Mouse digit tip regeneration is mediated by fate-restricted progenitor cells

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Regeneration of appendages is frequent among invertebrates as well as some vertebrates. However, in mammals this has been largely relegated to digit tip regeneration, as found in mice and humans. The regenerated structures are formed from a mound of undifferentiated cells called a blastema, found just below the site of amputation. The blastema ultimately gives rise to all of the tissues in the regenerate, excluding the epidermis, and has classically been thought of as a homogenous pool of pluripotent stem cells derived by dedifferentiation of stumps tissue, although this has never been directly tested in the context of mammalian digit tip regeneration. Successful digit tip regeneration requires that the level of amputation be within the nail bed and depends on expression of Msx1. Because Msx1 is strongly expressed in the nail bed mesenchyme, it has been proposed that the Msx1-expressing cells represent a pluripotent cell population for the regenerating digit. In this report, we show that Msx1 is dynamically expressed during digit tip regeneration, and it does not mark a pluripotent stem cell population. Moreover, we show that both the ectoderm and mesoderm contain fate-restricted progenitor populations that work in concert to regenerate their own lineages within the digit tip, supporting the hypothesis that the blastema is a heterogeneous pool of progenitor cells.

Regeneration of appendages occurs widely throughout the animal kingdom, although it is limited in higher vertebrates. Although many fish regenerate fins and a number of urodele and larval anuran amphibians regenerate entire limbs, in mammals such as humans and mice appendage regeneration is limited to the distal digits. Regeneration begins with the formation of a specialized regenerative epithelium across the plane of amputation that is required for regeneration to proceed (1, 2). Histological analysis of amphibian limb regenerates suggests that the regenerative epithelium forms by the migration of stump epidermal cells, although additional contributions from other cell populations have not been ruled out (3). The formation of the regenerative epithelium is followed by an accumulation of mesenchymal cells distal to the stump, which proliferate to form a bud-like structure known as the blastema. These blastema cells subsequently differentiate into the musculoskeletal and connective tissues of the regenerated appendage.

It is critical to understand the origin of blastema cells and the degree to which they represent a multipotent cell population. The blastema could be formed from a preexisting multipotent stem cell as found in planaria; however, vertebrate research suggests, at least in amphibian limb regeneration, that the majority of blastema cells are from dedifferentiation of the mature tissues of the stump (4, 5). In principle, blastema cells could remember their origin and only differentiate into the cell types from which they arose. Alternately, blastema cells could be multipotent, capable of transdifferentiation into all cell types of the regenerate. Indeed, transdifferentiation has been described in both axolotl tail regeneration (6) and amphibian limb regeneration experiments in which certain tissues were removed or irradiated (7, 8). However, recent lineage analyses after amputation of both the axolotl limb and the zebrafish fin strongly suggest that transdifferentiation does not significantly contribute to the regenerates, and that instead the blastemas are made up of lineage-restricted cell populations (9–11).

Digit tip regeneration has been reported in mammals including mice and juvenile humans (12, 13). Amputations of the terminal phalax through levels associated with the nail organ are capable of regeneration, whereas more proximal amputations are not. Intriguingly, mesenchymal nail bed cells in neonatal mice and humans express the transcription factor Msx1 (14, 15), which is a transcription factor expressed in the proliferative, undifferentiated regions of regenerating newt limbs (16, 17). This expression may reflect a functional importance of Msx1 in blastema formation and maintenance. Msx1 induces dedifferentiation and proliferation of cultured myotubes, which are ultimately capable of being redifferentiated down multiple lineages (18). Importantly, Msx1−/− digits do not regenerate in ex vivo embryo culture. Moreover, Msx1 is necessary for Bmp4 activity, which could rescue the ability of these Msx1−/− digits to regenerate (19). Msx1 expression is not observed in the digit tip blastema itself (20), suggesting that its critical function may be in the nail bed mesenchyme. Given the requirement of the nail bed for successful regeneration and the association of Msx1 with undifferentiated, multipotent cells in both developmental and regenerative settings, Msx1-expressing nail bed mesenchyme could represent a pluripotent stem cell population necessary for digit tip regeneration. Alternately, the Msx1-expressing nail bed mesenchyme could serve as a signaling center, producing critical factors required for regeneration.

To determine whether mouse digit tip regeneration involves the recruitment of multiple cell type determinant lineages or the formation of a pluripotent progenitor pool, and to answer the related question of whether these blastema cells are derived from a preexisting stem cell population or from dedifferentiation of mature tissues in the digit stump, we undertook a lineage analysis using inducible alleles of Cre recombinase expressed in specific tissues within the neonatal limb.

Results

Descriptive Analysis of the Process of Digit Tip Regeneration. To develop a context for our lineage studies, we established a time course of the early events of mouse digit tip regeneration. In preliminary experiments, we found that regeneration reliably occurs when the distal-most 400 μm are amputated on postnatal day 3 (PN3) CD1 neonatal mice (SI Text and Fig. S1). This represents ≈50% of the length of the PN3 distal phalanx and transverses the proximal nail bed, although digit size and regeneration

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can vary with strain (Fig. S2). CD1 mothers were used in all subsequent experiments, except where specifically noted.

