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REVIEW

Mammalian Regeneration and Regenerative Medicine

Ken Muneoka,* Christopher H. Allan, Xiaodong Yang, Jangwoo Lee, and Manjong Han

Mammals are generally considered to be poor regenerators, yet there are a handful of mammalian models that display a robust ability to regenerate. One such system is the regenerating tips of digits in both humans and mice. In vitro studies of regenerating fetal human and mouse digit tips display both anatomical and molecular similarities, indicating that the mouse digit is a clinically relevant model. At the same time, genetic studies on mouse digit tip regeneration have identified signaling pathways required for the regeneration response that parallel those known to be important for regeneration in lower vertebrates. In addition, recent studies establish that digit tip regeneration involves the formation of a blastema that shares similarities with the amphibian blastema, thus establishing a conceptual bridge between clinical application and basic research in regeneration. In this review we discuss how the study of endogenous regenerating mammalian systems is enhancing our understanding of regenerative mechanisms and helping to shed light on the development of therapeutic strategies in regenerative medicine. **Birth Defects Research (Part C) 84:265–280, 2008.** © 2008 Wiley-Liss, Inc.

Key words: regeneration; mammal; digit; finger; blastema; ossification

INTRODUCTION

In this posthuman genome/post-animal cloning era of modern biology, many have turned their attention to the prospect of controlling the regeneration of tissues or organs that do not regenerate in humans. Successes in this new field of Regenerative Medicine would have enormous impact on current medical practices and, as well, on the general quality of human life. Regenerative medicine is strongly influenced by breakthroughs in our understanding of organ and tissue formation during embryogenesis on the one hand, and on the body's endogenous ability to repair wounds following injury on the other. The goal of regenerative medicine is to be able to replace adult body parts on demand, and to this end we can identify three general avenues being taken for the development of novel regeneration therapies. The first is a cell based approach. This approach has grown largely from the successes in the use of hematopoietic stem cells in cell replacement therapies for the cure of blood diseases (Bhattacharya et al., 2008). The potential to expand into other organ systems is driven by (1) the potential of adult stem cells to participate in the formation of various organ systems when introduced in early embryos (Jiang et al., 2002), (2) the feasibility of transforming adult cells into pluripotent stem cells (Yamanaka, 2008), and (3) the isolation and characterization of adult multipotent stem cells from virtually every tissue of the body (Crisan et al., 2008). The second approach involves a bioengineering strategy in which a substrate or scaffold is introduced that can either be infiltrated by host cells (Badylak, 2007), or seeded with selected cells before implantation (Howard et al., 2008). This approach includes the in vitro engineering of specific tissues for use in transplantations, and in doing so sidesteps the problems associated with tissue morphogenesis and patterning that are key to the successful regeneration of injured body parts. However, it does introduce a secondary problem of integrating an engineered tissue with the host that still needs to be addressed (Khan et al., 2008).

The third approach is to study naturally regenerating models for comparison with nonregenerating injury wounds to discover critical

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factors necessary for a regeneration response, and this is the primary topic of this review. This approach represents a long history of experimental inquiry focused largely on invertebrate models that have enhanced regenerative ability (Sanchez Alvarado and Tsonis, 2006) and selected vertebrate groups that possess the ability to regenerate structures such as the limb and tail (Brockes and Kumar, 2005; Gardiner, 2005). Included in this category are studies on the developing appendages of mammals, birds, and frogs which possess regenerative ability that is lost as the animal matures (Muller et al., 1999). Although the leap between regenerating systems and human therapies may seem large, there is substantial evidence that signaling pathways important for regeneration have been conserved through evolution (Sanchez Alvarado, 2000; Brockes et al., 2001), and there are examples of specific signaling pathways or genes that are important for regeneration in both traditionally regenerating and nonregenerating organisms (Taylor et al., 1994; Yokoyama et al., 2000; Beck et al., 2003; Han et al., 2003). In addition, there are a handful of mammalian systems that can successfully regenerate. Studies of these systems are producing important insight into the feasibility of an enhanced endogenous regenerative response in humans, and also in the design of alternative strategies in regenerative medicine, particularly to address the problem of integration with host tissues (see Pendegrass et al., 2006). In this review we highlight studies on appendage regeneration in mammals, with particular emphasis on the regenerating digit tip, in the context of how such efforts may impact the development of successful therapies in regenerative medicine.

MAMMALIAN MODELS OF APPENDAGE REGENERATION

Appendage regeneration has been studied primarily in amphibians with a focus on limb or tail regeneration in adult urodeles (Brockes and Kumar, 2005; Tanaka, 2003) or larval anurans (Slack et al., 2008). While mammals lack similar regenerative capabilities, there are a handful of model systems in which a variation of appendage regeneration has been described, and these models provide a glimpse at the limitations and potential for regeneration in humans. These include the closure of excisional tissues in ears following hole punch in rabbits and mice (Metcalfe et al., 2006), the annual regeneration of antlers in deer (Price et al., 2005; Kierdorf et al., 2007), and the regeneration of amputated digit tips known to occur in humans and rodents (Han et al., 2005). Although the regenerative capability of mammals does not compare with that of amphibians, it is critical to keep in mind that these mammalian models provide insight into how successful regeneration can be accomplished within the context of a warm-blooded terrestrial animal with similarities to humans. Lessons learned from such examples are likely to provide important insight into how to effectively modify the human wound environment to elicit an enhanced regenerative response, or to establish a functional interface with a bioengineered or artificial organ or structure. The mammalian ear punch and the deer antler models have recently been reviewed (Price et al., 2005; Metcalfe et al., 2006; Kierdorf et al., 2007), so a very brief introductory overview is provided here and the reader is directed to these excellent reviews. We will focus most of our attention on the human and mouse digit models that we have explored over the past few years. On the one hand the mouse digit models share anatomical and molecular similarities with human fingertip regeneration making it clinically relevant, and on the other hand, digit tip regeneration is comparable to other well studied regeneration models in that wound healing culminates in the formation of a blastema that mediates the regeneration response.

EARS AND DEERS

The ears of some mammals are able to undergo scar-free healing and regeneration after an excisional hole punch that removes a cylindrical mass of tissue including epidermis, dermis, muscle and cartilage. This response in mammals was first characterized in rabbit ears and later shown to be a characteristic not restricted to lagomorphs (Williams-Boyce and Daniel, 1986). In recent years research on ear hole punch regeneration has been stimulated by the finding that different mouse strains display variability in this regeneration response (Clark et al., 1998; Kench et al., 1999; Li et al., 2001), raising the possibility that the genetic basis of this variation might be uncovered (Heber-Katz, 1999). In mice, a 2-mm diameter hole punch undergoes re-epithelization that involves epidermal closure from the two opposite surfaces of the ear, and centripetal filling in of the hole is driven by growth of a blastema-like structure that forms between the existing ear tissue and the wound epidermis. The MRL strain, also known as the healer strain, displays the highest level of regenerative ability described (Heber-Katz, 1999); however, even in this strain complete regeneration does not always occur (Rajnoch et al., 2003). The regeneration process is characterized by a wound healing response that involves formation of a specialized wound epidermis that integrates the epidermal layers from the inner and outer ear surfaces. This wound healing response is influenced by the nature of the injury and the degree of trauma ear tissues experience (Rajnoch et al., 2003). This has led to the suggestion that a high degree of trauma leads to a regenerative response, whereas a low degree of trauma results in a reparative response (Metcalfe et al., 2006). The existence of a dichotomous switch that triggers an epimorphic regeneration response

versus a wound healing response is an important consideration in the development of regeneration therapies and needs to be further investigated. The wound healing process results in the formation of a blastema-like structure that is continuous around the margin of the wound. The blastema is composed of proliferating cells and as it grows centripetally, the punch hole eventually closes and the epithelial surfaces fuse. Re-differentiation of ear cartilage occurs by extension of the existing cartilage sheet or by the differentiation of cartilaginous islands at the base of the blastema (Rajnoch et al., 2003). In some cases ectopic bone formation has been described during redifferentiation (William-Boyce and Daniel, 1986), suggesting that the cells involved in this response are multipotent and responsive to the wound environment.

