Limb Regeneration in Higher Vertebrates: Developing a Roadmap

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We review what is known about amphibian limb regeneration from the prospective of developing strategies for the induction of regeneration in adult mammals. Prominent in urodele amphibian limb regeneration is the formation of a blastema of undifferentiated cells that goes on to reform the limb. The blastema shares many properties with the developing limb bud; thus, the outgrowth phase of regeneration can be thought of as cells going through development again, i.e., redevelopment. Getting to a redevelopment phase in mammals would be a major breakthrough given our extensive understanding of limb development. The formation of the blastema itself represents a transition phase in which limb cells respond to injury by dedifferentiating to become embryonic limb progenitor cells that can undergo redevelopment. During this phase, rapid wound closure is followed by the dedifferentiation of limb cells to form the blastema. Thus, the regeneration process can be divided into a wound-healing/dedifferentiation phase and a redevelopment phase, and we propose that the interface between the wound-healing response and gaining access to developmentally regulated programs (dedifferentiation) lies at the heart of the regeneration problem in mammals. In urodele amphibians, dedifferentiation can occur in all of the tissues of the limb; however, numerous studies lead us to focus on the epidermis, the dermis, and muscle as key regulators of regeneration. Among higher vertebrates, the digit tip in mammals, including humans, is regeneration-competent and offers a unique mammalian model for regeneration. Recent genetic studies in mice identify the Msx1 gene as playing a critical role in the injury response leading to digit tip regeneration. The results from regeneration studies ranging from amphibians to mammals can be integrated to develop a roadmap for mammalian regeneration that has as its focus understanding the phenomenon of dedifferentiation. Anat Rec (Part B: New Anat) 287B:14–24, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

An amputation injury involving a limb in humans is a life-threatening and life-altering ordeal. This type of injury is immediately life-threatening due to blood loss resulting from damage to the vasculature supplying the limb, and it is life-altering because humans lack the ability to regenerate appendages following such injury. As we uncover more and more about how organs, such as the limb, form during normal development and about the incredible potential of stem cells to participate in organ formation, it is reasonable to begin to wonder how we might be able to enhance our own regenerative potential. From an experimental viewpoint, a key question we are immediately faced with is how can we begin to study regeneration in animals that cannot regenerate? The obvious answer is to study regeneration when and where it does occur, then translate those findings to a mammalian amputation wound. Three general approaches have been taken. The first is represented by studies that date back centuries and focus on uncovering the secrets to limb regeneration in urodele amphibians. The second takes advantage of a well-established fact...
that all tetrapod vertebrates can undergo successful regeneration responses during certain periods of their life cycle, generally during embryonic or larval stages. The third is to study adult mammalian regenerative responses, however modest, and one such response involves the regeneration of digit tips in mice. These various approaches have their individual strengths and weaknesses. However, it is heartening that these various paths often lead us to common answers.

Studies on various regeneration models describe a highly dynamic process in which the wound-healing response that results in the formation of a regeneration blastema interfaces with a redevelopment process that ultimately regenerates the limb (Bryant et al., 2002). In contrast, the wound-healing response following limb amputation in adult mammals has not been studied in great detail, but key events of nonamputation wound healing include the formation of a fibrin clot, a relatively slow reepithelialization, an inflammatory response that helps to populate the wound site, the formation of granulation tissue, the differentiation of fibroblasts to become myofibroblasts that contract the wound, and the deposition of parallel collagen bundles that form scar tissue (Martin, 1997). The contrast between the mammalian wound-healing response and a regenerative response in any animal model (including the mouse digit tip) is so great that it is often difficult to see any relationship beyond the moment of injury. This, along with an understanding that regeneration is a sequential process with later stages dependent on the successful completion of earlier stages, suggests that translational studies must focus on the earliest events of a regeneration response. In this review, we outline our current understanding of limb regeneration in adult urodèles, in anuran larva, and in developing amniote embryos, focusing on wound closure and dedifferentiation as two key early events in the process that must be altered to induce a mammalian regeneration response. We also review studies on the regeneration of digit tips as a mammalian regeneration model, and while the digit tip is only a small part of the limb, we propose that understanding early events in regenerating versus nonregenerating mammalian digits will provide important clues to solving the problem of regenerative failure of human limbs.