Over the first few days after amputation of the distal 400 μm of a PN3 digit, the epidermis closes across the wound. As has previously been noted for adult mouse digit tip regeneration, the steps of epidermal closure are the same from digit to digit, whereas the timing is variable (21). It is not unusual to observe 1- to 2-d variability in each regenerative stage among littermates or between different genetic backgrounds or drug treatment groups. Fig. 1 shows an idealized course of events. Immediately after amputation a superficial blood clot forms at the amputation site. Over the first few days after amputation of the distal 400 μm of a PN3 digit, the epidermis closes across the wound. As has previously been noted for adult mouse digit tip regeneration, the steps of epidermal closure are the same from digit to digit, whereas the timing is variable (21). It is not unusual to observe 1- to 2-d variability in each regenerative stage among littermates or between different genetic backgrounds or drug treatment groups.

**Fig. 1.** Lineage of Krt14-expressing keratinocytes during digit tip regeneration. (A–E) X-gal stained (blue) Krt14-CreESR;R26R-lacZ digit sections from 3 dpa to 1 wkPA. (F and G) TdTomato (red) fluorescence overlaying differential interference contrast brightfield (grayscale) images of Krt14-CreESR1;R26R-CAG-tdTomato digit sections at 2 wkPA and 3 wkPA. Epidermal retraction and proximal attachment to terminal phalanx occurs between 1 and 3 dpa (A and A’, arrows). Dotted line shows approximate plane of amputation. Dially exposed bone becomes integrated into clot (B, B’, C, and C’). Blastema formation occurs after epidermal closure (D and D’). Blasta- xia- a, bone; c, clot; ct, connective tissue; e, epidermis. (Scale bars, 100 μm.)

**Fig. 2.** Lineage of Sp7-expressing osteoblasts during digit tip regeneration. X-gal stained Sp7-tTA-tetO-EGFP::Cre;R26R-lacZ digit sections. (A and A’) Images at 2 wkPA show descendants in clot (arrow), bone, and blastema, but not the epidermis. (B and B’) Images at 3 wkPA show descendants in bone and connective tissue but not epidermis. Staining resembles normal 3 wk nonregenerative digit osteoblast contribution (C and C’). Insets in A’–C’ show 40× magnification. bl, blastema; b, bone; ct, connective tissue; e, epidermis. (Scale bars, 100 μm.)
The epidermis continues to consist of marked cells (tdTomato) at 2 wkPA and 3 wkPA (Fig. 1 F, F’, G, and G’), indicating that the marked keratinocytes that form the initial regenerative epithelium continue to maintain the epidermis as the digit tip regenerates. Hence, epidermal stem cells as well as mature keratinocytes of the regenerative epidermis are derived from the ectodermal tissue overlying the stump. Most importantly, our analysis of postamputation digits up to 3 wkPA does not show significant keratinocyte descendants in any tissue type other than the epidermis. Krt14-expressing keratinocyte descendants are not found in the clot, bone, or connective tissue, demonstrating that no transdifferentiation occurs from the ectodermal germ layer during digit tip regeneration. Conversely, we never see contribution to the regenerative epidermis from the various mesodermally derived cell populations analyzed below.

**Regenerated Bone Derives from Lineage-Restricted Preamputation Osteoblasts.** The distal 400 μm of the PN3 mouse digit consists primarily of bony and connective tissues, with no muscle or tendon present at this level. Moreover, there is no cartilage: the terminal phalanx forms during limb development by two discrete processes, endochondral ossification via chondrocytes limited to the growth plate of its proximal end, and direct ossification via osteoblasts at its distal tip (25). Although muscle, tendons, and cartilage are all absent from the amputation plane, it was nonetheless possible that these proximal tissues could contribute pools of cells that would migrate into the blastema after amputation injury. To test this, we addressed the possibility that chondrocytes contribute to the regenerated bone with the Col2a1-CreERT allele (26) with the R26R-lacZ Cre reporter line and found that Col2a1-expressing chondrocytes do not contribute to any tissues of the regenerated digit (SI Text and Fig. S3).

We turned our attention to the descendants of the pre- amputation osteoblasts, to verify that they give rise to the regenerating skeletal tissues and to test whether they remain lineage- restricted or dedifferentiate to give rise to multiple tissues in the regenerative digit. To address the contribution of postamputation osteoblasts to the regenerate, we used the Sp7-atac-tetO-EGFP::Cre allele (27) with the R26R-lacZ Cre reporter line. Sp7 is known to specifically mark osteoblasts in both endochondral and intramembranous bones. In this cross, Cre is expressed in osteoblasts throughout development until doxycycline is administered on PN0 and throughout the rest of the experiment to repress Cre expression. PN3 digits were amputated and regenerates were analyzed 2 wkPA and 3 wkPA. (Mice developed and regenerated significantly slower than animals from the other crosses in this report. In this set of experiments, all regenerative events are slightly delayed.) By 2 wkPA, Sp7-expressing osteoblast descendants are found in the blastema, the newly forming bone, and the clot at the distal tip of the regenerate, but not the epidermis (Fig. 2 A and A’). The large concentration of osteoblast descendants in the clot likely reflects the observed trapping of a fragment of the distal bone into the clot as the epidermis closes underneath it (Fig. 1). By 3 wkPA the clot has sloughed off, and the digit is largely regenerated, with preamputation osteoblasts contributing exclusively to the regenerated bone and the adjacent periosteum (Fig. 2 B and B’). In unamputated contralateral digits, Sp7-expressing osteoblasts are also found to contribute to these two tissues by 3 wk (Fig. 2 C and C’), thus indicating that preamputation osteoblasts serve as fate-restricted progenitors, only repopulating tissues in which they were normally expressed. Importantly, no x-gal stained cells were found in the epidermis, indicating that there is no transdifferentiation of mesodermally derived osteoblasts into the ectodermal lineage.