The annual regeneration of deer antlers represents another example of a naturally occurring regenerative response in mammals. Deer, along with many of their relatives, shed their antlers annually only to have them undergo a complex regenerative response that involves outgrowth from the pedicle, a bilateral bony protrusion of the frontal bone. Primary antler development occurs during puberty and in response to circulating levels of sex steroids. Antler development involves the initial formation of the pedicle from a specialized periosteum associated with the frontal bone. Pedicle outgrowth and elongation involves many developmental processes beginning with intramembranous ossification to initiate pedicle formation, and is followed by the formation of a distal endochondral growth zone with proximal ossification that continues until the antler is fully developed (Price et al., 2005). Antler shedding or casting normally occurs in the spring and is mediated by enhanced osteoclast activity at the distal region of the pedicle, leaving an open pedicle wound that forms a scab (Goss et al., 1992). The regeneration process is initiated by the closure of the epidermis over the pedicle wound to form a wound epithelium. Beneath the wound epithelium is a dermal layer overlaying a fibrous perichondral layer that is continuous with the periosteal layer of proximal bone. Just proximal to the fibrous perichondrium at the distal end of the regenerating antler is a mesenchymal growth zone (also called the reserve mesenchyme) where cells are actively dividing, and is arguably the antler blastema (Faucheux et al., 2004; Li et al., 2005; Kierdorf et al., 2007). The cells of the mesenchymal growth zone appear to be derived from the pedicle perichondrium and these cells display characteristics of mesenchymal stem cells (Rolf et al., 2008). Proximal to the mesenchymal growth zone are regions containing, in distal to proximal order, chondroprogenitor cells, chondroblasts, hypertrophic chondrocytes, and bone, undergoing endochondral ossification similar to the developing antler. In addition to bone tissue, antler regeneration involves the regeneration of epidermis and its derivatives, dermis, and vasculature.

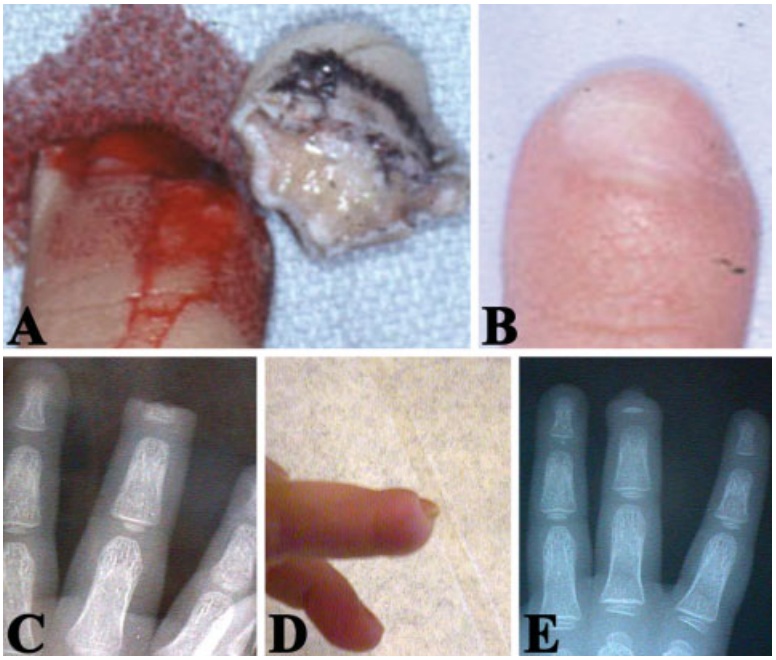

Figure 1. Fingertip regeneration in humans. (**A, B**) A fingertip injury of a 7-year old girl resulted in an amputation at the base of the nail. The injury was treated conservatively with dressing changes and after 8 weeks the fingertip regenerated (From Stocum DL. Regen Biol Med 2006, 394, copyright 2006, Elsevier, reproduced by permission.). (**C**–**E**) A fingertip injury of a 2-year old child resulted in an amputation at a level proximal to the nail. (C) Radiograph at the time of injury indicated that the level of amputation was through the proximal region of the terminal phalangeal bone. (D) The amputation injury was treated conservatively with dressing changes and after 10 months the fingertip healed without significant scarring and a nail rudiment was present. The fingertip had a normal contour and sensibility had returned. (E) Radiographic evidence after healing showed that there was no re-growth of the terminal phalangeal bone and indicated that a regenerative response was not stimulated. (From Han M, Yang X, Lee J, et al. Dev Biol 2008, 315:125–135, Copyright 2008, Elsevier, reproduced by permission.).

FINGERTIP REGENERATION IN HUMANS

The prospect of developing strategies for enhancing regenerative ability in humans is encouraged by clinical observations that the human fingertip is capable of a regenerative response (Fig. 1A, B). While the initial descriptions of fingertip regeneration were made in children (Douglas, 1972; Illingworth, 1974), they were followed by descriptions of fingertip regeneration in adults as well (Lee et al., 1995). The key for human

fingertip regeneration is to treat the amputation wound in a conservative manner, e.g., clean and dress the wound so as to allow it to heal by secondary intention (i.e., without assisted wound closure). It is thought that such conservative treatment in humans promotes the formation of a wound epidermis that is required for the initiation of a regenerative response. Appendage regeneration in amphibians and in embryonic limbs of birds has been shown to have a similar requirement of a specialized wound epidermis for a successful regenerative response (see Muller et al., 1999). Nevertheless, the actual closure of the amputation wound itself in humans is a very slow process, and much of the regenerative growth and remodeling associated with the regeneration response occurs before the completion of wound healing. Thus, it is safe to conclude that continuity of the wound epidermis is not a prerequisite for regeneration, but it is unclear now what role the wound epidermis plays in human regeneration.

Fingertip regeneration in humans is reported to be restricted to the distalmost, or terminal phalangeal element and associated with the nail organ (Illingworth, 1974). The clinical use of the term regeneration is not strictly defined so the clinical description of amputation injuries often involve cosmetic and neurological assessment of soft tissue repair, whereas regenerative studies in animal models generally focus on the restoration of skeletal tissue in addition to soft tissue (Han et al., 2008). Using bone regrowth as definitive evidence for a regenerative response, there is a subset of clinical reports that document fingertip regeneration after conservative management of amputation wounds in both children (Vidal and Dickson, 1993) and adults (Lee et al., 1995). Thus, it is clear that human fingertips display a true regenerative response that establishes the foundation upon which we can begin to explore ways to enhance the regenerative response. As a first step, we provide evidence from a case report that begins to define the proximal extent of regenerative capabilities (Han et al., 2008). This case report involved an amputation injury in the proximal region of the terminal phalangeal bone that was conservatively treated, and because there was X-ray documentation at the time of injury and after the healing response was completed, there is clear indication that a regenerative response that included bone regrowth did not occur (Fig. 1C, D, E). Thus, despite the fact that cosmetic healing and good sensibility of the fingertip was restored, this case report begins to identify the proximal boundary of regenerative ability in humans. Understanding the physical boundaries of regenerative potential in humans is an important first step toward developing a protocol that has predictive value for the treatment of amputation injuries. What is needed is a concerted effort to better document the limits of this amazing regenerative response in humans that would entail radiographic analysis of amputation injuries before and after healing to establish a database that can be used both for predicting clinical outcome and for experimental studies (see below). Since injuries to the hand alone represent ~30% of all reported injuries (Oleske and Hahn, 1992; Angermann and Lohmann, 1993), and there are ~19,000 reported digit amputations per year in the United States alone (Sorock, 1993), it should be possible to establish such a database in a relatively short time frame. This would be the first step toward developing an understanding of the limitations of human regenerative ability and, as well, for the development of therapies to enhance this amazing response.

DIGIT TIP REGENERATION IN MICE

A valuable experimental model for human fingertip regeneration is the digit tip in rodents. The mouse digit tip includes the terminal phalangeal bone which is surrounded by connective tissue and encased within a nail (Fig. 2A). Like human fingertips, regenerative ability is level specific within the terminal phalanx. In adult mice amputations midway through the terminal phalanx result in a robust regenerative response, whereas amputation in which more than 3/4th of the bone is removed fails to mount a response (Neufeld and Zhao, 1995; Han et al., 2008). Because the mouse digit is relatively small, the physical distance between these two amputation injuries is less than 1 mm and the tissue composition of both regenerating and nonregenerating wounds is similar, making this a good model to investigate the cause of regenerative failure. The fact that the mouse digit tip is able to regenerate makes it a very unique part of the body, and we have made considerable effort to characterize its developmental anatomy as well as its regenerative potential.