**MAMMALIAN REGENERATION: IS IT FEASIBLE?**

We begin by examining whether or not a goal of inducing a regenerative response in humans is a reasonable goal. It has long been known that some vertebrates, e.g., salamanders, have incredible regenerative powers. The first question that arises is whether regenerative ability that is unique to a specific group of animals evolved along a particular phylogenetic path or whether regenerative ability was lost in many groups, including humans, and retained in others. In other words, is regenerative ability the result of a gain of function in some groups or the loss of function in other groups. A cursory look at regenerative ability across phylogenetic groups leads to the conclusion that regenerative potential declines with the evolution of complexity (Sanchez Alvarado, 2000). Thus, simple organisms like Hydra and planaria have tremendous regenerative capabilities, whereas vertebrates tend to regenerate poorly. This suggests that the absence of regenerative ability in mammals is a loss of function, perhaps a consequence of physiological adaptations important for survival following injury. At the same time, the observation that fingertips of children have regenerative capacity indicates that the ability for a modest level of regeneration was retained in a context-specific format even in higher mammals. The general conclusion that a reduction in regenerative potential in humans is an example of a lost function can be viewed as a positive indicator for efforts aimed at inducing, or rather restoring, a regenerative response.

Another aspect of regeneration biology that supports this general conclusion is the observation that regenerative potential is enhanced in developing systems, and that the process of limb regeneration, where it occurs, is mechanistically very similar to the process of limb development (Muneoka and Sassoon, 1992). In other words, there are component parts of the regeneration process that can be considered redevelopment (Bryant et al., 2002). Not only can developing and regenerating cells interact with one another to build a limb (Muneoka and Bryant, 1982, 1984), there are numerous examples of genes whose expression during development and regeneration are identical and other genes whose expression is largely similar (Gardiner et al., 2002). These studies provide support for the idea that the genetic machinery to regenerate a limb is available in humans, and that regeneration-competent animals have maintained access to reactivating these developmental programs. Certainly, the tremendous successes we have witnessed in animal cloning demonstrate the feasibility of reactivating developmental programs in adult cells (Wilmut et al., 2002).

The prospect of enhanced limb regeneration in humans is encouraged also by the observation that many of the tissues of the adult limb have an inherent capacity to undergo a limited regenerative response to injury. Muscle regeneration is perhaps the best-studied example, and in this case, regeneration involves the proliferation and differentiation of a stem cell population (satellite cells) associated with mature muscle (see review by Carlson in this issue). In addition, bone tissue has limited repair potential in that new bone is formed from mesenchymal stem cells located in the bone marrow to repair a break or fracture. Tendons and ligaments also undergo limited regenerative healing (Molloy et al., 2003). Other tissues of the limb, such as the skin, are less successful in their response to injury and heal imperfectly. However, there is indication that significant advances in inducing scar-free healing of adult skin wounds are on the horizon (Ferguson and O’Kane, 2004). The fact that individual injured tissues of the human limb have the capacity to undergo a regenerative response indicates the existence of rudimentary repair processes that might be enhanced to initiate a limb regeneration response.

The feasibility of a human regeneration response may be limited by the physical size of the human limb. Limb formation occurs in the embryo when...
all of the organ systems are small and the physical dimensions of the limb bud may play an active role in guiding pattern formation and morphogenesis (e.g., diffusion, cell migration, cell-cell interactions). When considering the size of a human arm in comparison to the human forelimb bud and the fact that regeneration reutilizes developmental mechanisms, the size differential between the two may make it impossible to mount a regenerative response. On the other hand, studies on the regenerating salamander limb show that there is a period of active cell migration that results in the accumulation of a blastema at the center of the amputation surface (Gardiner et al., 1986); thus, regardless of the initial size of the amputated limb stump, a small blastema of cells forms. In addition, if we consider the regeneration of tails that occurs in some large reptiles as providing relevant information on the role that size may play in regeneration, the regeneration of an alligator tail that approximates the cross-sectional diameter of a human arm or leg has been noted in the wild (Fig. 1). These considerations lead us to conclude that the induction of human regeneration need not be constrained by the size of the adult limb.

**AMPHIBIAN LIMB REGENERATION: A MODEL OF REGENERATION**

Urodele amphibians, such as newts and salamanders, have the ability to regenerate their limbs following amputation anytime during their life cycle. Regeneration is a local response of the cells of the limb stump and results in a perfect replacement limb regardless of the level of amputation. Following amputation of the limb, the regeneration process involves a number of stages, including the closure of the wound by the epidermis, the dedifferentiation of stump tissue to release cells, the transformation of the wound epithelium into a specialized structure called the apical epithelial cap (AEC) that is required for limb outgrowth, the migration of dedifferentiated cells to the center of the wound where a blastema forms, and the proliferation of blastema cells (Stocum, 1995; Tsonis, 1996). Once formed, the blastema is thought to be similar to the limb bud as it goes through morphogenesis, pattern formation, and redifferentiation. Like the limb bud, outgrowth of the blastema involves interactions between the blastemal mesenchymal cells and the AEC.