**Mx1 Is Expressed More Broadly than Previously Appreciated.** The necessity of Mx1 for successful digit tip regeneration (19) led us to evaluate its expression pattern throughout the process, to ensure that we have a complete understanding of the tissues involved. Using the Mx1-nlacZ knockin line (28), we analyzed

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**Fig. 3.** Expression of Mx1 during digit tip regeneration. Mx1-nlacZ x-gal stained digit sections during regeneration. PN3 (A) and 3 wk (B) unamputated digits show expression in dorsal and ventral dermis and throughout bone and sweat glands. (C and C’) Expression at 3 dpa is in normal stump domains and in the clot. (D and D’) Expression at 5 dpa is in the clot and a few bone cells where epidermis is closing under the clot (arrow). (E and E’) Expression at 1 wkPA remains in the clot but is absent from the blastema. (F and F’) Expression at 3 wkPA returns to a nonregenerative expression pattern and resembles B. Epidermis is outlined in higher-magnification pictures. bl, blastema; b, bone; c, clot; e, epidermis; np, nail plate; sg, sweat gland. (Scale bars, 200 μm.)

Because the Krt14-dependent Cre genetically causes recombination and hence activation of the reporters exclusively in the PN0 epidermal cells and is not active during regeneration, x-gal staining allows for lineage analysis of stomp keratinocytes throughout regeneration. At 3 dpa, marked cells are seen throughout the epidermis surrounding the lateral stump and extending to the retracted edge of the epidermis, where it reattaches to the bone, just proximal to the amputation plane (Fig. 1 A–C). By 6 to 7 dpa, stump-derived keratinocytes are clearly incorporated into the newly formed epidermis (Fig. 1 D, E, D’, and E’). From these experiments we find that most, if not all, of the regenerated epithelium is descended from preamputation keratinocytes. As seen at 3 dpa, there are a few unlabeled cells in the regenerative epidermis, likely reflecting incomplete penetrance of the Cre activity, although we cannot rule out a minor contribution from a distinct cell pool. The epidermis continues to consist of marked cells (tdTomato) at 2 wkPA and 3 wkPA (Fig. 1 F, F’, G, and G’), indicating that the marked keratinocytes that form the initial regenerative epithelium continue to maintain the epidermis as the digit tip regenerates. Hence, epidermal stem cells as well as mature keratinocytes of the regenerative epidermis are derived from the ectodermal tissue overlying the stump. Most importantly, our analysis of postamputation digits up to 3 wkPA does not show significant keratinocyte descendants in any tissue type other than the epidermis. Krt14-expressing keratinocyte descendants are not found in the clot, bone, or connective tissue, demonstrating that no transdifferentiation occurs from the ectodermal germ layer during digit tip regeneration. Conversely, we never see contribution to the regenerative epidermis from the various mesodermally derived cell populations analyzed below.

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**Mx1 Is Expressed More Broadly than Previously Appreciated.** The necessity of Mx1 for successful digit tip regeneration (19) led us to evaluate its expression pattern throughout the process, to ensure that we have a complete understanding of the tissues involved. Using the Mx1-nlacZ knockin line (28), we analyzed
preamputation PN3 digit tips and found the nail bed mesenchymal expression to be the largest and most homogenous domain of expression (Fig. 3A). Msx1 was also expressed in the lateral dermis (still contiguous with the nail) as well as, to a lesser degree, the ventral dermis (not associated with the nail) (Fig. 3A), domains that were not previously reported. Additionally, many lacZ-positive cells are found within the proximal articular cartilage, the growth plate, tendon attachment points, and the periostium (Fig. 3A), all of which have been previously noted for long bones (29). Msx1 is also expressed within the sweat glands of the ventral fat pad, blood vessels, and hair follicles, but these tissues are not directly analyzed in this study.

During regeneration there are dynamic changes to the expression of Msx1 at the amputation plane. By 3 dpa the expression of Msx1 in the stumps seems unchanged, but a new expression domain forms in the clot surrounding the piece of trapped bone, in which there are also sparse Msx1-expressing cells (Fig. 3C and C'). At 5 dpa Msx1 expression is still maintained in the clot, and additional expression can now be found in the bone, perhaps osteoclasts (29), specifically where the epidermis is closing under the clot (Fig. 3D and D'). By 7 dpa the epidermis has closed, and the blastema has formed. Msx1 expression remains in the clot and is absent from the blastema (Fig. 3E and E'). By 2 wkPA the clot has sloughed off, and by 3 wkPA (Fig. 3F and F') regeneration is complete and the expression of Msx1 once again resembles that of an unamputated control digit (Fig. 3B).