Developmental Anatomy of the Digit Tip

The primary structure of the mature digit tip is the terminal phalangeal bone (P3), which is laterally flattened and has a triangular shape with its base articulating with the subterminal phalangeal element (P2) and its apex forming a sharp point (Fig. 2B, C). The P3 bone contains a bone marrow region localized to the base of the element and associated with a small oblong canal that is contiguous with the lateral connective tissues. There are insertion sites for the dorsal extensor tendon and ventral flexor tendon in the proximal region, and collateral ligaments join P2 and P3 laterally. The terminal phalangeal bone is encased within a nail organ that comes to a sharp distal point extending well beyond the tip of the terminal phalanx. The nail covers the dorsal and lateral surfaces of P3 but is not contiguous ventrally. The nail organ consists of the nail matrix proximally that supplies cells to the nail bed which is overlain by the nail plate. Mouse nail growth is continuous through-

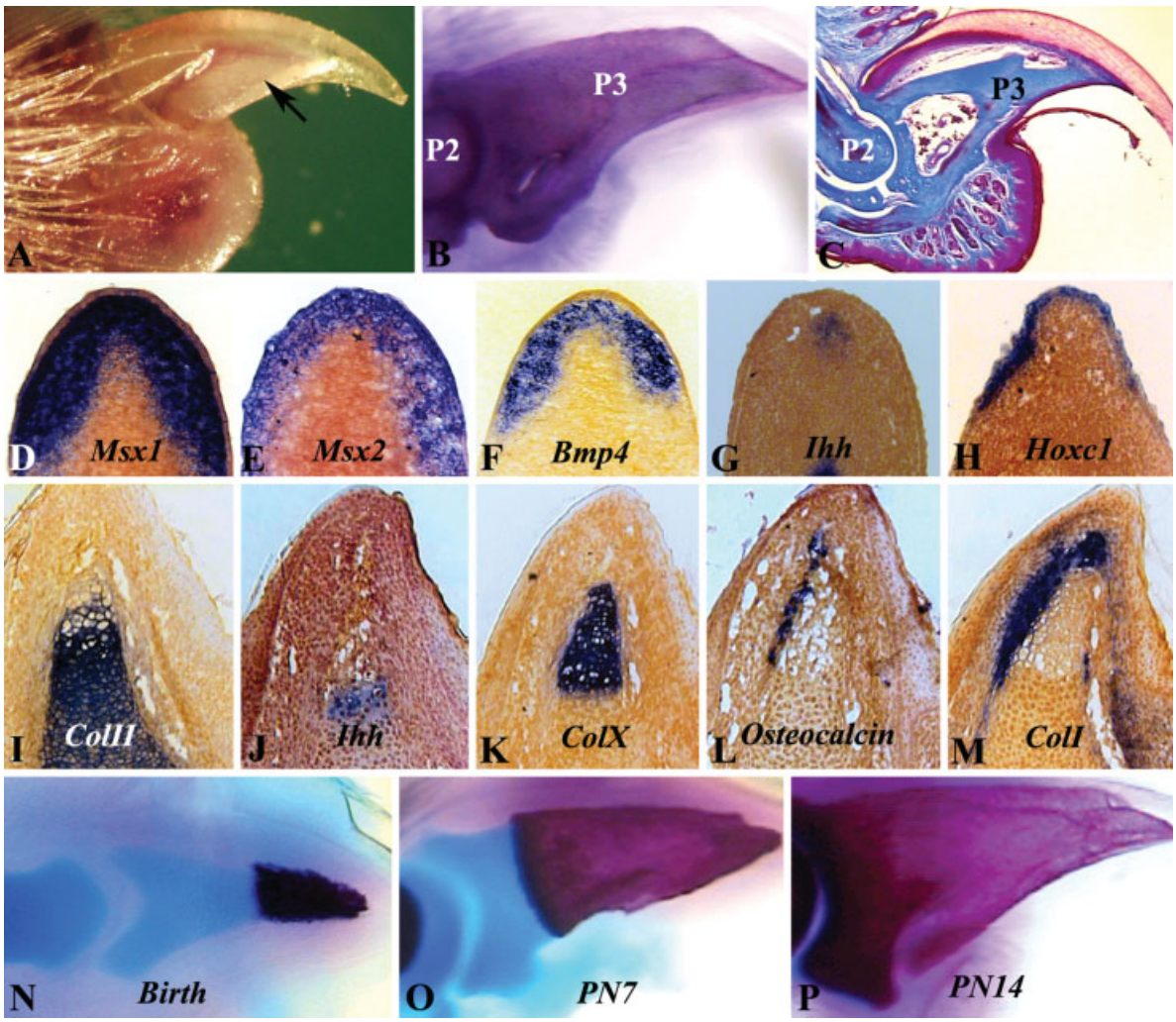

Figure 2. Developmental anatomy of the mouse digit tip. (**A**–**C**) Mature mouse digit tips. (A) A digit tip showing that the terminal phalange is encased within a nail dorsally and laterally. The terminal phalangeal bone is visible through the nail (arrow). (B) Whole mount terminal phalangeal (P3) bone stained with Alizarin Red S showing a sharp point at the apex. Proximal end of the P3 bone articulates with the subterminal phalangeal (P2) bone. (C) Sagittal sectioned sample stained with Mallory's triple stain. The loose connective tissue between the nail bed and the bone contains cells that appear fibroblastic. (**D**–**H**) Gene expression in the developing digit tips of E14.5 embryos. (D) *Msx1* is expressed in the apical mesenchymal cells surrounding the forming terminal phalanx. (E) *Msx2* is expressed in the apical epidermis and in mesenchymal cells subjacent to the epidermis. (F) *Bmp4* is expressed in apical mesenchymal cells in a domain similar to that of *Msx1*. (G) *Ihh* is expressed in digit tip cells, initiating endochondral ossification of the terminal phalanx. (H) The nail organ marker, *Hoxc13*, is expressed in the distal epidermis associated with presumptive nail tissue. (**I**–**M**) Gene expression in the developing digit tips at birth. (I–K) Expression of cartilage-specific genes. (I) Type II collagen (*Col II*), a marker for proliferating chondrocytes; (J) Indian hedgehog (*Ihh*), a marker for prehypertrophic chondrocytes; (K) Type X Collagen (*Col X*), a marker for hypertrophic chondrocytes. (L–M) Expression of osteoblast-specific genes. (L) *Osteocalcin* transcripts are first expressed at the apex of the terminal phalangeal bone then extend along the dorsal surface; (M) Type I collagen (*Col I*) is first expressed in a similar pattern. (**N**–**P**) Whole-mount skeletal staining of postnatal digit tips stained with Alizarin Red S and Alcian Blue. Chondrogenic tissue stains blue and osteogenic tissue stains red. Ossification begins from the distal tip at birth (N) and progresses in a proximal direction. (O) At postnatal day 7 (PN7), the distal ¾ of the terminal phalangeal bone has initiated ossification and by PN14 (P) ossification has commenced along the entire proximal-distal length of the bone. [B,C, I–P (From Han M, Yang X, Lee J, et al. Dev Biol 2008, 315:125–135, Copyright 2008, Elsevier, reproduced by permission.); D–H (From Han M, Yang X, Farrington JE, et al. Development 2003, 130:5123–5132, Copyright 2003, Company of Biologist, reproduced by permission.)]

out life with nail loss occurring apically. The ventral surface of the digit tip is covered with a thin layer of keratinized epidermis that extends to the distal tip where it is thickened and contiguous with the dorsal nail epidermis. Proximally, the ventral epidermis is contiguous with the thickened interdigitating epidermis of the ventral fat pad. Between the nail bed and the terminal phalanx is a layer of loose connective tissue that surrounds the bone. This layer of connective tissue is more prominent along the dorsal and lateral surfaces by comparison to the ventral, and there are regions where the connective tissue cells are organized perpendicular to the bone surface and appear to attach the nail bed

to the bone surface. The loose connective tissue consists primarily of fibroblasts and cells associated with the vascular system.

Developmentally, the digit tip is first identified as an autonomous structure at embryonic day 14.5 (E14.5) when the P2-P3 interphalangeal joint becomes visible. At this stage, there are several genes that are specifically expressed by cells of the digit tip that distinguishes this region from the proximal phalangeal elements (Fig. 2D–H). For example, the expression of *Hoxc13* in cells of the dorsal epidermis marks the forming nail organ (Godwin and Capecchi, 1998), *Msx2* is expressed in the apical epidermis and also in cells of the most distal mesenchyme (Reginelli et al, 1995), and *Msx1* and *Bmp4* are expressed in a larger domain of the distal mesenchyme (Reginelli et al., 1995; Han et al., 2003). Other genes known to be expressed at the developing mouse digit tip include *Dlx5* (Acampora et al., 1999), *Bambi* (Grotewold et al., 2000), and *Dachshund* (Hammond et al., 1998; Davis et al., 1999). The distal specific expression of these genes reinforces the conclusion that the digit tip is a unique structure both in development and in regeneration. The uniqueness of the digit tip in birds and mammals has recently been reviewed by Casanova and Sanz-Ezquerro (2007) in the context of digit evolution.

