf-10 is likely to be involved in this capacity. © 2000 Academic Press

INTRODUCTION

Anuran amphibians exhibit different degrees of capacity for limb regeneration at different stages of their life cycle. They therefore serve as excellent materials to investigate the essential difference between regenerative limbs and nonregenerative limbs. Xenopus can completely regenerate hindlimb buds prior to the onset of metamorphosis, but the regenerative capacity declines gradually as metamorphosis proceeds (Dent, 1962; Muneoka et al., 1986). Limbs in froglets and adults do not regenerate any recognizable structures but form a hypomorphic structure after amputation (Skowron and Komala, 1957). It has been shown that the ontogenetic decline of regenerative capacity is due to intrinsic changes in the limb bud itself, which occur as the tadpole undergoes metamorphosis (Sessions and Bryant, 1988).

The vertebrate limb bud is 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; Messenger, 1976) as well as for outgrowth of developing limb buds (Saunders, 1948; Zwilling, 1956; Summerbell, 1974). It is unclear, however, whether it is the epidermal or mesenchymal cells that control the regenerative capacity in the anuran limb bud. Although there are various possibilities suggesting that the loss of regenerative capacity in anuran limb buds is due to changes in the mesenchymal cells, epidermal cells, or both (Stocum, 1995), no direct evidence of this has yet been presented. The results of some transplantations with regard to this issue have been reported (Gidge and Rose, 1944; Carlson, 1982), but they are not conclusive. We therefore focused on the task of clarifying whether it is the epidermal or the mesenchymal tissue that controls the regenerative capacity in anuran limb buds.

Several FGFs have been shown to play important roles in
epidermal–mesenchymal interactions required for limb initiation and elongation in chick and mouse embryos (see Martin, 1998, for review). fgf-8 is expressed in the apical ectodermal ridge (AER), required for limb bud elongation, and is thought to be the endogenous AER factor for the growth of mesenchymal cells in the mouse (Ohuchi et al., 1994; Crossley and Martin, 1995) and chick embryo (Crossley et al., 1996; Vogel et al., 1996). fgf-8 is also expressed in the apical epidermis of Xenopus limb buds (Christen and Slack, 1997; Yokoyama et al., 1998). These studies suggest that FGF-8 could be a key molecule that mediates the function of the apical epidermis to the mesenchyme in regenerating limb buds. On the other hand, a recent study by Ohuchi et al. (1997) has shown that fgf-10 is expressed in the prospective chick limb mesoderm and also in the mesenchyme of established limb buds. Ohuchi et al. (1997) also showed that FGF-10 induces fgf-8 expression in the adjacent ectoderm and that fgf-10 expression of the mesenchyme is maintained by FGF-8. Furthermore, it has been shown that fgf-10-deficient mice cannot form both fore- and hindlimbs and cannot induce fgf-8 expression around the presumptive site of limb bud initiation (Min et al., 1998; Sekine et al., 1999). These results suggest that FGF-8 and FGF-10 mediate epidermal–mesenchymal interactions required for limb bud outgrowth and that these FGFs have critical roles not only in limb development but also in limb regeneration.

The goal of the present study was to determine whether it is the epidermis or the mesenchyme that controls the regenerative capacity of the Xenopus limb bud and to demonstrate the relationship between regenerative capacity and two key molecules, fgf-10 and fgf-8, in order to clarify the molecular mechanism of limb regeneration. For this purpose, we cloned a Xenopus homolog of fgf-10 and examined fgf-10 and fgf-8 expression in developing and regenerating limb buds. We then performed reciprocal transplantations between regenerative limb buds and nonregenerative ones in order to investigate the regenerative capacity of Xenopus recombinant limb buds composed of the epidermis of regenerative limb buds and the mesenchyme of nonregenerative limb buds or vice versa. We found that mesenchyme, not epidermis, controls the regenerative capacity of limb buds and that fgf-10 and fgf-8 can be reexpressed in regenerative recombinants but not in nonregenerative ones, suggesting that these two molecules reflect the regenerative capacity of limbs.