**Cells Expressing Msx1 in the Preamputation Digit Contribute Broadly to the Regenerate.** To explore the contribution of Msx1-expressing cells to the regenerate, we used the Msx1-CreERT2 tamoxifen-inducible Cre allele (30) with the R26R-CAG-tdTomato Cre reporter line* (24). Intraperitoneal tamoxifen was given to PN0 pups, followed by digit tip amputation on PN3. By 1 wkPA (Fig. 4A–A'), descendants of Msx1-expressing cells are found in the clot (Fig. 4A' and A'') as well as the blastema (Fig. 4A''), but not the epidermis. The descendants found in the clot are consistent with the Msx1 clot expression domain (Fig. 3C–E) and may be the same population of cells. The descendants found in the blastema, however, represent a cell population that does not actively express Msx1 (Fig. 3E), indicating a change in gene expression and perhaps cell type as the cells enter and proliferate in the blastema. By 2 wkPA (Fig. 4B–B') the clot is mostly lost, and Msx1 descendants are found within the bone and dermis, but not the epidermis. At 3 wkPA (Fig. 4C–C') Msx1 descendants have heterogeneously populated the bone and dermis of the regenerate, resembling the normal expression pattern of Msx1 at this stage (Fig. 3F).

Importantly, Msx1 descendants were not found within the epidermis, once again indicating that there is no transdifferentiation between the mesodermal and ectodermal germ layers. Because Msx1 is expressed in a diverse set of cell types before amputation, we cannot conclude that Msx1-expressing cells do not transdifferentiate into another Msx1-expressing cell type. However, it is clear that the set of cell types expressing Msx1 before amputation is congruent to the cell types to which their derivatives contribute. Because Msx1 is expressed more broadly than just the nail bed mesenchyme, and because no alternative unambiguous markers exist for this tissue, we cannot determine the fate of these specific cells in the regenerate. Thus, it remains unclear whether the nail bed mesenchymal cells give rise exclusively to the regenerated nail bed mesenchyme and whether they represent a true cell type or simply a dense accumulation of dermal cells.

**Regeneration Is Impaired by Removal of Fibrous Clot.** In our lineage analyses we found that Sp7 and Msx1 descendants populate the clot after amputation (Fig. 2A and 4A). To address whether the fibrin clot at the distal tip of the regenerating digit serves a regenerative function in addition to classical wound healing, we removed the clot at time points correlating with the closure of the regenerative epithelium. For technical reasons the clot could not be removed cleanly before 6 dpa. Regeneration, as assayed by bone outgrowth, is compromised compared with control regenerates, when the clot was removed at 6 dpa or 7 dpa (P < 2.205e−14 and P < 0.0001, respectively), but not 8 dpa (P > 0.416) (Fig. 5). Moreover, removal of the clot at 6 dpa often produces small regenerates that near the size of a nonregenerated digit (Fig. 5). Whereas at 1 wkPA are in blastema (A'') and clot (A'') but not epidermis (A–A''), (B–B') Descendants at 2 wkPA are found in bone and dermis but not epidermis. (C–C') Descendants at 3 wkPA have reestablished normal Msx1 expression pattern of the distal tip. (A–C) Msx1 expression with no overlay. (A–A'', B, B'', C, and C') Differential interference contrast brightfield images (grayscale) with tdTomato (red) fluorescent overlay. bl, blastema; b, bone; cl, clot; d, dermis; e, epidermis; np, nail plate. (Scale bar, 100 μm.)

*The R26R-lacZ reporter allele used in some of the previous experiments generally displays robust expression when activated via Cre-mediated recombination and was used interchangeably with the R26R-CAG-tdTomato reporter. However, in some tissues reporter expression is much weaker and hence the use of the R26R-CAG-tdTomato allele was favored, as was the case here (Fig. S4). Although less dramatic, the staining from the R26R-lacZ reporter allele is consistent with the R26R-CAG-tdTomato results described below.
importance of clot removal because puncturing the skin may lead to new clot formation, possibly rescuing regeneration.

**Discussion**

**Fate-Restricted Progenitor Model of Mouse Digit Tip Regeneration.** Using tissue-specific, inducible Cre alleles, we were able to show that the neonatal digit tip regenerates via fate-restricted progenitors derived from the preamputation tissue. First we established that there is no transdifferentiation between tissue layers during regeneration. Following Krt14-marked keratinocytes during regeneration, we were able to show that although the regenerative epithelium is derived from preexisting stump keratinocytes, these cells do not contribute to any other lineage in the regenerate and remain fate-restricted to the epidermis. In complementary experiments, we show that mesodermally derived tissues, marked by Sp7 and Msx1, contribute only to mesoderm lineages, never ectoderm. Moreover, Sp7-expressing osteoblasts from the limb only contribute to skeletal tissues (bone and periosteum) in the regenerate, indicating that at least these blastema cells are lineage restricted. Altogether, the lineage data we present are consistent with and support reports of fate-restricted progenitor cells contributing to the regenerates of zebrafish fin, *Xenopus* tail, and axolotl limb (9–11, 31); and the emerging model that transdifferentiation between germ layers or tissue types does not occur during appendage regeneration. Additionally, these results indicate that digit tip regeneration may be more analogous to other examples of appendage regeneration than previously believed.

During the preparation of this manuscript, Rinkevich et al. (32) published a report focusing on cell lineage restriction during adult mouse digit tip regeneration, using a similar approach to that taken here. Their conclusion, that lineage-restricted progenitor cells repopulate the digit tip without transdifferentiation, corroborates the findings in our report. As in our study, Rinkevich et al. demonstrated that the ectoderm of the regenerate is derived from preamputation progenitor cells and that these cells do not contribute to any other lineage or germ layer. Moreover, they showed that no mesodermally derived tissue transdifferentiates into an ectodermal fate and all cells remain faithful to their original lineage.