The earliest events of the urodele injury response distinguish the pathway leading to regeneration from a response leading to wound healing and scar formation in mammals. The first involves the epidermis, which is able to close the amputation wound in a matter of hours. This is dramatically different by comparison to mammalian wounds that take multiple days to close. Once the wound is closed, the wound epithelium undergoes changes in morphology and gene expression to form the AEC, which directs the outgrowth of limb mesenchymal cells during morphogenesis. The AEC is considered to be homologous to the apical ectodermal ridge (AER) of the developing limb bud (Christensen and Tassava, 2000). The second regeneration-specific event concerns the origin of the mesenchymal cells that form the blastema. Differentiated cells of the stump respond to the wound microenvironment by dedifferentiating into mesenchymal cells that accumulate to form the blastema. A dedifferentiation response is not seen in mammalian wounds. The source of blastema cells has been investigated by a number of different laboratories and virtually all tissues present in the limb stump appear to contribute, to varying degrees, to the blastema and the regenerated limb (Steen, 1968; Namenwirth, 1974; Dunis and Namenwirth, 1977; Muneoka et al., 1986a; Echeverri et al., 2001; Brockes and Kumar, 2002).

Following dedifferentiation, regenerating cells are classified as mesenchymal and undifferentiated (i.e., appearing embryonic); they display embryonic characteristics such as migratory behavior, differential adhesion and readily undergo cell division, and they reexpress many developmentally regulated genes, including members of the Hoxa and Hoxd gene clusters, Msx1, Msx2, and Shh (Gardiner et al., 1999). Their migratory and proliferative behaviors play a key role in the accumulation of the blastema at the center of the wound (Gardiner et al., 1986; Muneoka et al., 1986a), and their differential adhesive character plays a role in distinguishing their proximal-distal position in the limb (Crawford and Stocum, 1988). The reexpression of developmental genes warrants the classification of later stages of regeneration as redevelopment (Bryant et al., 2002). The process of limb regeneration is complex and not fully understood, but it is clear that it is the early events of wound healing and dedifferentiation that distinguish the amphibian from the mammalian response. It is therefore appropriate to focus on these early stages of the amphibian regeneration response to provide clues for designing a roadmap to mammalian regeneration.

**CELL DEDIFFERENTIATION: HALLMARK OF REGENERATION RESPONSE**

Understanding the cellular and molecular mechanisms involved in dedifferentiation is important for thinking about cell reprogramming that is necessary for inducing a regeneration response (Brockes and Kumar, 2002; Tanaka, 2003). Since blastema cells share characteristics with limb bud cells and reexpress genes known to function in limb development, characterizing the dedifferentiation of any given tissue must involve cytological changes associated with the transformation to a mesenchymal phenotype, changes in transcriptional activity, and changes in cell behaviors. The dedifferentiation process is likely to be uniquely different for each of the tissues contributing cells to the blastema, and we are unable to find a suitable definition of dedifferentiation in the literature that can be applied to all tissues. We propose to define dedifferentiation simply as the regression of a cell from a differentiated state to an embryonic state (Fig. 2A). As simple as this definition sounds, in order to be characterized as a dedifferentiated cell, a differentiated cell must be followed as it transforms into an embryonic state and the criteria for defining this transformation must involve the downregulation of differentiation-specific genes associated with the upregulation of embryonic genes. Cell
behaviors such as cell migration, differential adhesion, and cell proliferation may also be associated with dedifferentiation; however, cell behavior alone cannot define dedifferentiation since these characteristics are not necessarily embryo-specific. Associated with the injury response, a number of regeneration-specific genes will also be upregulated (Carlson et al., 2001; Christen et al., 2003). A subset of these genes may be linked to the initiation of the dedifferentiation process, another subset of genes may be inhibitory for dedifferentiation, and a final subset may be completely unrelated. This simple model for dedifferentiation can be used to characterize the cellular response to amputation injury of either regeneration-competent or regeneration-incompetent limbs. For regeneration-incompetent limbs, we can use developmental genes to guide experimental modification of the wound response to encourage the dedifferentiation of any specific tissue.

The urodele blastema itself is composed of cells that express embryonic genes; thus, it seems clear that both epidermal and mesenchymal components represent dedifferentiated cells as long as cell marker studies link these cells to some adult tissue compartment (Fig. 2B). The cells of the wound epithelium are known to come from the epidermis and they go on to differentiate into the regenerated epidermis (Riddiford, 1960; Hay and Fischman, 1961), so the evidence indicates that the epidermis dedifferentiates to form the AEC. The formation of the mesenchymal component of the blastema is more complex because multiple tissue types provide cells that contribute to the blastema, and quantitative data for only a few tissues are available. Nevertheless, it is clear that the relative contribution from different tissues in the stump is not equivalent and that some tissues, such as the dermis, overcontribute, while other tissues, such as skeleton and muscle, undercontribute (Tank and Holder, 1979; Muneoka et al., 1986a; Echeverri et al., 2001). In this view, the formation of the blastema involves the dedifferentiation of multiple tissues to form embryonic tissues that subsequently interact with one another to redevelop the limb structure. Since limb development itself is dependent on epithelial-mesenchymal interactions, the timing of dedifferentiation in the various tissues must be coordinated for a successful regenerative response (Fig. 2B).