Ossification of the mouse digit tip initiates just before birth (Han et al., 2008). Gene expression studies indicate that hypertrophic chondrocytes begin to mature between E17.5 and E18.5 (birth), with the onset of *ColX* expression at the distal tip. At this stage proliferating chondroblasts identified by *ColII* expression are localized to the proximal half of P3 and there is a band of prehypertrophic cells identified by Ihh expression between the *ColX* and *ColII* expression domains (Fig. 2I–K). During this same period ossification is initiated at the digit tip in association with the *ColX* domain as evidenced by histological staining and expression of marker genes for ossification, *Osteocalcin* and *ColI* (Fig. 2L, M). As the digit tip matures, ossification progresses in a distal to proximal direction (Fig. 2N–P).

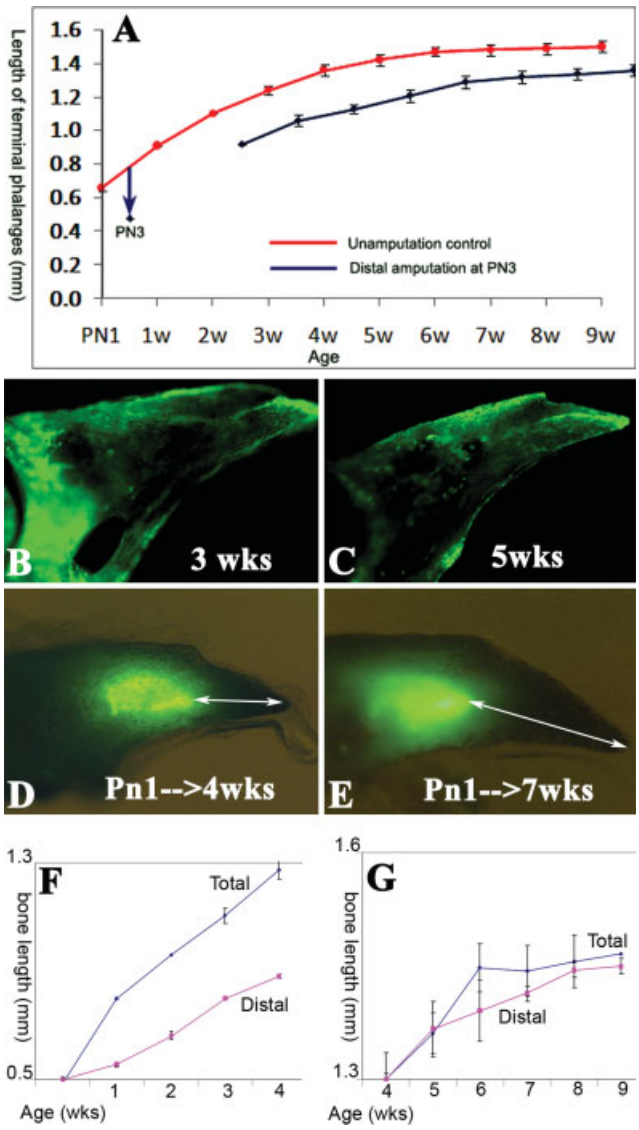

Figure 3. Ossification and growth of the terminal phalangeal (P3) bone. (**A**) Growth curve of P3 bone. The terminal phalangeal bone continues to elongate during the postnatal period and reaches its mature length at 8 weeks. The lower curves show that following amputation at PN3, the terminal phalangeal bone never catches up with unamputated controls. (**B**–**G**) Calcein incorporation into the P3 bone. (B) Calcein labeling for one day identifies two ossification centers at 3 weeks of age, one associated with the proximal growth plate and one at the distal tip. (C) By 5 weeks of age only the distal ossification center is observed. (D) Calcein incorporation is used as a vital marker to identify existing bone by long-term labeling studies. New bone deposition distal to the calcein label identifies bone deposition that has occurred since the initial labeling (white arrows). Calcein was injected at PN1 and analyzed at 4 weeks (D) and 7 weeks (E) of age. (F) By measuring the length of the terminal phalangeal bone and the proximal-distal length of new bone deposition, we show that both proximal and distal ossification centers contribute equally to bone elongation from birth until 4 weeks of age. (G) Similar analysis of the period between 4 and 9 weeks of age indicate that lengthening of the terminal phalanx results solely from the distal ossification center. [A–E (From Han M, Yang X, Lee J, et al. Dev Biol 2008, 315:125–135, Copyright 2008, Elsevier, reproduced by permission.)]

In mice, elongation of the terminal phalanx continues until it reaches a mature length at 8 weeks of age (Fig. 3A). Elongation rate is rapid during the first 3–4

weeks following birth, after which the rate of elongation levels off until the mature length is reached (Han et al., 2008). Calcein is a marker that incorporates into newly forming bone and pulse labeling is used to identify regions of the bone that are undergoing active ossification (Suzuki and Mathews, 1966). Calcein pulse labeling of the terminal phalanx indicates that the early stages of bone growth (before 4 weeks) are associated with two ossification centers, one proximal and one distal (Fig. 3B), whereas by 5 weeks of age only the distal ossification center remains (Fig. 3C). The proximal ossification center is linked to the proximal endochondral growth plate which closes at 3–4 weeks of age, and the distal ossification center results in appositional bone growth similar to that occurring during diametrical growth of the bone collar. Because ossification by appositional growth is typically linked to growth of the bone collar in long bones, it seems reasonable to consider the terminal phalangeal bone as equivalent to the proximal half of a subterminal phalanx with its central collar region constricted to form the apical tip of P3. Calcein can also be introduced as a vital marker of existing bone to quantitate ossification that takes place after its introduction; in this case newly formed bone is unlabeled (Fig. 3D, E). Measurements of distal ossification by comparison to the elongation of the entire terminal phalanx show that during the period from birth to 4 weeks of age about half the length of the terminal phalanx results from distal ossification (Fig. 3F). After 4 weeks of age the distal ossification center is responsible for 100% of terminal phalanx elongation (Fig. 3G).