**Insight into Human Adult Digit Tip Regeneration.** Appendage regeneration occurs in teleost fish and urodele amphibians throughout life, yet only larval anurans are capable of limb regeneration. Similarly, mice can regenerate their digit tips throughout life, whereas humans lose the ability with age. Hypotheses on the lack of human adult digit tip regeneration include the following: preferential inflammation response over regenerative response in adults, tumor suppressor gene-mediated inhibition of progenitor cell hyperproliferation in postneonatal tissues, age-dependent progenitor cell depletion, and neonatal and adult digit tip regeneration occurring by two discrete processes (of which humans have lost the latter). Although the majority of these hypotheses are beyond the scope of this work, our data combined with other mouse digit tip reports, can provide insight into whether the neonate and adult processes, as exemplified in the mouse, are the same.

It is becoming increasingly clear that neonatal and adult mouse digit tip regeneration are much more similar than previously appreciated. Fernando et al. (21) showed that during regeneration in adult mice the terminal phalanx regenerates via direct ossification, consistent with the study by Han et al. (20), in which the same was found during neonatal regeneration. Our report builds upon these commonalities and reveals that the regenerative epithelium of the neonatal postamputation digit tip forms at a variable rate and includes a fragmentation of the terminal bone at a more proximal level, highly similar to what is described for the adult. Moreover, our data provide evidence against a single pluripotent blastema cell type and support a mechanism of multiple fate-restricted progenitor populations participating in neonatal digit tip regeneration, which is consistent with what Rinkevich et al. (32) found during adult mouse digit tip regeneration. Collectively, the data support a single process used both during neonatal and adult mouse digit tip regeneration, and the question remains what the fundamental difference is in adult human digit tips that prevents successful regeneration.

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Why Is Msx1 Necessary for Digit Tip Regeneration? The requirement of Msx1, coincident with its expression in the nail bed, led to the hypothesis that the Msx1-expressing nail bed mesenchymal cells could serve as a stem cell population for the regenerate. Our data show that although Msx1 cells do broadly contribute to the blastema and their own lineages in the regenerates, they do not act in isolation, and multiple other progenitor populations actively participate in the process. This suggests that the necessity of Msx1 during regeneration may be due to the signaling properties of the cells rather than the strict necessity of the descendant population, although a combination of the two remains possible.

Msx1 has been shown to be an important mediator of Bone Morphogenetic Protein (BMP) signaling during digit tip regeneration (19), and it follows that Msx1-expressing cells may be necessary to mediate BMP activity after amputation. In this report we show that Msx1 is expressed in the distal clot. The clot has not received much attention in regard to digit tip regeneration because it is a structure that forms routinely upon injury to the mammalian skin, but by lineage analyses we show that Msx1 and Sp7 descendants reside within the clot, suggesting a functional role during regeneration, possibly beyond typical wound healing. By removing the clot, we provide preliminary data in support of this hypothesis. In urodele amphibians, successful limb regeneration depends on mitogenic signals provided by the nerve. In contrast, mouse digit tip regeneration proceeds even in the absence of innervation (33), suggesting that if these are analogous processes, the necessary signals are coming from another source. Agrawal et al. (34) provide support for this hypothesis by showing that at nonregenerative levels in digits of adult mice, application of ECM degradation products to the wound, as an artificial source of signals, led to the accumulation of stem-like cells at the plane of amputation. Collectively there is clearly a delicate balance between wound healing and regeneration, and although clot formation is typically associated with a wound healing response, it will be interesting to further explore the specific function(s) the clot serves during digit tip regeneration.

Materials and Methods

Mouse Strains and Alleles. Msx1-nlacZ mice were described previously (28). The Msx1-CreERT2 allele was generated by introducing the CreERT2 coding sequence (35) (a kind gift of Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France) at the initiator ATG site of Msx1 by homologous recombination (30). Additional inducible Cre mouse strains Sp7-TA-tetO-EGFP::Cre (27), Krt14-CreER(T2) (22), and Col2a1-CreERT (26) were obtained from Jackson Laboratories (strains 006361, 005107, and 006774). Cre reporter alleles R26R-CAG-tdTomato (24) and R26R-lacZ (23) were also obtained from Jackson Laboratories (strains 007905 and 003474). All crosses were performed with compound heterozygous males carrying both the inducible Cre driver and Cre reporter alleles and a CD1 wild-type female with the exception of the Sp7-TA-tetO-EGFP::Cre allele, which was crossed directly to the R26R-lacZ reporter.

Digit Manipulations. All mouse breeding and manipulations were done in accordance with the Harvard Medical School Institutional Animal Care and Use Committee. Mouse digit tip amputations were done on PNS as previously reported (20). A dissecting microscope (Leica M26) fitted with an eyepiece reticle was used for digit amputations. Pups were cryoanesthetized before 400 μm of hindlimb digits 2, 3, and 4 were amputated using microdissection spring scissors (FST 15003-08). In all animals, right hindlimb digits were left unamputated as controls. One dose of 0.05 mg/kg s.c. buprenorphine was given as postprosurgical analgesia.