During any regeneration event, the final outcome involves the differentiation-specific genes and the upregulation of embryonic genes, and changes in cell behavior. This model does not preclude the upregulation of wound-healing genes whose expression may or may not be linked to the dedifferentiation process. The model simply provides guidelines for characterizing an injury response to determine whether or not dedifferentiation occurs. B: The process of regeneration is shown with respect to the dedifferentiation model outlined in A. The epidermis and nonepidermal stump tissues dedifferentiate to form the blastema. The epidermis responds to amputation injury and contributes 100% of the cells that comprise the wound epidermis. The wound epidermis dedifferentiates to become the AEC and begins expressing developmental genes such as Fgf8 and Dlx3. While all of the nonepidermal stump tissues are thought to contribute to the blastema, the relative contribution from different tissues is not equivalent. The dermis represents approximately 19% of the nonepidermal stump cells but contributes approximately 43% of the cells in the blastema. Muscle tissue comprises the majority of the nonepidermal stump cells (approximately 65%) and contributes an estimated 17% of the blastema. Of the remaining 40% of the blastema, skeletal tissue contributes approximately 2% and the remainder is speculated to come from nondermal stump fibroblasts. The end result of the dedifferentiation process is the formation of the AEC and the blastema mesenchyme that interact with one another during the redevelopment phase of regeneration.

Figure 1. A group of juvenile American alligators (Alligator mississippiensis) approximately 3–4 feet in length is shown resting a short distance off a hiking trail in the Barataria Preserve of Jean Lafitte National Historical Park and Preserve (approximately 17 miles south of New Orleans). According to park rangers, one alligator (center) had suffered an amputation injury and was in the process of regenerating its tail (inset). The diameter of the tail is estimated as being approximately the same size as the diameter of a human arm or leg. This observation suggests that the size of the wound surface following amputation injury does not necessarily present physical limitations for the initiation of a regeneration response. Photographed in November 2004 by Carol A. Burdsal.

Figure 2. A: The process of dedifferentiation is defined as the regression of a cell from a differentiated state to an embryonic state. This is shown in relation to a simple linear model of development. During development, a cell obtains patterning information, goes through morphogenesis, becomes determined, and undergoes terminal differentiation, and in response to injury the dedifferentiation process causes the cell to regress to any level along this pathway. Dedifferentiation can be characterized by the downregulation of differentiation-specific genes, the upregulation of embryonic genes, and changes in cell behavior. This model does not preclude the upregulation of wound-healing genes whose expression may or may not be linked to the dedifferentiation process. The model simply provides guidelines for characterizing an injury response to determine whether or not dedifferentiation occurs. B: The process of regeneration is shown with respect to the dedifferentiation model outlined in A. The epidermis and nonepidermal stump tissues dedifferentiate to form the blastema. The epidermis responds to amputation injury and contributes 100% of the cells that comprise the wound epidermis. The wound epidermis dedifferentiates to become the AEC and begins expressing developmental genes such as Fgf8 and Dlx3. While all of the nonepidermal stump tissues are thought to contribute to the blastema, the relative contribution from different tissues is not equivalent. The dermis represents approximately 19% of the nonepidermal stump cells but contributes approximately 43% of the cells in the blastema. Muscle tissue comprises the majority of the nonepidermal stump cells (approximately 65%) and contributes an estimated 17% of the blastema. Of the remaining 40% of the blastema, skeletal tissue contributes approximately 2% and the remainder is speculated to come from nondermal stump fibroblasts. The end result of the dedifferentiation process is the formation of the AEC and the blastema mesenchyme that interact with one another during the redevelopment phase of regeneration.
tion of blastema cells into the various tissue types originally present in the amputated structure. The cycle of dedifferentiation and redifferentiation raises the possibility that cells may be able to transdifferentiate into cell types that differ from their tissue of origin, and there is evidence that this type of plasticity does occur during limb and tail regeneration (Casimir et al., 1988; Lo et al., 1993; Kumar et al., 2000; Echeverri and Tanaka, 2002). Many regeneration studies have focused on transdifferentiation as a measure of cell plasticity and these studies assume that in order to transdifferentiate, a cell must first dedifferentiate. This may not be the case. In a recent study, Sustar and Schubiger (2005) demonstrated that transdifferentiation of regenerating Drosophila imaginal disk cells does not involve dedifferentiation at all, but instead a direct modification of the differentiation process. While it still remains possible that transdifferentiation in vertebrates involves cell dedifferentiation and redifferentiation, we believe that it is critical to define dedifferentiation independent of transdifferentiation because it is the dedifferentiation process that distinguishes regenerating limbs from nonregenerating limbs.