**MATERIALS AND METHODS**

**Gene Cloning and in Situ Hybridization**

A partial cDNA encoding Xenopus fgf-10 was obtained by PCR with mRNA extracted from whole Xenopus embryos at stage 37–39 (Nieuwkoop and Faber, 1956) using degenerate primers that are complementary to amino acids conserved in other vertebrates, YNHLOGD and QMFVAlN. The PCR product was cloned into the pCR-Script vector (Stratagene) and sequenced. To synthesize an antisense RNA probe, this plasmid was linearized with EcoRII and transcribed with T3 RNA polymerase (Boehringer Mannheim). Whole-mount in situ hybridization in the Xenopus limb bud was performed as described by Endo et al. (1997). Developing or 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, Elkhart, IN), and serially sectioned at 10 μm. Non-RI in situ hybridization in sections was carried out as described by Yoshiida et al. (1996).

**Manipulation and Transplantation (Transplant Combinations Are Shown in Fig. 4)**

Xenopus tadpoles were allowed to develop until they reached the appropriate stages (stage 51–52 and 56). For manipulation of limb buds, the tadpoles were anesthetized with 1:5000 ethyl-3-aminobenzoate (Aldrich) dissolved in Holtfreter's solution. We prepared small tadpoles at stage 56 by thyroxine (T4) treatment to obtain mesenchymal grafts, since normal stage 56 limb buds are too large to be grafted onto stage 52 host limb buds. We added thyroxine, which promotes metamorphosis, in water (5 μg/mL; according to La Mesa et al., 1995) and reared stage 52–53 tadpoles in this solution until they reached stage 56. Excised whole hindlimb buds (stages 51–52 and 56) were washed with Holtfreter's solution and then treated with 0.05% EDTA in Ca/Mg-free Holtfreter's solution for 30 min to loosen epidermal–mesenchymal adhesion. After the epidermis was removed mechanically, the limb bud mesenchyme was grafted to a hindlimb stump freshly amputated at the presumptive knee level of host tadpoles (stages 56 and 52). The grafted mesenchyme was held in place with tungsten pins. The alignment (anteroposterior, dorsoventral, and proximodistal) of the graft was in accordance with that of the host limb stump. The operated tadpoles were reared in 30% Holtfreter's solution for 3 days and then in water. After 5–7 days, the tadpoles were anesthetized and examined microscopically to confirm that the grafted mesenchyme had been covered with host epidermis. Just after this examination, these grafted limbs were amputated at the presumptive knee level (with reference to the fate map by Tschemi, 1957) of the grafts in order to analyze the regenerative capacities of recombinant limb buds composed of grafted mesenchyme and host epidermis. 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.

**Chimera Analysis**

Recombinant limb buds from stage 51–52 Xenopus laevis mesenchyme and stage 56 Xenopus borealis epidermis were prepared for chimeric analysis. Cell contribution was determined by differential quinacrine staining of nuclei, according to Thiébaud (1983). Recombinant limbs were fixed in Carnoy's for 30–60 min. Fixed limbs were dehydrated in butanol, embedded in paraffin, and cut at 8 μm into serial sections. The sections were stained with quinacrine (Thiébaud, 1983) and analyzed by fluorescence microscopy (Olympus BH-2).
RESULTS

Cloning of Xenopus fgf-10 and Its Expression during Limb Development

To understand the molecular aspects of epidermal-mesenchymal interactions and the capacity for limb regeneration, we focused on two key molecules, fgf-10 and fgf-8. We first cloned a partial cDNA of fgf-10 from Xenopus. An alignment of the predicted Xenopus FGF-10 amino acid sequences shows 85, 80, 83, and 83% identity to the chick, mouse, rat, and human FGF-10, respectively (Fig. 1A). The spatial and temporal expression patterns of fgf-10 mRNA were examined during Xenopus limb development by whole-mount and sectioned in situ hybridization. fgf-10 was strongly expressed in the distal region of limb buds at early stages (stages 51 and 52, Figs. 1B and 1C). This expression was gradually reduced and only weak expression was detected at stage 53 (Fig. 1D), and the expression disappeared by stage 56 (Fig. 1E). Sectioned in situ hybridization revealed that fgf-10 is expressed not in the epidermis but only in the distal mesenchyme of limb buds (Figs. 1F and 1G), as was reported in chick limb buds (Ohuchi et al., 1997).

fgf-10 and fgf-8 Expressions in Regenerating Limb Buds

To confirm that limb buds at different stages have different regenerative capacities, we amputated stage 51–52 and stage 56 limb buds at the presumptive knee level, and we observed the cartilage patterns in the resultant regenerates (Fig. 2, Table 1). Almost all of the stage 52 limb buds regenerated completely (12/15, Fig. 2A), while some of the stage 56 limb buds did not regenerate any structures (6/18, Fig. 2B) and others regenerated only a small hypomorphic structure (12/18, Fig. 2C). Based on these results, stage 51–52 and stage 56 limb buds were referred to as regenerative and nonregenerative limb buds, respectively.