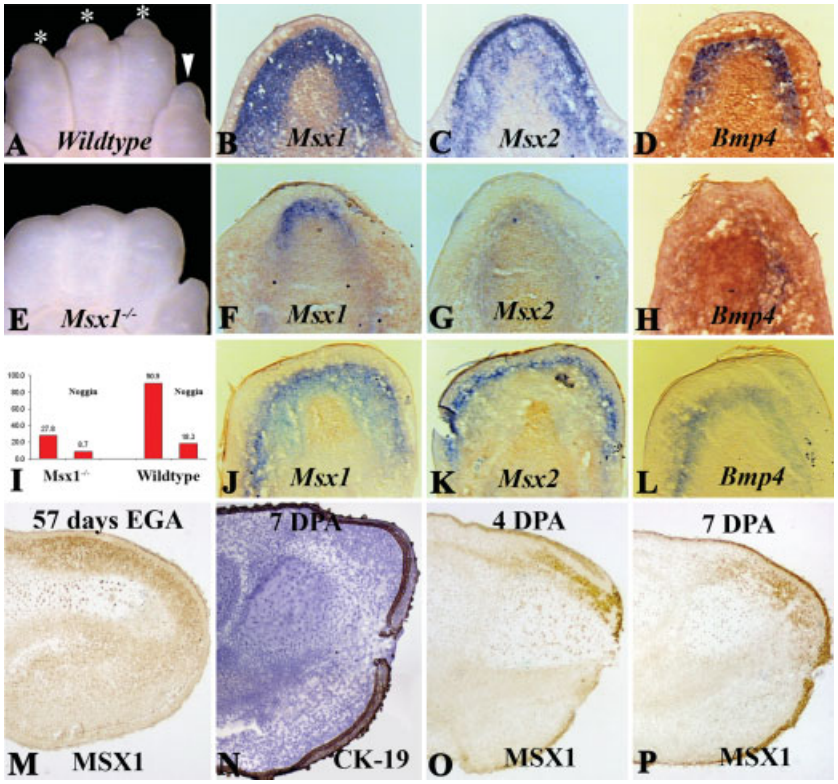

Figure 4. Regeneration response in the fetal digit tips of mice and humans. (**A**): The central digits (digits 2, 3 and 4) that were amputated at E14.5 exhibit normal regeneration response. Note that the regenerated digit tips (*asterisk*) are little bit shorter than a non-amputated control digit tip (*arrowhead*). (**B**–**D**) In situ hybridization of frontal sections at 2 days after amputation showing expression of *Msx1* (B), *Msx2* (C), and *Bmp4* (D). (**E**) Digits of *Msx1*$^{-/-}$ mutant mice display a regeneration defect. (**F**–**H**) In situ hybridization of frontal sections at 2 days after amputation. (F) A nonfunctional transcript of *Msx1* is expressed at the amputation wound. (G, H) *Msx2* and *Bmp4* transcripts are not upregulated in the failed regeneration response. (**I**) Treatment of wildtype and *Msx1*$^{-/-}$ mutant digits with the BMP antagonist, NOGGIN, inhibits the regeneration response. Despite the absence of a regeneration response by NOGGIN treatment, stump tissues maintain expression of *Msx1* (**J**), *Msx2* (**K**) and *Bmp4* (**L**), suggesting that all three genes are upstream of the NOGGIN inhibitory effect in digit regeneration. (**M**–**P**) Embryonic human digits initiate a regeneration response in vitro. (M) A fingertip from a human embryo with an estimated gestational age (EGA) of 57 days displays MSX1 immunostaining that is localized to the nail forming region. (N) Amputated fingertips cultured for 7 days postamputation (DPA) initiate a regeneration response, forming a blastema at the wound site (cytokeratin-19 immunostaining). (O) Amputated fingertips cultured for 4 DPA and immunostained for MSX1 show upregulation in dorsal mesenchymal tissue. (P) At 7 DPA, blastemal cells stain positive for MSX1. [A–H, J–L (From Han M, Yang X, Farrington JE, et al. Development 2003, 130:5123–5132, Copyright 2003, Company of Biologist.); M–P (From Allan CH, Fleckman P, Fernandes RJ, et al. Wound Repair Regen 2006, 14:398–404, Copyright 2006, Wound Healing Society.)]

Embryonic Digit Tip as a Model for Mammalian Regeneration

The developing limb bud and digits of higher vertebrates possess enhanced regenerative capabilities by comparison to adults, and represent models for investigating regenerative responses and regenerative decline associated with maturation (Muneoka and Sassoon, 1992; Muller et al., 1999). For example, the limb buds of rats and mice have been shown to partially regenerate following amputation in vitro (Deuchar, 1976; Lee et al., 1991) and in vivo (Wanek et al., 1989), and the embryonic mouse digit tip undergoes a rapid and complete regenerative response in utero (Reginelli et al., 1995) and in vitro (Han et al., 2003). In mice, the proximal limit of regenerative capability is associated with the proximal extent of the *Msx1* expression domain during digit development (Reginelli et al., 1995). During embryonic digit tip regeneration a number of genes specifically expressed at the apex of the developing digit are upregulated, suggesting that these genes, *Msx1*, *Msx2*, *Bmp4*,

Hoxc13, and *Ihh* (Fig. 4A–D), are important for the regeneration response. These genes are not expressed at a proximal digit amputation wound that is normally nonregenerating (Reginelli et al., 1995; Han et al., 2003). We have analyzed the *Msx1* mutant (Satokata and Maas, 1994) and the *Msx2* mutant (Satokata et al., 2000) to determine whether either of these genes are playing a functional role in the regeneration response (Han et al., 2003). Our studies show that the *Msx1* mutant, but not the *Msx2* mutant, displays a regeneration phenotype (Fig. 4E), suggesting that *Msx1* is playing a critical role in the regeneration response (Han et al., 2003). The distal tip of the *Msx1* mutant digit following amputation behaved like a proximal level amputation, resulting in a truncated digit phenotype and a failure to upregulate the distal digit marker genes (Fig. 4F–H). Since the *Msx1* mutant digit does not have a limb phenotype in development, these results suggest that *Msx1* is functioning in a regeneration-specific manner. We also found that *Bmp4* expression in the digit tip was controlled by the combined action of *Msx1* and *Msx2*, and we therefore carried out studies to determine whether exogenous BMP4 could rescue the *Msx1* regeneration phenotype. Indeed, we found that BMP4 rescued digit tip regeneration in a dose-dependent manner and expression of all distal digit marker genes was upregulated in this induced regenerative response. To corroborate this finding, we used the BMP antagonist, Noggin, to determine if BMP signaling was required for digit regeneration in wildtype digits, and found that treating amputated digits with exogenous Noggin inhibited the regeneration response (Fig. 4I). Gene expression studies demonstrated that Noggin treatment did not affect *Msx1*, *Msx2*, or *Bmp4* expression (Fig. 4J–L), suggesting a linear pathway in which *Msx1*/*Msx2* regulated *Bmp4* and that BMP4 played a key role in controlling the regeneration response (Han et al., 2003). In other studies we have investigated the role of *Dlx5* in embryonic digit regeneration (J. Lee, unpublished data). *Dlx5* is expressed in the apical ectoderm and mesenchyme in the E14.5 digit tip in a domain that overlaps with *Msx2* (Acampora et al., 1999; Han et al., 2003). In amputation studies we do not find a regeneration phenotype in amputated homozygous *Dlx5* mutant embryo digit tips. In addition, we have tested mutant embryos lacking both the *Dlx5* and *Msx2* genes, and in response to digit tip amputation, these embryos can also successfully regenerate. Thus, we can conclude that both *Msx2* and *Dlx5* are not essential for embryonic digit tip regeneration even though both are prominently expressed in the forming digit tip.

In separate studies on embryonic human digits, Allan et al. (2006) established that cultures of embryonic human digits under serum-free conditions were able to go through early stages of a regenerative response. Digits tested were from embryos with an estimated gestational age (EGA) of 53–117 days and were maintained in culture from 4 to 28 days. MSX1 expression was analyzed immunohistochemically and was found in control digits to be expressed in the connective tissue between the nail bed and the terminal phalangeal bone in digits up to 70 days EGA (Fig. 4M). An analogous expression domain of *Msx1* is observed in the late mouse embryo and early neonatal digit tips (Reginelli et al., 1995). Amputated 57 day EGA digits initiated a regenerative response with the formation of a blastema like structure (Fig. 4N) and MSX1 expression was found to be associated with the regenerating apical cells (Fig. 4O, P; Allan et al., 2006). These results provide evidence that human digit tissues share gene expression and injury responses with that of the mouse digit, and point to the *MSX1* gene as a candidate regulator in the control of a human regeneration response.

The *Msx* genes are implicated in other models of regeneration and/or cell renewal. In the regenerating urodele limb, *Msx* genes are down-regulated in the mature limb, and following limb amputation both *Msx1* and *Msx2* are re-expressed during regeneration then down-regulated after redifferentiation (Crews et al., 1995; Simon et al., 1995). *Msx* gene expression during regeneration is largely similar to developmental expression: *Msx1* is expressed by mesenchymal cells, whereas *Msx2* is expressed by both mesenchymal and apical epidermal cells (Carlson et al., 1998; Koshiba et al., 1998). *Msx1* is also expressed in association with limb regeneration in developing *Xenopus* limbs (Endo et al., 2000) and in association with FGF-induced regeneration of the amputated chick wing bud (Taylor et al., 1994; Kostakopoulou et al., 1996). In these cases the re-expression of *Msx1* is initiated at the wound surface and establishes an expression domain in the regenerate that is similar to the developing limb. *Msx1* has been shown to be required for tail regeneration in *Xenopus* (Beck et al., 2003) and fin regeneration in Zebrafish (Thummel et al., 2006). Developmental studies show that *Msx1* acts to inhibit differentiation of a variety of cell types (Hu et al., 2001) and there is evidence that over-expression of *Msx1* can induce myotube dedifferentiation in vitro (Odelberg et al., 2000). Transcription studies show that the *Msx1* protein functions as a transcriptional repressor acting with a TATA binding protein (Catron et al., 1995; Zhang et al., 1996, 1997) and the linker histone, H1b, to inhibit differentiation-specific gene expression (Lee et al., 2004). Since we find that *Msx1* expression is required for digit regeneration, is acting in a regeneration-specific manner, and is upregulated during the regeneration process, we speculate that it is functioning to repress an activity that is normally inhibitory for a regenerative response. Our BMP4 rescue data suggests that the