Muscle Tissue

Muscle represents a highly differentiated cell type in which the mature cell is a multinucleated syncytium that forms from the fusion of myoblast precursors. During limb development in amniotes, myoblasts migrate from somitic tissue to populate the limb bud, where they take up residence and differentiate to form all of the muscle tissue in the limb. In urodeles, dedifferentiation of multinucleated myotubes and myoblasts has been demonstrated in vitro and in vivo and involves fragmentation of the multinucleated syncytium and cellularization to form mitotically active uninucleated cells that enter the blastema (Brockes and Kumar, 2002). There is evidence that these muscle-derived blastema cells are not strictly myogenic and appear to be capable of transdifferentiation into cartilage (Casimir et al., 1988; Lo et al., 1993; Kumar et al., 2000). The muscle dedifferentiation response has been studied extensively in vitro and requires the activity of the Msx1 gene for fragmentation (Kumar et al., 2004), and the transition from quiescent to mitotically active cells is induced by thrombin (Tanaka et al., 1999).

The demonstration that myofibers can dedifferentiate provides a striking example of cellular plasticity in limb regeneration, and there is evidence that mammalian myotubes can undergo a similar dedifferentiation response in vitro (see review by Odellberg in this issue). In mammals, however, muscle tissue regeneration occurs routinely but is not associated with a dedifferentiation response; instead, myogenic stem cells called satellite cells provide a cellular source for the regeneration response (see review by Carlson in this issue). It is also well known that normal-appearing limbs that lack muscle tissue are formed during development or regeneration when muscle precursor cells are prevented from participation in limb outgrowth (Kiely and Chevallier, 1979; Holder, 1989). These studies indicate that the contribution of myoblasts in limb development or muscle tissue-derived blastema cells in limb regeneration is not required for limb outgrowth per se, but they are required for forming the limb musculature. These studies also highlight the plasticity of the blastema itself to organize a regenerative response in the absence of contribution from a major tissue source. What is currently unclear is to what extent cells derived from myofibers dedifferentiate into myoblasts, i.e., their normal precursor, versus transdifferentiate into other cell types.

Dermal Tissue

A number of studies identify the cells of the dermis as being extremely important for forming the blastema, for contributing to different regenerated tissues, and for organizing the regenerated limb pattern. Virtually all of these studies involved tissue grafting of skin that contains both dermis and epidermis; however, it has long been established that epidermal cells only contribute to the epidermis of the regenerate and not to the underlying blastema cells (Riddiford, 1960; Hay and Fischman, 1961). The dermis as a tissue is primarily composed of fibroblastic cells but also contains other cell types such as pigment cells and cells associated with vascular and neuronal innervation. In skin grafting studies, it is assumed that dermal fibroblasts are the responsible cell type because not only do they predominate, but also because fibroblasts have been shown to exit the dermis, migrate across the amputation wound, and participate in blastema formation (Endo et al., 2004). A critical role for the dermis in the regeneration response is indicated based on regeneration rescue experiments following X-irradiation. In these studies, limbs that are regeneration-inhibited by X-irradiation are rescued by grafting of a nonirradiated dermis with the demonstration that a normal, muscle-free, limb regenerates (Dunis and Namenwirth, 1977; Loveheurx, 1983; Holder, 1989). Since no other tissue tested is capable of inducing a normally patterned regeneration response, these studies identify the cells of the dermis as being critical for the regeneration response. Dermis has also been shown to play a key role in organizing the limb pattern that regenerates; thus, manipulation of the dermis can inhibit the regeneration response (Tank, 1983) or induce an extraordinary regeneration response involving the formation of supernumerary digits (Carlson, 1975; Rollman-Dinsmore and Bryant, 1982).

Contribution from the dermis to the regenerate has been demonstrated by following marked cells into different tissues of the regenerate, including cartilage, joint connective tissue, and general connective tissue (Dunis and Namenwirth, 1977). Quantitative data for the contribution of dermis to the blastema indicate that dermal cells make up, on average, 43% of the blastema, representing an overcontribution by more than twofold (Muneoka et al., 1986a). However, there was dramatic variation in contribution to the blastema when considering individual samples in this study, with individual blastemas composed of more than 75% of the cells derived from the dermis (a fourfold overcontribution) and other blastemas with less than 20% contribution. Thus, as we saw with muscle tissue, the blastema displays a level of plasticity that can accommo-
date considerable variation in the relative contribution from the various tissue types in the limb stump, and the data suggest that a fixed level of cellular participation from stump tissues is not a requirement.