The reason for the decrease in regenerative capacity as limb development proceeds may be a lack of some molecular interactions during the process of limb regeneration. To investigate whether FGF-10 and FGF-8 are involved in this phenomenon, the expression of fgf-10 in the mesenchyme and fgf-8 in the epidermis was analyzed in those blastemas. Thus, stage 52 and 56 limb buds, which represent regenerative and nonregenerative limb buds, respectively, were amputated at the presumptive knee level (see Fig. 1), and fgf-10 and fgf-8 expressions were examined. The fgf-10 expression domain in the distal region of the stage 52 limb bud was completely removed by this amputation.

For regenerative limb buds at stage 52, examination was performed 5 days after amputation, as all stage 52 limb buds form blastemas within 5 days after amputation. fgf-10 expression was detected in the distal mesenchyme (Figs. 3A and 3B), and fgf-8 was expressed in the inner layer of the thickened apical epidermis (Figs. 3C and 3D). On the other hand, the expression of neither fgf-10 nor fgf-8 was detected in stage 56 limb buds at 3 days (Figs. 3E and 3G), 5 days (Figs. 3I and 3K) or 10 days (data not shown) after amputation. Notably, a significant structure of thickened epidermis without fgf-8 expression, reminiscent of an apical ectodermal cap in regenerating urodele limbs (Wallace, 1981), was observed 3 days after amputation (Figs. 3F and 3H), and this structure degenerated by the fifth day (Figs. 3J and 3L).

Taken together, these results demonstrate that expression of both fgf-10 and fgf-8 corresponds to differential regenerative capacities between stage 51–52 and stage 56, suggesting the important roles of these genes in epidermal-mesenchymal interactions during limb regeneration.

Recombinants with Stage 51–52 Mesenchyme and Stage 56 Epidermis (Type A Recombinant)

Analysis of fgf-10 and fgf-8 expression in the later stage blastemas (stage 56) revealed that some defects in molecular interactions, including the expression of both fgf-10 and fgf-8 genes in the mesenchyme and epidermis, occur. However, it is still not clear whether it is the mesenchyme or the epidermis that is responsible for the decrease in regenerative capacity. In order to solve this issue, we decided to construct some types of recombinants by swapped combinations of mesenchyme and epidermis (Fig. 4).

We prepared recombinant limbs composed of the epidermis from nonregenerative limb buds and mesenchyme from regenerative limb buds (type A recombinants). For this purpose, stage 51–52 whole limb bud mesenchyme was grafted onto a host hindlimb stump that had been freshly amputated at stage 56. When these recombinant limbs were allowed to develop without amputation, they frequently formed a complete cartilage pattern of hindlimbs (7/10, Fig. 5A). To confirm that the host epidermis covers the grafted mesenchyme, the cell contribution from host and graft tissues was analyzed 5 days after grafting, by using a chimera between X. laevis and X. borealis (Fig. 6). This analysis revealed that the epidermis of host hindlimb buds (X. laevis) covered the graft mesenchyme (X. borealis), resulting in chimeric recombinant limbs.

These recombinant limbs were amputated at the presumptive knee level to examine their regenerative capacities. Most of them regenerated completely, as normal 51–52 limb buds did (8/10, Fig. 5B, Table 1). Supernumerary limbs were sometimes formed near the boundary between the host and the grafted mesenchyme of developing (6/10) or regenerating (6/10) limbs (see asterisks in Figs. 5A and 5B). There may have been some disparity of positional value because of mismatch of size between graft and host tissues, resulting in intercalary interactions that led to the formation of supernumerary limbs. Alternatively, the mismatch itself may have formed uncovered space in the amputated plane and it may have evoked a regenerative response from the grafted mesenchyme, resulting in the formation of...
supernumerary limbs. Some of the recombinant limbs that formed a complete distal pattern had shortened or partially deformed proximal structures (femur or tibia/fibula; data not shown) (2/7 in developed limbs; 3/8 in regenerated limbs). These results suggest that mesenchyme derived from a regenerative limb bud can regenerate as well as