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References

Supporting Information

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SI Text

Mouse Neonatal Digit Tip Regeneration is Robust and Level Dependent. To reliably assess the lineage of cells during mouse neonatal digit tip regeneration, we first sought out the optimal amputation level for regeneration as well as the most robust strain for digit tip regeneration. Successful mouse digit tip regeneration has already been shown to be reliant on the level of amputation, whereby distal amputations of up to 50% of the terminal phalanx will regenerate but proximal amputations containing 67–75% of the terminal phalanx typically will not (1, 2). Using the average CD1 postnatal day 3 (PN3) terminal segment length as 800 μm, we amputated digits 2–4 at different fixed levels for multiple litters. Measuring from the distal tip, cuts were made at 200 μm, 264 μm, 400 μm, 528 μm, and 706 μm with all pups in a given litter amputated at the same level. At 6 wk after (post) amputation (wkPA) all digits were analyzed, confirming that the level of regenerative failure exists within the proximal third of the neonatal digit tip (Fig. S1). On the basis of these results all regenerative assays in this report are performed by amputating half of the terminal phalanx (400 μm), thus allowing for reliable yet significant regeneration.

To assure that no common inbred mouse strain had a more robust regenerative response than the outbred CD1 strain, we performed a comparative mouse strain regeneration assay. Distal amputations (400 μm) were done on CD1, C57BL/6, DBA, BALB/c, and C3H PN3 pups (Fig. S2). This experiment demonstrates that regeneration occurred in all of the strains tested, yet there was a range in outcomes. Eighty-five percent of the digits analyzed from CD1 mice regenerates to 91–100% of unamputated size. The smaller regenerates found in the remaining 15% are most likely due to a combination of technical variation in amputation plane and variation in the regeneration process itself. All other strains tested were capable of successful regeneration to unamputated digit size, albeit at much different rates (C3H: 74%; BALB/c: 52%; C57BL/6: 29%; DBA/2: 17%). Analysis of 2D digit area of 3-wk-old unamputated digit controls revealed variation in the terminal phalanx size (C3H: 228,908 pixels (px); CD1: 227,994px; BALB/c: 201,224px; C57BL/6: 202,723px; DBA/2: 186,262px) presumably already present at PN3. Distal amputations (400 μm) were clearly not comparable among strains, and this amputation level may near the non-permissive zone on the smaller digits. For this report we chose to use CD1 mice when possible but verified that intercrossing this strain with alleles on different backgrounds would not affect regeneration.

Col2a1-Expressing Chondrocytes Do Not Participate in Digit Tip Regeneration. Col2a1-CreERT;R26R-lacZ mice were used to track Col2a1-expressing chondrocytes during digit tip regeneration. Col2a1 is a well-established marker of differentiating chondrocytes. Strain variation amputation experiments used C57BL/6, DBA2, C3H, BALB/c, and CD1 WT mice from Charles River Laboratories (strains 007905 and 003474). Cre reporter alleles R26R-lacZ were crossed directly to the R26R-tetO-EGFP::Cre allele (7), Krt14-CreESR1 (8), and Col2a1-CreERT (9) were obtained from Jackson Laboratories (strains 006361, 005107, and 006774). Cre reporter alleles R26R-CAG-tetTomato (10) and R26R-lacZ (11) were also obtained from Jackson Laboratories (strains 007905 and 003474). All crosses were performed with compound heterozygous males carrying both the inducible Cre driver and Cre reporter alleles and a CD1 WT female, with the exception of the Sp7-tTA-tetO-EGFP::Cre allele, which was crossed directly to the R26R-lacZ reporter.

Regeneration Studies. To assure that no common inbred mouse strain had a more robust regenerative response than the outbred CD1 strain, we performed a comparative mouse strain regeneration assay. Distal amputations (400 μm) were done on CD1, C57BL/6, DBA, BALB/c, and C3H PN3 pups (Fig. S2). This experiment demonstrates that regeneration occurred in all of the strains tested, yet there was a range in outcomes. Eighty-five percent of the digits analyzed from CD1 mice regenerates to 91–100% of unamputated size. The smaller regenerates found in the remaining 15% are most likely due to a combination of technical variation in amputation plane and variation in the regeneration process itself. All other strains tested were capable of successful regeneration to unamputated digit size, albeit at much different rates (C3H: 74%; BALB/c: 52%; C57BL/6: 29%; DBA/2: 17%). Analysis of 2D digit area of 3-wk-old unamputated digit controls revealed variation in the terminal phalanx size (C3H: 228,908 pixels (px); CD1: 227,994px; BALB/c: 201,224px; C57BL/6: 202,723px; DBA/2: 186,262px) presumably already present at PN3. Distal amputations (400 μm) were clearly not comparable among strains, and this amputation level may near the non-permissive zone on the smaller digits. For this report we chose to use CD1 mice when possible but verified that intercrossing this strain with alleles on different backgrounds would not affect regeneration.