The dermis of the urodele limb is composed primarily of fibroblastic cells that form a loose connective tissue layer underlying the epidermis. Virtually all of the tissues in the limb are associated with connective tissue composed of fibroblasts, and it has been suggested that these cells form a blueprint of the limb pattern (Gardiner et al., 2002). Since the dermis is at the limb periphery and the blastema forms at the center of the amputation wound, dermal cells must migrate across the amputation surface. This migration response is first observed within days of the injury (Gardiner et al., 1986; Endo et al., 2004) and represents the first indication of fibroblast dedifferentiation in blastema formation. This transformation of a mature dermal fibroblast to a migrating fibroblast has been described but has not been characterized in detail, but should prove informative for comparison to the mammalian wound-healing response that fails to regenerate. In mammals, dermal fibroblasts invade the wound and differentiate, or possibly transdifferentiate, into myofibroblasts that function transiently to contract the wound (Martin, 1997). In summary, there is convincing evidence for the involvement of dermal cells in blastema formation and in directing limb patterning; however, the details of their dedifferentiation response to a blastema cell remain poorly understood. Such studies are hampered by our lack of understanding of the characteristics of the mature urodele fibroblast. These studies do identify the dermal fibroblasts in mammalian limbs as a potential target for manipulating the wound-healing response to induce regeneration.

**Cartilage**

Another limb tissue for which there is histological evidence of dedifferentiation is cartilage. Cell marking studies demonstrate that pure cartilage tissue at the amputation wound dedifferentiates to form proliferating blastema cells that later contribute to skeletal and other connective tissue cells of the regenerate (Steen, 1968; Namenwirth, 1974; Muneoka et al., 1986a). Beyond these early cell marking studies, the dedifferentiation of cartilage in limb regeneration has not been extensively studied. However, mammalian chondrocyte dedifferentiation has been investigated in the context of a number of arthritic diseases, where the chondrocyte response to damage of surrounding extracellular matrix results in a dedifferentiation response (Sandell and Aigner, 2001). Chondrocytes are characterized by their production of matrix components, including type II collagen andaggrecan; maintaining this differentiated profile is critical for the long-term maintenance of healthy cartilage. The dedifferentiation of chondrocytes is observed in vivo in association with some forms of arthritis and in vitro when chondrocytes are enzymatically dispersed from their extracellular matrix. Dedifferentiated chondrocytes can be categorized by their production of a splice variant of type II collagen that is characteristic of chondrocyte progenitor cells (Oganesian et al., 1997). An alternative model is the in vitro dedifferentiation of chondrocytes into a fibroblast phenotype associated with the downregulated expression of type II collagen and the upregulation of type I collagen. The in vitro conversion of chondrocytes to a fibroblast phenotype has been shown to be induced by the activation of the canonical Wnt signaling pathway (Hwang et al., 2005). The downregulation of type II collagen in chondrocytes, in conjunction with the upregulation of type I collagen, could represent an example of transdifferentiation to a fibroblast phenotype since the expression of type I collagen is not characteristic of chondrocyte precursor cells during development. The downregulation of type II collagen without the upregulation of type I collagen can be induced with retinoic acid and is regulated by BMP signaling (Benya and Padilla, 1986; Nishihara et al., 2003); thus, these two events appear to be regulated independently. Retinoic acid is known to have dramatic effects on limb regeneration in amphibians (Maden, 1982), and there is evidence that chondrocyte dedifferentiation is specifically influenced by retinoic acid treatment (Maden and Keeble, 1987; Ju and Kim, 1994). Thus, one possibility is that the injury response of chondrocytes in mammals versus urodèles may center on the stimulation of transdifferentiation into fibroblasts versus the dedifferentiation into a mesenchymal phenotype. This interpretation may also underlie the effect that retinoid therapy has on models of rheumatoid arthritis (Beehler et al., 2004).

**WOUND HEALING AND DEDIFFERENTIATION**

Amputation of the mature limb transects a number of distinct tissues, including the epidermis, dermis, muscle, nerve, blood vessels, and bone, as well as the loose connective tissue that surrounds these tissues, thus exposing all of these tissues at the wound surface. In urodèles, the initial response to the injury is the formation of a fibrin clot that covers the wound surface and provides a substrate on which peripheral epidermal cells migrate to close the wound (Donaldson et al., 1985, 1987). This process is initiated by cells at the wound edge extending lamellipodia and actively moving across the wound, and more proximal cells similarly extending lamellipodia behind these lead cells (Mahan and Donaldson, 1986; Duncan et al., 2002). Urodele wound closure occurs incredibly fast; in young axolotls, an amputation wound is closed within 4 hr (Carlson et al., 1998), and in the adult newt, wound closure is completed in less than 12 hr (Repesh and Oberpriller, 1978). By comparison to a similar-size mammalian wound, for example, an amputated mouse digit that takes multiple days to close, the speed of urodele wound closure is extraordinary. Some of the outcomes of rapid wound closure include minimizing tissue damage, minimizing infection and an inflammation response, and rapid stabilization of the wound microenvironment.