![Image of amino acid sequence comparison and expression diagrams]

**FIG. 1.** (A) Comparison of amino acid sequence of FGF-10 (abbreviations: x, Xenopus; c, chick; m, mouse; r, rat; h, human). (B–G) fgf-10 expression in developing limb buds at stage 51 (B), stage 52 (C), stage 53 (D), and stage 56 (E). (F) In situ hybridization on a section of stage 52 limb bud (only distal region). (G) Higher magnification view of (F). a, anterior; p, posterior. Arrowheads show the presumptive knee level (amputation level) of limb buds. Bars, 250 μm for (B), (C), (D), and (E), and 50 μm for (F) and (G).
develop even if it is covered with the epidermis from a nonregenerative limb bud.

Recombinant with Stage 56 Mesenchyme and Stage 52 Epidermis (Type B Recombinant)

For the following recombination, small limb buds of stage 56 tadpoles were made using thyroid hormones (T4). These tadpoles were about the same size as normal stage 52 tadpoles, but their limb shape resembled that of a stage 56 limb bud. When they underwent amputation, they exhibited regenerative response similar to that of a normal stage 56 tadpole (Fig. 2D, see also Table 1). In order to prepare recombinant limbs composed of the epidermis from regenerative limb buds and mesenchyme from nonregenerative limb buds (type B recombinants), the whole mesenchyme of a T4-treated stage 56 limb bud was grafted onto a stage 52 host hindlimb stump. As in type A recombinants, chimera analysis confirmed that the host epidermis covered the grafted mesenchyme (data not shown). The resultant recombinant limbs formed a complete cartilage pattern of hindlimbs (6/6, Fig. 5C, Table 1) if they further developed without amputation, as was the case in type A recombinants. This indicates that a stage 52 host is sufficient for the growth of recombinant limbs.

When these recombinant limbs were amputated at the presumptive knee level, some of them (9/11) underwent wound healing without any regeneration and others (2/11) regenerated only a hypomorphic structure (Fig. 5D, Table 1), indicating that these recombinant limbs have only the regenerative capacity of a normal stage 56 limb bud. These results suggest that the epidermis derived from a regenerative limb bud cannot promote regeneration with the mesenchyme from a nonregenerative limb bud. Such limb buds often formed some small supernumerary structures (asterisk in Fig. 5D: 2/6 in developed limbs, 5/11 in regenerated limbs) near the boundary between the host and the recombinant limb.

fgf-10 and fgf-8 Expression in the Recombinant Limbs

To gain more insight into the question of whether FGF-10 and FGF-8 are involved in the regeneration that occurred in the recombinant limbs, fgf-10 and fgf-8 expression in type A and type B recombinant limbs was examined 3 days after amputation at the presumptive knee level. Although fgf-10 expression was never detected in the blastema of stage 56 limb buds (Figs. 3E and 3I), strong fgf-10 expression was detected in the blastema of recombinants with stage 51–52 mesenchyme and stage 56 epidermis (type A recombinant; Fig. 7A). Although strong expression of fgf-8 was also detected in the blastema of recombinants with stage 51–52 mesenchyme and stage 56 epidermis (type A recombinant; Fig. 7A). Although strong expression of fgf-8 was also detected in the blastema of type A recombinant limbs (Fig. 7B), the expression of neither fgf-8 nor fgf-10 was observed in the blastema of type B recombinants (Figs. 7C and 7D). We examined fgf-8 expression in type B recombinants 6 and 12 days after amputation, but no expression was detected (data not shown). The expression of fgf-10 and fgf-8 in the type A recombinants (Figs. 7A and 7B) should be involved in the blastema, not in the supernumerary limbs, of the recombinants since this expression was detected far from the boundary between the host and the recombinant limb.
DISCUSSION