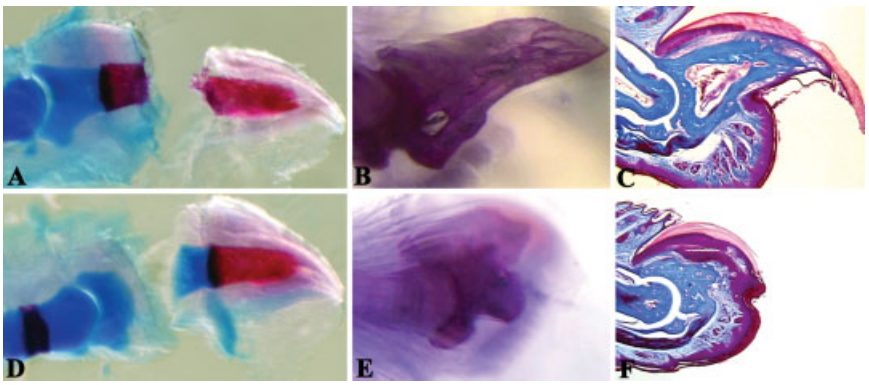

Figure 5. Digit tip regeneration in neonatal mice. Amputations were carried out at a distal level through bone (**A**) and at a proximal level through cartilage (**D**) at postnatal day 3 (PN3). After 6 weeks, digits were analyzed using whole-mount bone stain with Alizarin Red S (**B, E**) and histological analysis with Mallory's triple stain (**C, F**). Distal amputations regenerate anatomically normal digit tips (B, C), however, proximal amputations show no signs of regeneration (E, F).

Bmp4 is a target gene for such an anti-regeneration activity.

Postnatal Digit Tip Regeneration

The regeneration of neonatal and adult digit tips is thought to be largely equivalent (Neufeld and Zhao, 1995), although regeneration of neonatal digit tips occurs during a time of rapid skeletal elongation. Amputation midway through the terminal phalanx results in a regeneration response (Fig. 5A–C), whereas amputation through the proximal region fails to mount a regenerative response (Fig. 5D–F). These two amputation planes transect similar tissues yet the repair response differs dramatically. Digit tip regeneration can be described in terms of distinct phases that are characteristic of other regeneration models such as the urodele amphibian limb (Gardiner et al., 2002). After distal amputation the regeneration response is characterized by an initial wound closure response that requires multiple days, despite the relatively small surface area of the amputation injury (Fig. 6A, B). The slow rate of epidermal closure following amputation is similar to the human response and unlike the rapid re-epithelialization that occurs during amphibian limb regeneration (Carlson et al., 1998). During the wound healing phase there is considerable erosion of the amputated stump bone resulting from enhanced osteoclast activity (Revardel and Chebouki, 1987), which extends the actual limit of regeneration to a more proximal level so as to include the eroded bony tissues. A similar region of tissue remodeling during amphibian limb regeneration is associated with the upregulation of MMPs that are thought to mediate this effect (Yang et al., 1999). Similarly, microarray studies of digit tip regeneration in mice identify several upregulated histolytic genes, including MMPs, associated with this phase of regeneration (Chadwick et al., 2007).

The second phase involves blastema formation. The question of whether or not a ''blastema'' forms in mammals is addressed below, and for purposes of discussion the blastema is simply defined as an aggregation of proliferating cells involved in the regeneration process. Based strictly on histological observations and cell proliferation studies the blastema appears to form from two sources: (1) the migration of connective tissue cells across the amputation wound (Neufeld et al., 2004), and (2) cells arising from the marrow cavity of the skeletal stump. In nonregenerating proximal digit amputation, the skeletal stump forms a periosteum across the injury site and undergoes ossification resulting in a truncated cap of the amputated bone. The blastema, on the other hand, appears to integrate cells derived from the connective tissue surrounding the bone with cells derived from the marrow to form a proliferation center associated with the regeneration response (Fig. 6C, D; Han et al., 2008). The cell contribution to the blastema has not yet been documented, and this is an area of research that is critically important for our understanding of mammalian regeneration. The third phase of regeneration involves the differentiation of regenerated structures, i.e., bone, connective tissue, and nail. The regrowth of bone is most critical because it structurally defines the regenerate. For example, nail regrowth continues in proximally amputated digits but the regrown nail lacks anatomical structure and simply covers the truncated stump (Fig. 5F). In the neonate, bone regrowth following amputation occurs between 7 and 14 days postamputation and proceeds by direct ossification, i.e., without expression of any endochondral marker genes in the distal digit region where ossification is commencing (Fig. 6E). Osteoblasts present at the interface between the blastema and the bone stump at 7 days postamputation suggest that the blastema is organizing and perhaps contributing to the regenerated new bone (Fig. 6F). There is a burst of ossification that occurs between 7 and 14 days postamputation that restores the characteristic pattern of the P3 bone and completes the regenerative response (Fig. 6G).

THE MAMMALIAN BLASTEMA

One of the hallmarks of limb regeneration in urodele amphibians is the formation of a blastema of undifferentiated cells that proliferate, go through morphogenesis, and differentiate to replace structures lost by amputation (Bryant et al., 2002; Brockes and Kumar, 2005). The blastema is a transient phase in regeneration that has been described in terms of the

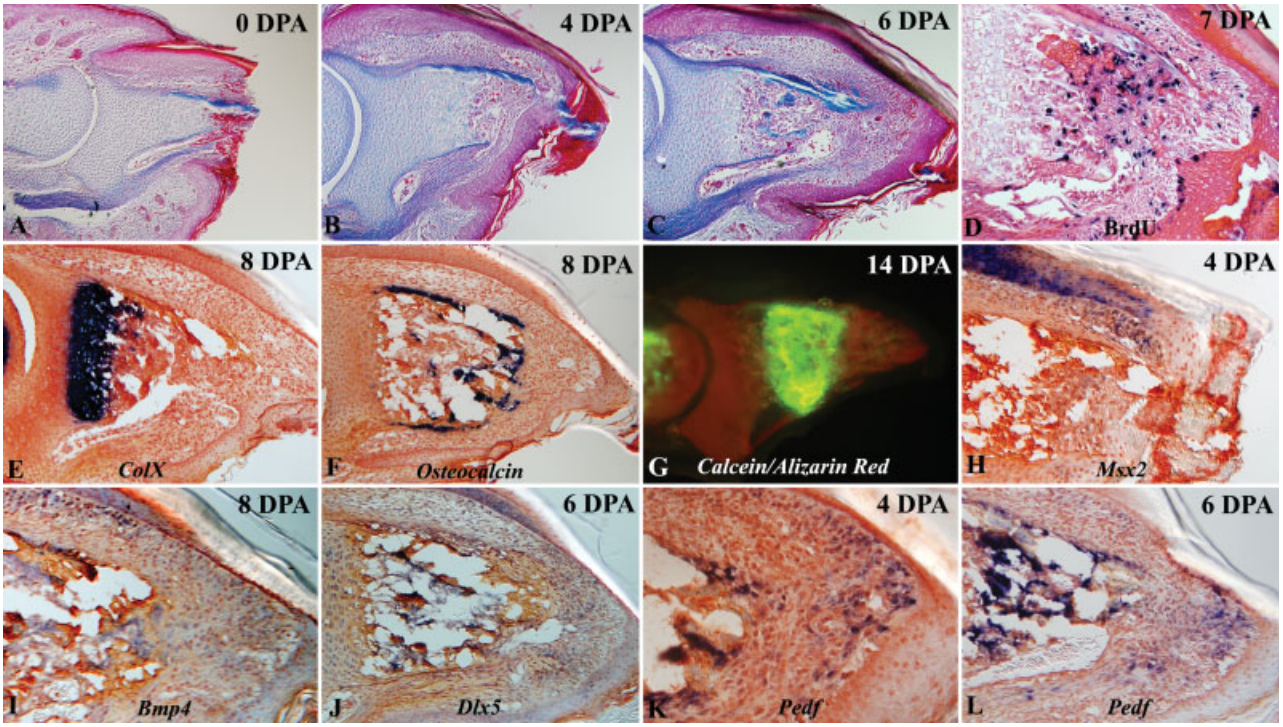

Figure 6. Histological and gene expression analyses of regenerating digit tips. (**A**–**C**) Histological sections of regenerating digit tips stained with Mallory's triple stain at the time of amputation (A), 4 days postamputation (DPA) (B) and 6 DPA (C). Note the formation of a blastema by 6 DPA. (**D**) BrdU incorporation at 7 DPA shows robust proliferation in the connective tissue and the bone stump. (**E, F**) In situ hybridization analyses documenting the expression patterns of a hypertrophic chondrocyte marker (E), *Type X Collagen* (*Col X*), and an osteoblast maker (F), *Osteocalcin*. Note the absence of chondrogenic marker gene transcripts associated with the blastema indicating that the regeneration response involves direct ossification. (**G**) Calcein was used as a vital label to identify ossification in the stump 1 week after amputation and Alizarin Red S was used to stain bone after 2 weeks. Differentiation of the regenerate is largely completed by 2 weeks after amputation. (**H**) *Msx2* expression is induced in the dorsal connective tissue during wound healing stages but is absent in the blastema (not shown). (**I**) *Bmp4* transcripts are present at the distal tip of the blastema and also in the dorsal connective tissue. (**J**) *Dlx5* is expressed in cells at the base of the blastema and also in the marrow region of the stump. (**K, L**) *Pedf* is specifically expressed in the bone marrow and distal apex of the blastema underneath the wound epidermis during regeneration.