After wound closure, the wound epithelium begins to thicken and eventually forms an AEC that is required for a regenerative response. Covering the amputation wound surface with mature skin completely inhibits the
regeneration response, demonstrating that the wound-healing response and AEC formation are required for regeneration (Mescher, 1976). The AEC is a transient epidermal structure that appears to function much like the AER in directing limb outgrowth during development in amniotes (Christensen and Tassava, 2000). The AEC induces ectopic regenerative outgrowths in much the same manner an ectopic AER induces ectopic outgrowth of the limb bud (Thornton and Thornton, 1965; Saunders et al., 1976). Removal of the AER from a limb bud results in the inhibition of limb outgrowth, and removal of the AEC similarly inhibits the regenerative response (Dearlove and Stocum, 1974; Saunders, 1998). One major difference between the AER and the AEC is that the AEC readily regenerates, whereas the AER is nonregenerative. The nonregenerative character of the AER has been shown to be causally linked to regenerative failure in the developing limbs of higher vertebrates (Hayamizu et al., 1994).

The AER function is largely mediated by its production of FGFs, in particular FGF4 and FGF8 (Sun et al., 2002). The urodele AEC is known to express Fgf8 (Han et al., 2001; Christensen et al., 2002) and to accumulate FGF1 and FGF2 peptides (Mullen et al., 1996; Dungan et al., 2002; Giampaoli et al., 2003), but there are few functional studies that test the role of FGFs in regeneration. Details of the expression pattern of Fgf family members suggest that there are key differences between the AER of amniote limb buds and the AEC of urodele regenerating limbs. For example, in the AER, Fgf8 and Fgf4 are specifically expressed by AER cells, whereas Fgf8, but not Fgf4, is expressed in the AEC (Han et al., 2001; Christensen et al., 2002). In addition, Fgf8 is also expressed in mesenchymal cells of the distal blastema in contrast to the AER-specific expression of Fgf8 in the limb bud (Han et al., 2001). FGF2 is immunolocalized to the AEC and the AER; however, during limb development FGF2 is also immunolocalized to the limb bud mesenchyme (Savage et al., 1993; Giampaoli et al., 2003). In regeneration, it is generally thought that Fgf1 and Fgf2 are transcribed in nerve cell bodies and transported to nerve terminals present in the AEC, where they are secreted (Mullen et al., 1996; Dungan et al., 2002). Thus, while both the AER and the AEC are sources of FGFs and appear to function in a similar manner, there appears to be clear differences in which FGFs are produced and how they accumulate in the distal epithelium.

The epidermis is not generally considered to be a tissue that undergoes dedifferentiation and redifferentiation during limb regeneration. However, there is evidence that this does occur. In fact, because the epidermis does not undergo transdifferentiation (Riddiford, 1960; Hay and Fischman, 1961), the epidermis is a relatively simple system in which to consider the dedifferentiation process. The relevant epidermal cell is the basal stem cell that is mitotically active, stationary, and committed to form epidermal cells by asymmetric division. In response to injury, these basal cells cease proliferating (Gardiner and Bryant, 2005) and initiate a migration response to cover the wound surface rapidly. This response is curiously reminiscent of the behavior of ectodermal cells during gastrulation. This type of wound closure occurs regardless of the type of injury, i.e., amputation versus skin wound. After wound closure, however, the wound epithelium of an amputation injury dedifferentiates to become the AEC and functions to direct limb outgrowth during the regeneration process. It is interesting that the reexpression of embryonic genes such as Fgf8 and Dlx3 in the AEC does not occur until after a blastema forms, thus suggesting that the final stages of epidermal dedifferentiation may be dependent on interactions with the dedifferentiating mesenchyme. After outgrowth of the blastema, these cells presumably undergo redifferentiation to form the basal stem cells that populate the regenerated epidermis.

REGENERATION DURING LIMB DEVELOPMENT

Limb regeneration has also been studied in developing limbs that display a progressive loss of regenerative ability with maturation. The developing anuran limb has been best studied. The loss of regenerative ability as the limb develops indicates that developing cells can mount a regenerative response to injury and suggests that a fundamental change in this response is linked to regenerative failure in adults. We have previously proposed a model in which multiple changes, or regeneration barriers, occurring sequentially during development are responsible for regenerative failure (Muller et al., 1999). There is now clear evidence that one of these barriers involves the initiation of an FGF signaling feedback loop between the wound epithelium and the injured limb tissue (Yokoyama et al., 2001).

The developing hindlimb of Xenopus laevis has the capacity to regenerate after amputation; however, this ability is progressively lost as the limb matures (Dent, 1962; Muneko et al., 1986b). Although Xenopus limb buds do not possess a morphologically distinct AER, Fgf8 is expressed in a manner that is analogous to the AER-specific expression of amniote limb buds, and it is reexpressed during limb bud regeneration (Christen and Slack, 1997). Thus, the Xenopus limb bud is distinct from the amniote limb bud in that the AER equivalent structure is able to reform following limb bud amputation, and limb regeneration is induced. Amputation at later limb bud stages fails to form a blastema and Fgf8 is not reexpressed (Christen and Slack, 1997; Yokoyama et al., 2000). The lack of Fgf10 expression by limb stump cells is also associated with regenerative failure (Yokoyama et al., 2000), and this is of interest because during amniote limb development, Fgf8 expression by the ectoderm and Fgf10 expression by the mesenchyme form a cross-regulatory loop that is essential for limb outgrowth (Martin, 1998). Treatment of later-stage amputated limbs with FGF8 results in a limited regenerative response; however, treatment with FGF10 induces significant regeneration (Yokoyama et al., 2001). Associated with induced regeneration is the reexpression of Fgf8 and Fgf10, suggesting that FGF10 treatment jump-started the Fgf8-Fgf10 cross-regulatory loop, thus initiating the regenerative response.