Mesenchyme Controls Regenerative Capacity of the Limb Bud

We have shown that fgf-10 expression in developing and regenerating limbs corresponds to the regenerative capacity of the limb. Moreover, young regenerative mesenchyme in type A recombinants could completely regenerate even under an epidermis derived from an old nonregenerative limb bud, and this recombination induces the expression of two key molecules, fgf-10 and fgf-8, in the blastema. Mesenchymal tissue derived from stage 51–52 limb buds in these recombinants originally possesses regenerative capacity because this tissue can regenerate completely after amputation (Fig. 2A) and reexpress fgf-10 (Figs. 3A and 3B). Interestingly, this mesenchyme induced fgf-8 expression in the overlying epidermis (Fig. 7B) derived from a limb bud at stage 56, by which time the limb bud loses its regenerative capacity, and never reexpressed fgf-8 (Figs. 3G, 3H, 3K, and 3L). On the other hand, type B recombinants regenerated only hypomorphic structures (Fig. 5D). This type of recombination failed to induce either fgf-10 or fgf-8 expression (Figs. 7C and 7D). These results strongly suggest that the conditions for including fgf-10 expression in the mesenchyme determine the regenerative capacity of a recombinant limb. We conclude that the regenerative capacity of a limb bud depends on the mesenchyme, not the epidermis.

Role of fgf-10 and fgf-8 Expression in a Regenerating Limb Bud

In the chick limb bud, fgf-10 expression in the underlying mesenchyme can induce fgf-8 expression in the apical epidermis (Ohuchi et al., 1997; Yonei-Tamura et al., 1999), and the interactions between FGF-10 and FGF-8 mediate epidermal-mesenchymal interactions required for limb elongation (Ohuchi et al., 1997). It has been shown that fgf-8 is expressed in the apical epidermis of developing Xenopus limb buds (Christen and Slack, 1997; Yokoyama et al., 1998). Expression analysis of the developing Xenopus limb bud confirmed that fgf-10 is expressed in the distal mesenchyme at the early stage (Fig. 1), as it is in the chick limb bud (Ohuchi et al., 1997). It is assumed, therefore, that epidermal-mesenchymal interaction mediated by FGF-10 and FGF-8 could be conserved also in amphibian limb buds.

In higher vertebrates such as birds and mammals, a regeneration response can be observed only when they are amputated within the region of Msx-1, a homeobox-containing gene expressed in mesenchymal cells subjacent to the distal ectoderm in mouse limb buds (Reginelli et al., 1995). Limb buds amputated through a more proximal plane can regenerate small and incomplete distal structures when exogenous FGF is applied to the stump tissues in chick limb buds (Taylor et al., 1994; Kostakopoulou et al., 1996). Without application, they cannot regenerate any structure. Msx-1 is also expressed in the distal region of Xenopus limb buds, underneath the apical epidermis expressing fgf-8 (Christen and Slack, 1998). In Xenopus, however, limb regeneration does not correlate with regions of Msx-1 expression nor need exogenous FGF application because stage 52 limb buds, amputated at the plane more proximal to the fgf-10, fgf-8, and Msx-1 expression domains, can regenerate completely without exogenous FGF application (Fig. 2A, Table 1). Furthermore, when stage 52 limb buds were amputated, the limb bud stump could restore sufficient fgf-10 and fgf-8 expression and reinitiate molecular interaction between FGF-10 and FGF-8 (Figs. 3A and 3C). On the

<table>
<thead>
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<th>Type of limb bud</th>
<th>Total No. of limb buds</th>
<th>Wound healing (without regeneration)</th>
<th>Pattern formed</th>
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</thead>
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<tr>
<td>St. 52</td>
<td>15</td>
<td>2</td>
<td>Complete</td>
</tr>
<tr>
<td>St. 56</td>
<td>18</td>
<td>6</td>
<td>Incomplete</td>
</tr>
<tr>
<td>T4-treated St. 56</td>
<td>11</td>
<td>5</td>
<td>Complete</td>
</tr>
<tr>
<td>St. 51-52 mes + St. 56 epi (development)</td>
<td>10</td>
<td>0</td>
<td>Complete</td>
</tr>
<tr>
<td>St. 51-52 mes + St. 56 epi (regeneration)</td>
<td>10</td>
<td>0</td>
<td>Complete</td>
</tr>
<tr>
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<td>6</td>
<td>0</td>
<td>Incomplete</td>
</tr>
<tr>
<td>St. 56 mes + St. 52 epi (regeneration)</td>
<td>11</td>
<td>9</td>
<td>Complete</td>
</tr>
</tbody>
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TABLE 1
Developmental and Regenerative Capacity of Limb Buds