characteristics of cells with respect to both their tissue of origin as well as their ultimate fate in regeneration. Thus, for example, we know that the amphibian blastemal cells (1) arise from either dedifferentiation of, and/or stem cells present in, mature tissues, (2) appear undifferentiated and express developmental genes during the blastema phase, (3) proliferate, and (4) differentiate in either a homotypic or heterotypic (metaplastic) manner (Brockes and Kumar, 2002; Han et al., 2005; Morrison et al., 2006). There is currently no mammalian counterpart to the urodele blastema, and because there exists a growing interest in developing strategies to induce regenerative responses in mammals, particularly humans, it is both necessary and important to identify parallels with, as well as deviations from, the best characterized regenerating systems.

Many models for mammalian appendage regeneration involve the formation of a proliferating aggregate of undifferentiated cells that undergoes differentiation to form a regenerated structure. This cell aggregate is often described as "blastema-like" because it does not have all of the characteristics of the classical amphibian blastema that mediates appendage regeneration. The term blastema is used generally to describe a cell aggregate involved in development (e.g., blastema condensations during skeletal formation, metanephrogenic blastema during kidney development) or regeneration (e.g., osteoblastic blastema in fracture healing); however, in limb regeneration, the term has been redefined to include characteristics of urodele blastema cells, and/or their interactions with the overlying epidermal layer. For example, the regeneration blastema has been defined as (1) a structure derived from the dedifferentiation of cells at the amputation wound, (2) arising through epithelial-mesenchymal interactions, and (3) contains intrinsic morphogenetic information (see Carlson, 2005, 2007). Some have even proposed that regeneration itself is defined as a response that must involve the dedifferentiation of cells at the amputation wound (Kostakopoulou et al., 1996). With respect to mammalian regeneration, this strict definition of a regeneration blastema impairs the utility of the blastema concept

simply because it presumes that mammalian regeneration must proceed via mechanisms similar to amphibians. Whether or not this is true remains to be seen. Indeed, we argue below that there are likely multiple ways to regenerate a single structure, and if a blastema is involved, it follows that the characteristics of the blastema must not be constant. Thus, there is value in broadly defining the blastema as an anatomical structure so as to bring regeneration studies under a common umbrella where we can discover similarities and differences when comparing regeneration events of distinct animal groups (e.g., amphibians versus mammals). We therefore favor the simple definition of a regeneration blastema as an aggregate of proliferating undifferentiated cells involved in the regeneration of a lost body part.

All three mammalian regeneration models described earlier (ear punch, antler, and digit tip) regenerate via a blastema. In ear punch regeneration, the blastema forms around the circumference of the hole punch and it grows inward progressively filling in the hole with tissue. Several genes and proteins are expressed in the blastema, including BMP2 (Urist et al., 1997), the EGF family member *Pref-1* (Samulewicz et al., 2002), and the matrix metalloproteinases (MMP), MMP2 and MMP9 (Gourevitch et al., 2003). In addition, an angiopoietin-related growth factor (AGF) expressed in injured skin promotes regeneration in the transgenic mouse model (Oike et al., 2003). The blastema of the regenerating deer antler is the proliferating mesenchymal region called the mesenchymal growth zone or the reserve mesenchyme (Price et al., 2005; Kierdorf et al., 2007). These cells express the mesenchymal stem cell marker STRO-1, and have been shown to be multipotent when challenged in in vitro differentiation assays; thus, it is proposed that the blastema is stem cell derived (Rolf et al., 2008). In vivo, these cells give rise to chondrocytes that undergo hypertrophy and are invaded by osteocytes following an osteogenic process that recapitulates development (Faucheux et al., 2004). Cultures of antler blastemal cells provide for a way to characterize the control of blastema growth and differentiation (Price et al., 1994; Sadighi et al., 1994). Factors shown to enhance cell proliferation in vitro include IGF-I, IGF-II, FGF2, and PTHrR, and in vivo support for this proliferative effect comes from immunohistochemical studies showing that the corresponding receptors (IGFR, FGFR, and PPR) are expressed in the blastema (Price et al., 1994; Sadighi et al., 1994; Barling et al., 2004; Faucheux et al., 2004; Lai et al., 2007). Other studies provide evidence that key developmental signaling pathways, such as canonical *Wnt* signaling (Mount et al., 2006), BMP signaling (Barling et al., 2005) and retinoic acid signaling (Allen et al., 2002), are also playing a critical role in antler regeneration.

The regenerating mouse digit tip forms a blastema of proliferating cells that later undergoes direct ossification to restore the distal region of the terminal phalanx (Revardel and Chebouki, 1987; Neufeld, 1992). While the cellular origins of the blastema remain to be explored, studies of the neonatal blastema identify the loose connective tissue surrounding the terminal phalangeal bone and the marrow-forming region as areas where enhanced cell proliferation is occurring in association with the regenerative response (Han et al., 2008; see Fig. 6D). The digit blastema is characterized by its continuity both with the connective tissue surrounding the stump bone and with the stump bone itself. Unlike digit amputation at a proximal (nonregenerating) level where a periosteum forms a distal cap associated with bone truncation (Neufeld, 1985), the smooth integration of the regenerated bone tissue with the stump is a likely outcome of the stump-blastema continuity, and the extensive remodeling of the stump that occurs during the wound healing phase appears to play a role. The integration of the stump tissues with the regenerate is a research topic that has not received much attention, yet this interface is clearly important for success in functional tissue engineering and regenerative medicine. In regenerating systems, this interface represents a site where terminally differentiated cells of the stump must functionally interact with the undifferentiated cells of the regenerate. Further studies on this topic will prove to be important for the development of therapeutic strategies critical for functional tissue engineering.

Recently, we characterized the expression of digit development markers during neonatal digit tip regeneration using in situ hybridization (Han et al., 2008). *Msx1* and *Msx2* are expressed in the distal digit tip during development and are prominently expressed during embryonic digit tip regeneration (Fig. 4). In the neonatal digit *Msx1* is expressed in the dorsal connective tissue, whereas *Msx2* is expressed in the nail epidermis. Following digit tip amputation we see *Msx1* expression upregulated in association with the healing dorsal connective tissue, and in this same region we find *Msx2* expression induced (Fig. 6H). This upregulation of *Msx* genes is transiently associated with the wound healing response since we do not observe expression of either gene in the blastema. We note that the early induction of *Msx2* has also been observed following amputation during amphibian limb regeneration (Carlson et al., 1998), thus perhaps *Msx2* may be serving a parallel function. Once the blastema has formed, we find *Bmp4* transcripts expressed in the dorsal connective tissue, in the forming bone, and in the distal blastemal mesenchyme (Fig. 6I). In the unamputated digit, *Bmp4* expression is restricted to the forming bone, so it appears to be upregulated specifically in the blastema and dorsal connective tissue. We also find the homeodomain containing gene *Dlx5* expressed in the blastema. *Dlx5* is expressed in

both the epidermis and mesenchyme of the developing digit tip in a pattern similar to *Msx2*, and genetic studies indicate that neither *Dlx5* nor *Msx2* are required for regeneration (see above). *Dlx5* is also known to play a critical role in osteogenesis (Holleville et al., 2007), thus its expression during postnatal digit regeneration is probably associated with the onset of ossification by regenerating cells. In the blastema, *Dlx5* expression is restricted to the proximal regions and lies between the *Bmp4* domain and the skeletal stump (Fig. 6J). We also find genes expressed in the blastema that are not expressed during digit development. For example, pigment epithelium-derived factor (*Pedf*), a secreted protein with neurotrophic activity (Ramirez-Castillejo et al., 2006), is expressed in the bone marrow, and is prominently expressed in the blastema (Fig. 6K, L). To date, *Pedf* is the earliest gene that we have specifically localized to the forming blastema. Cells expressing *Pedf* localize to the apex of the blastema just underlying the wound epidermis. It is not expressed in proximal amputation wounds that fail to regenerate (not shown), suggesting that it may be playing a key role in the regeneration response. Because *Pedf* is prominently expressed in both bone marrow and blastema, one possibility is that *Pedf* expressing cells in the bone marrow contribute to the establishment of the blastema. In addition, the neurotrophic activity of PEDF may be linked to maintaining damaged neurons following amputation and during the regeneration process. In conclusion, the digit tip blastema cells display characteristics similar to the developing digit tip, but also characteristics that are unique to regeneration.