Col2a1-Expressing Chondrocytes Do Not Participate in Digit Tip Regeneration. Col2a1-CreERT;R26R-lacZ mice were used to track Col2a1-expressing chondrocytes during digit tip regeneration. Col2a1 is a well-established marker of differentiating chondrocytes. Mothers were delivered tamoxifen by gavage such that PN0 pups received tamoxifen via nursing, thus lacZ marking Col2a1-expressing chondrocytes in the proximal growth plate of the distal phalanx. PN3 digits were amputated and analyzed for Col2a1 descendant contribution to the regenerate at 1 wkPA. At this stage we found no contribution from Col2a1-expressing chondrocytes to the regenerating digit (Fig. S3 B and B'). X-gal stained chondrocytes continued to reside within the growth plate, far proximal to the amputation plane, and never migrated or divided into the regenerating tissue. At later stages, no Col2a1-expressing chondrocyte descendants were observed outside of the growth plate, including the clot, blastema, bone, skin, or connective tissue (Fig. S3B and B'). Moreover, lineage of Col2a1-expressing chondrocytes in contra-

lateral control unamputated digits shows a similar x-gal staining pattern, indicating that postnatal chondrocytes are not contributing to the normal development of the terminal phalanx (Fig. S3C). In contrast, postnatal chondrocytes still contribute to the long bone of the more proximal digital segment (Fig. S3D). Although in principle cartilage-derived cells could have transdifferentiated into osteoblasts, the lack of chondrocyte contribution to the regenerate is consistent with the finding that bone regrowth occurs by direct ossification in both neonatal and adult digit tip regeneration (2, 3).

SI Materials and Methods

Mouse Strains and Alleles. Amputation level regeneration studies used CD1 WT mice (Charles River Laboratories strain 022) and were harvested 6 wkPA. Strain variation amputation experiments used C57BL/6, DBA2, C3H, BALB/c, and CD1 WT mice from Charles River Laboratories (strains 027, 026, 025, 028, and 022) and were harvested 3 wkPA. Msx1-lacZ mice were described previously (4). The Msx1-CreERT2 allele was generated by introducing the CreERT2 coding sequence (5) (a kind gift of Pierre Chamoun, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France) at the initiator ATG site of Msx1 by homologous recombination (6). Additional inducible Cre mouse strains Sp7-tTA-tetO-EGFP::Cre (7), Krt14-CreESR1 (8), and Col2a1-CreERT (9) were obtained from Jackson Laboratories (strains 006361, 005107, and 006774). Cre reporter alleles R26R-CAG-tetTomato (10) and R26R-lacZ (11) were also obtained from Jackson Laboratories (strains 007905 and 003474). All crosses were performed with compound heterozygous males carrying both the inducible Cre driver and Cre reporter alleles and a CD1 WT female, with the exception of the Sp7-tTA-tetO-EGFP::Cre allele, which was crossed directly to the R26R-lacZ reporter.

Recombinase Induction. For tamoxifen-inducible alleles, 20 mg/mL tamoxifen (Sigma) in corn oil was administered at 3 mg/40 g mouse body weight. Cre induction of Col2a1-CreERT and some Krt14-CreESR1 PN0 pups was done by gavage of the nursing mother, thus allowing for tamoxifen delivery through the mother’s milk. PN0 pups from Msx1-CreERT2 and some Krt14-CreESR1 experiments were dosed with tamoxifen by IP injections. For the Sp7-tTA-tetO-EGFP::Cre allele, 1 mg/mL doxycycline in 5% sucrose drinking water was administered to the mother and nursing babies from PN0 throughout the remainder of the experimental time course. Number of regenerates analyzed and mode of Cre induction for each experiment are summarized in Table S1.

Digit Manipulations. All mouse breeding and manipulations were done in accordance with the Harvard Medical School IACUC. Mouse digit tip amputations were done on postnatal day 3 as previously reported (2). A dissection microscope (Leica MZ6) fitted with an eyepiece reticule was used for digit amputations. Pups were cryoanesthetized, and 400 μm of hindlimb digits 2, 3, and 4 was amputated using micродissection spring scissors (Fine Science Tools 15003-08). In all animals, right hindlimb digits were left unamputated as controls. One dose of 0.05 mg/kg s.c. buprenorphine was given as postsurgical analgesia.

IP ketamine/xylazine was used to anesthetize mice to remove the fibrous clots from the regenerating digits at 6, 7, or 8 dpa. Sterile forceps were used to mechanically remove the clot structure without puncturing the newly formed underlying epidermis. If bleeding was induced, it was noted as an unsuccessful attempt, although the regenerative data reflect all of the animals and not just
those self-reported as successful. Nonregenerate digits were generated by removing the nail at 6 dpa.

**Tissue Histology.** To analyze regenerated tissues, animals were euthanized, and digits were collected for sectioning and reporter expression analysis. Digits expressing tdTomato were fixed in 4% paraformaldehyde and digits expressing lacZ were fixed in 0.2% gluteraldehyde. All digits were processed through a sucrose gradient over 2 d (5–30%) and embedded in OCT (Tissue-Tek). Twenty-micrometer sections were made using a Leica Cryostat CM3050S, air-dried for 30 min, and then immediately processed for reporter expression. Tissues expressing tdTomato were washed in PBS, counterstained with DAPI, and coverslipped with Fluormount-g aqueous mounting medium (Southern Biotech). Tissues expressing lacZ were stained by a standard X-gal staining protocol (12), with the exception that slides were stained overnight at room temperature in the dark. X-gal–stained slides were dehydrated through an ethanol gradient, counterstained with eosin, cleared with HistoclearII (National Diagnostics), and mounted with HistoMount (National Diagnostics). Sections were imaged on a Zeiss axiophot light microscope.