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FIG. 3. fgf-10 and fgf-8 expression in regenerating limb buds. Limb buds were amputated at stage 52 (A–D), or stage 56 (E–L), and examined for fgf-10 (A, E, I) and fgf-8 (C, G, K) in serial sections. (B, D, F, H, J, and L) Phase-contrast photograph of (A), (C), (E), (G), (I), and (K), respectively. Arrows indicate amputation level. Arrowheads indicate thickened epidermis. d, dorsal; v, ventral. Bars, 250 μm.
other hand, a stage 56 limb bud cannot reexpress either molecule (Figs. 3E, 3G, 3I, and 3K), suggesting that a stage 56 limb bud cannot reinitiate or maintain molecular interactions mediated by FGF-10 and FGF-8. Therefore, sufficient reexpression of fgf-10 and fgf-8 and molecular interactions between them may be necessary for complete limb regeneration, as they are for chick limb bud initiation and elongation. The stage 56 limb bud 3 days after amputation, however, formed a thickened epidermis (arrowheads in Figs. 3F and 3H), which looks like an apical epidermal cap in the regenerating urodele limb (Wallace, 1981). A thickened epidermal structure, therefore, can be formed morphologically even in a nonregenerative limb bud, but it may not be functional because of the lack of fgf-8 expression. Expression of fgf-10 and fgf-8 in type A recombinant limb buds suggests that mesenchyme derived from regenerative limb buds reexpresses fgf-10 (Fig. 7A) by itself and then induces fgf-8 expression in the overlying epidermis (Fig. 7B) derived from nonregenerative limb buds, which would never reexpress fgf-8 after amputation without recombination (Figs. 3G and 3K). It should be emphasized that fgf-10 expression in the mesenchyme appears to be a critical cue for limb regeneration.

On the other hand, in type B recombinants, mesenchyme derived from a nonregenerative limb cannot induce fgf-8 expression even in the epidermis from a regenerative limb bud (Fig. 7D). fgf-10-deficient mice cannot form fore- and hindlimbs nor express fgf-8 in the epidermis of the presumptive limb region (Min et al., 1998; Sekine et al., 1999). Considering these observations, it is possible that this is because the mesenchyme has already lost the competence to express fgf-10. Alternatively, the mesenchyme might be unable to express FGF receptor genes that mediate FGF-8/FGF-10 interaction. Among FGFRs, FGFR2c can bind to FGF8 (MacArthur et al., 1995; Ornitz et al., 1996) and is present in the mesenchyme of developing mouse limb buds (Orr-Urtreger et al., 1991, 1993; Peters et al., 1992), while FGF-10 can bind only to FGFR2b (Igarashi et al., 1998), which is present in the surface ectoderm of mouse limb buds (Orr-Urtreger et al., 1991, 1993). FGFR2-deficient mice (which lack both FGFR2b and FGFR2c) cannot form limb buds, and the expression of fgf-8 is absent in the presumptive limb ectoderm and the expression of fgf-10 is downregulated in the underlying mesoderm (Xu et al., 1998). Taken together, the results of these studies suggest that FGFR2 is essential for interactions between FGF-10 and FGF-8. It is possible that the mesenchyme of nonregenerative limb buds cannot express fgf2c, and this failure may cause the downregulation of fgf-10 and fgf-8. Otherwise, some structures (e.g., impermeable ECM or a cell layer) formed by mesenchymal tissues may physically prohibit the interactive loop between FGF-10 and FGF-8, while mesenchymal cells have the ability to express FGF receptors and ligands.