EVOLUTION OF REGENERATION IN MAMMALS

The evolution of regeneration in the animal kingdom has been addressed largely within the context of animals that possess a high degree of regenerative capabilities, i.e., invertebrates and lower vertebrates (fish and amphibians). For these animals there is indirect evidence that the ability to regenerate is an ancient attribute of metazoans that is linked to developmental mechanisms, and as such, is non selectable (Brockes et al., 2001). It is further proposed that regeneration is an evolutionary remnant of the process of asexual reproduction (Sanchez Alvarado, 2000). It seems likely however, that the wound healing component of the regeneration process that does not involve a reiteration of development is adaptive and has undergone considerable evolution among metazoans. Since wound healing is the initial phase of any regeneration response, it would seem that an important consideration for our understanding of regeneration lies at the interface between an evolving wound healing process and conserved developmental mechanisms. The process of intercalary growth (French et al., 1976; Bryant et al., 1981), for example, may represent a mechanism that has successfully evolved in amphibians and invertebrates to span this interface (Gardiner et al., 1995).

The flip side of the view that regenerative ability is a conserved mechanism in animals that can regenerate is that negative selection for regeneration can explain why many groups of animals, including mammals, lack the extensive regenerative capacity enjoyed by others. It is here where most discussions on the evolution of regeneration ends; the animal kingdom is divided into regenerators and nonregenerators, and the discussion moves to addressing the question of what might be the circumstances surrounding the loss of regenerative ability in mammals. Indeed, a prevalent view is that the loss of regenerative ability is linked to a more urgent selective need of developing an adaptive immune system to combat pathogens invading injured tissue that evolved at the expense of regenerative ability (Mescher and Neff, 2005). However, this view leaves no place for endogenous regeneration models in mammals, because how can we systemically forfeit regenerative ability yet retain it in select parts of the body? Regeneration in mammals is real and how it fits into our thinking about the evolution of regeneration turns out to be critical for understanding how to move forward in regenerative medicine. For example, it is likely that regenerative ability evolved secondarily from a nonregenerative state in some mammals (see below), so we can look upon these regeneration models as examples of how nature got into the regenerative medicine business and came up with a successful product. Similarly, if regenerative ability was selectively retained in some parts of the body, how is it that these parts of the body can become refractory to a strong negative selective force? The answers to these questions may be quite profound and could have a major impact on the development of strategies for how we can successfully implement regenerative therapies.

Deer antler regeneration is perhaps the best example of an evolved regenerative response as fossil records clearly indicate that antler formation and regeneration evolved from a nonregenerative precondition (Goss, 1969). The antler develops postnatally as an outgrowth of the frontal bone and its formation and regeneration follow similar processes. The frontal bone forms during embryogenesis by intramembranous ossification and the initiation of antler outgrowth involves direct ossification; however, antler elongation itself involves an unusual form of endochondral ossification in which the growth plate is localized at the apex of the outgrowth. These observations clearly demonstrate that the evolution of antler formation/regeneration is not linked to the developmental mechanisms involved in frontal bone formation, but to developmental mechanisms

used to form elongated bony structures. Thus, the evolution of antlers involves the activation and modification of developmental mechanisms utilized in other parts of the body (e.g., long bones) and not those involved in frontal bone formation itself. One conclusion that can be drawn from this is that when regenerative ability evolved, it was not constrained by the developmental history of the tissue responding to the injury.

It is unclear whether the digit tip in mice represents a model of an evolved regenerative response, one in which regenerative ability has been maintained, or both. The vertebrate digit has undergone tremendous anatomical modification, including digit elongation, shortening, and reduction in number, so it is clear that the digit is under strong selective pressure. *Bmp4* is expressed in developing and regenerating digit tips and required for embryonic digit regeneration (Han et al., 2003), so its activity at the digit tip could very well be a target for adaptive selection. It is worth noting that BMP4 is known to play a critical role in the evolution of tooth formation in birds (Chen et al., 2000) and, as well, is responsible for variation in beak morphologies (Abzhanov et al., 2004; Wu et al., 2004). Thus, the expression of *Bmp4* in both digit development and regeneration, along with its close association with other evolving vertebrate structures, is consistent with the idea that digit tip regeneration has been maintained during vertebrate evolution. On the other hand, the regeneration of the postnatal digit tip involves intramembranous ossification of the terminal phalangeal bone (Han et al., 2008), a process that is not related to the developmental process of endochondral ossification that originally forms the limb skeleton. Thus, if digit tip regeneration is an example of a maintained response, then why would it utilize a developmental process that is completely novel for digit formation? Indeed, the intramembranous ossification of the regenerated bone suggests that digit tip regeneration represents an evolved regenerative response with cells at the amputation injury utilizing a developmental process that is novel for the developing limb. One possibility is that digit tip regeneration has been evolutionarily conserved because it is adaptive, but the process itself has evolved as well. We note that both intramembranous ossification and endochondral ossification are involved in bone formation during long bone fracture healing, thus both processes have evolved during bone healing and remodeling (Schindeler et al., 2008). It is also interesting to note that deer antler regeneration evolved from bone that undergoes direct ossification during embryogenesis to form the antler by endochondral ossification, so these two regeneration models appear to have evolved in opposite directions.

CONCLUSION

By comparison to other well studied regeneration models such as the amphibian limb, the study of appendage regeneration in mammals remains at an immature stage. Regeneration in mammals is very real despite the fact that it has clear limitations. In multiple mammalian models, the wound healing response results in the formation of a blastema of proliferating cells that mediates the regeneration response. The source of the cells that forms the blastema remains unknown, although there is evidence that these cells display characteristics associated with mesenchymal stem cells. At the same time there is also evidence that connective tissue fibroblasts also participate in blastema formation, thus the mammalian blastema may be composed of cells derived from multiple sources. Although the formation of the blastema in different mammalian models appears to share similarities, the mechanisms guiding re-differentiation during regeneration seem to be quite diverse. We interpret this observation to suggest that the process of re-differentiation during regeneration has evolved away from the mechanisms that guided their initial development, i.e., regeneration itself is not constrained by existing development models. This suggests that in mammals, regeneration is not refractory to adaptive selection, but that it is a process that continues to evolve. Sorting out aspects of regeneration that are evolutionarily conserved versus those that have evolved will prove to be important as we begin to consider human application based on discoveries from animal models.

The anatomy of the mammalian response suggests that a spatial system of positional information is required to guide regeneration, although we have no understanding of the nature of such a system in mammals. Because the regenerative response is limited, we do not have anatomical assays available to explore the role that position plays in regeneration. However, we are encouraged from studies of human fibroblasts which display expression profiles that vary with position in the body (Chang et al., 2002; Rinn et al., 2006). Since limb fibroblasts play a critical role in establishing the urodele blastema and in organizing the system of position information required to effect a successful regenerative response (Gardiner et al., 2002), the finding that analogous cells in adult humans maintain a similar positional memory is suggestive that the most essential component for a complex regenerative response in humans is largely intact. Finally, the role of fibroblasts in a mammalian injury response is generally linked to fibrosis and the production of scar tissue, yet in regenerating models these same cells are viewed as important contributors and play an organizing role in the regenerated pattern. Thus, it is our contention that understanding and re-directing the response of these cells, in particular, in a non-regenerating mammalian wound is a critical first step in transforming a wound healing response to a regenerative response.

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