**Skeletal Staining.** All digits analyzed by whole mount skeletal staining were dehydrated through an ethanol gradient, counterstained with eosin, cleared with HistoClearII (National Diagnostics), and mounted with HistoMount (National Diagnostics). Sections were imaged on a Zeiss axiophot light microscope.

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12. Vasioukhin V, Lehoczky et al. (2011) Skeletal stained wild-type CD1 6-wk regenerates resulting from varied levels of amputation at PN3. (A) Unamputated control digit. (B–F) Regenerated digit tips after PN3 amputation of one-quarter (B, 200 μm), one-third (C, 264 μm), half (D, 400 μm), two-thirds (E, 528 μm), and greater than two-thirds (F, 700 μm). All amputation planes distal to 528 μm (two-thirds) successfully regenerated, whereas cuts proximal to that level fail to mount a regenerative response. Each digit shown represents the average regenerative outcome from an entire litter amputated at the same level.
Fig. S2. Comparison of digit tip regeneration among mouse strains. Digital 2D area was measured for all regenerates and unamputated controls of the strains CD1, C3H, C57BL/6, BALB/c, and DBA/2. Each graph presents number of digits analyzed as well as average unamputated control digit area. The y axis is number of digits, and the x axis is amount of regeneration represented as a percentage of the average unamputated digit area. All strains had digits with complete regeneration (91–100%), but C57BL/6, BALB/c, and DBA/2 had higher rates of regenerative failure, possibly due to smaller unamputated control digit size.

Fig. S3. Col2a1-expressing chondrocytes do not contribute to the regenerating digit tip. X-gal stained Col2a1-CreERT;R26R-lacZ digit sections cut parallel to the nail. (A) Wild-type 1 wkPA regenerating digit to provide relative reference points for B–D. (B and B') Digits harvested 1 wkPA show x-gal stained chondrocytes within the proximal growth plate, far from the plane of amputation, indicating no contribution to any lineage of the regenerate. (C) Contralateral control digit shows no postnatal chondrocyte contribution to the normally developing terminal bone, as is seen in more proximal segments (D). b, bone; ct, connective tissue; e, epidermis. (Scale bars, 100 μm.)
Fig. S4. Irregular punctate staining from the R26R-lacZ Cre reporter allele. X-gal stained distal digit nail bed. (A) Perinuclear x-gal staining of the dermis in tamoxifen-induced Msx1-CreERT2;R26R-lacZ mouse. (B) X-gal staining is not found in any tissue of a tamoxifen-induced R26R-lacZ littermate. b, bone; d, dermis; e, epidermis. (Scale bars, 50 μm.)

Fig. S5. Scatterplot of clot removal data. Raw data from clot removal experiment scattered rather than box plots. Data illustrate that there may be technical limitations to the assay yet still underscore a clear effect of clot removal.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stage(s) analyzed</th>
<th>No. regenerated digits analyzed</th>
<th>Mode of Cre induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krt14-CreESR1;R26R-lacZ</td>
<td>3–6 dpa</td>
<td>21</td>
<td>i.p. tamoxifen</td>
</tr>
<tr>
<td></td>
<td>1 wkPA</td>
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<td>i.p. tamoxifen</td>
</tr>
<tr>
<td></td>
<td>2 wkPA</td>
<td>6</td>
<td>Gavage tamoxifen</td>
</tr>
<tr>
<td></td>
<td>3 wkPA</td>
<td>9</td>
<td>Gavage tamoxifen</td>
</tr>
<tr>
<td>Krt14-CreESR1;R26R-tdToma</td>
<td>2 wkPA</td>
<td>6</td>
<td>i.p. tamoxifen</td>
</tr>
<tr>
<td></td>
<td>3 wkPA</td>
<td>3</td>
<td>i.p. tamoxifen</td>
</tr>
<tr>
<td>Col2a1-CreERT;R26R-lacZ</td>
<td>1 wkPA</td>
<td>9</td>
<td>Gavage tamoxifen</td>
</tr>
<tr>
<td></td>
<td>2 wkPA</td>
<td>12</td>
<td>Gavage tamoxifen</td>
</tr>
<tr>
<td></td>
<td>3 wkPA</td>
<td>8</td>
<td>Gavage tamoxifen</td>
</tr>
<tr>
<td>Sp7-tTA-tetO-EGFP::Cre;R26R-lacZ</td>
<td>2 wkPA</td>
<td>18</td>
<td>Doxycycline in water</td>
</tr>
<tr>
<td></td>
<td>3 wkPA</td>
<td>9</td>
<td>Doxycycline in water</td>
</tr>
<tr>
<td>Msx1-CreERT2;R26R-tdToma</td>
<td>1 wkPA</td>
<td>21</td>
<td>i.p. tamoxifen</td>
</tr>
<tr>
<td></td>
<td>2 wkPA</td>
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<td>i.p. tamoxifen</td>
</tr>
<tr>
<td></td>
<td>3 wkPA</td>
<td>12</td>
<td>i.p. tamoxifen</td>
</tr>
<tr>
<td>Msx1-lacZ</td>
<td>2–6 dpa</td>
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<tr>
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<td>30</td>
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</tr>
<tr>
<td></td>
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</table>

Summary of animals used in this report, with detailed information regarding mode of Cre induction (where appropriate), the stages at which regenerates were analyzed, and the number of regenerated digits that were analyzed for each time point. dpa, days after (post) amputation.