FIG. 4. Schematic diagram illustrating experimental procedure for preparing recombinant limbs. The arrow indicates the hindlimb bud of the Xenopus tadpole. To determine whether epidermis or mesenchyme controls the regenerative capacity of the Xenopus limb bud, two types of recombinant limbs were made. In experiment A, the whole stage 51–52 limb bud was excised, the epidermis was removed, and then, naked mesenchyme was grafted onto the stage 56 hindlimb stump amputated at the presumptive knee level. After grafting, epidermis of stage 56 host migrated and covered the grafted mesenchyme, and therefore, recombinant limbs composed of stage 51–52 mesenchyme and stage 56 epidermis (type A recombinant) were formed. In experiment B, whole stage 56 (T4-treated) mesenchyme was grafted onto the stage 52 host hindlimb stump amputated at the presumptive knee level. After grafting, epidermis of stage 52 host covered stage 56 mesenchyme, and therefore, recombinant limbs composed of stage 56 mesenchyme and stage 52 epidermis (type B recombinant). 5–7 days after recombination, we amputated these two types of recombinant limbs at the presumptive knee level in order to analyze regenerative capacities. meso, mesenchyme; epi, epidermis.
Differences between Regenerative and Nonregenerative Limbs

Developing chick limb buds cannot regenerate the AER in the ectoderm (Saunders, 1948; Summerbell, 1974) or any skeletal structures (Muneoka and Sassoon, 1992) after amputation. When an amputated limb bud stump was combined with an AER, one or two cartilage elements of host origin were formed in the distal region (Watanabe et al., 1993), suggesting that the AER enables the differentiated

FIG. 5. Development and regeneration of recombinant limbs. (A and B) Recombinant limbs composed of stage 51–52 mesenchyme and stage 56 epidermis. (C and D) Recombinants composed of stage 56 mesenchyme and stage 52 epidermis. (A and C) Allowed to develop without amputation. They formed complete cartilage patterns. (B and D) Amputated at presumptive knee level. Note that the recombinant limb in (D) regenerates only an incomplete cartilage structure like a spike, while the recombinant in (B) regenerates a complete cartilage pattern. Arrowheads indicate host–graft boundary. Black arrows, amputation level. White arrows, recombinant limb. Asterisks, extra limbs. Bars, 1 mm.

FIG. 6. Chimeric analysis of recombinant limb. (A) A recombinant limb composed of stage 51–52 mesenchyme (X. borealis) and stage 56 epidermis (X. laevis). (B and C) Higher magnification photograph of distalmost region in the same recombinant. (B) In ordinary light. (C) In fluorescent light. Note that borealis mesenchymal cells (shown by mottled staining of nucleus) were covered with laevis epidermal cells (shown by uniformly bright staining). Arrowheads and arrows indicate host–graft boundary and epidermal cell layer, respectively. Bars, 100 μm.

FIG. 7. fgf-10 (A) and fgf-8 (B) expression in recombinant limb bud with stage 51–52 mesenchyme and stage 56 epidermis and fgf-10 (C) and fgf-8 (D) expression in recombinant with stage 56 mesenchyme and stage 52 epidermis 3 days after amputation. Arrowheads indicate host–graft boundary. Arrows show amputation level. Bars, 250 μm.
mesenchyme to make more distal structures. The ability of distalization in the mesenchyme is lost progressively as the limb bud develops, and the proximal mesenchyme cannot form distal structures or maintain a thickened structure of the AER (Saunders et al., 1959). These results remind us that the regenerative capacity of a Xenopus limb bud declines progressively as the developmental stage proceeds and that a nonregenerative limb bud (stage 56) cannot maintain a thickened epidermal structure (Figs. 3 and 3L). Thus, the mechanism of progressive change in the mesenchyme of a Xenopus limb bud, which is responsible for the loss of regenerative capacity as the developmental stage proceeds, might be the same as that of the loss of regenerative ability in the proximal mesenchyme of the chick limb bud. These progressive changes may involve the inability of mesenchymal tissues to reinitiate or maintain fgf-10 and fgf-8 expression in limb buds. Recent studies have revealed that fgf-10-deficient mice cannot express fgf-8 or shh, which specifies the anteroposterior axis of the limb (Riddle et al., 1993) and results in truncation of limbs (Min et al., 1998; Sekine et al., 1999). These observations suggest that fgf-10 expression and interactions between FGF-10 and FGF-8 are indispensable for sufficient limb pattern formation.

The results of this study indicate that fgf-10 and fgf-8 expression correlates with the regenerative capacity of the Xenopus limb bud and suggest that sufficient interactions between these two molecules may be therefore necessary to support more complete regeneration in the case of nonregenerative limbs. Further investigations into the molecular mechanisms of the change in the regenerative capacity of Xenopus limb bud mesenchyme should ultimately enable us to regenerate nonregenerative limbs in higher vertebrates such as birds or mammals.

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