Postmetamorphic Xenopus limbs display a hypomorphic regenerative response that involves blastema formation; however, the regenerate con-
sists of a tapering unsegmented cartilaginous spike surrounded by skin (Dent, 1962). During this hypomorphic regeneration response, Fgf8 is re-expressed, as is Msx1, a mesenchymal gene known to be responsive to the AER; thus, regenerative failure at this stage cannot be attributed to the lack of a functional AEC (Endo et al., 2000; Slack et al., 2004). Instead, Shh re-expression by posterior mesenchymal cells is absent during hypomorphic regeneration, suggesting that an additional regeneration barrier involves the regulation of patterning genes (Endo et al., 2000). These observations suggest not only that there are multiple regeneration barriers, but that they can also be stage-specific.

**Chick Limb Bud**

The developing limb bud of the chick embryo has also been used as a model to investigate regenerative failure. Unlike the anuran limb bud, amputation of the chick limb bud results in no regenerative response whatsoever. However, it is also well established that surgically removing only the AER results in limb truncations and that the AER fails to regenerate during wound healing after amputation. AER removal results in localized apoptosis and eventual limb truncation (Dudley et al., 2002), both of which can be rescued by treatment with a number ofFGF family members, including FGF2, FGF4, and FGF8 (Martin, 1998). FGF4 and FGF8 are known to be mitogenic for limb bud cells (Niswander and Martin, 1993; Kawakami et al., 2003), in addition to their role as cell survival factors (Dudley et al., 2002; Sun et al., 2002). FGF4 produced by the AER acts as a chemotactant for limb- and digit-forming cells (Li and Muneoka, 1999; Nog-Muller and Muneoka, 2000), and the data suggest that organized cell migration is critical for limb bud morphogenesis (Omi et al., 2002; Nog-Muller et al., 2004). While removal of the AER causes limb truncation, the presence of an ectopic AER redirects outgrowth and stimulates the formation of additional limb structures. Ectopic treatment of an intact limb bud with FGF2 or FGF4 does not induce additional limb structures (Li et al., 1996; Li and Muneoka, 1999), thus suggesting that the outgrowth-inducing effect of the AER must be more complex than simply providing FGFs.

With respect to limb regeneration, one significant difference between the AER and the AEC is that the limb buds of higher vertebrates are unable to regenerate the AER if it is damaged or removed following amputation. Amputation of the chick limb bud, which removes the AER and the distal mesenchyme, results in a unique wound-healing response involving the formation and contraction of an actin cable network that laces the ectodermal cells of the wound edge together to close the wound like a purse string (Martin and Lewis, 1992). A regenerated AER does not form and the limb that develops is truncated. Treatment of the amputation surface with FGF2 or FGF4 induces a regenerative response without inducing the regeneration of an AER (Taylor et al., 1994; Kostakopoulou et al., 1996), suggesting that FGF application replaces AER function at the wound surface. Consistent with this interpretation, genes known to be AER-dependent, such as Msx1, Hoxd13, and Shh, are induced in regenerating limb buds (Kostakopoulou et al., 1996, and data not shown). Studies where various tissues are placed into an excavated site under the AER to test for regenerative ability (Hayamizu et al., 1994) show that regenerative potential is lost in a stage-specific and position-specific manner during chick limb development (data not shown). These studies suggest that regenerative failure in developing amniote limbs results from an inability of the healing ectoderm to regenerate the AER, but if an AER (or its functional equivalent) is provided, amputated limb bud tissues respond in much the same manner as the anuran limb bud.

Studies on regenerative decline during the maturation of the limb point to defects in regenerating a functional AER, or its regeneration-specific equivalent, the AEC. Since the later stages of limb regeneration require a functional apical epidermis to direct limb outgrowth, it follows that understanding how to modify the wound-healing response following amputation in higher vertebrates to reexpress functional AER genes such as Fgf8 and Fgf4 represents an important first step toward inducing a regenerative response in higher vertebrates.

**DIGITAL TIP REGENERATION IN MICE**

The only part of the mature mammalian limb that possesses regenerative capabilities is the tips of the digits. This discovery was made first in children with the observation that simply treating fingertips amputations by dressing changes resulted in good cosmetic healing, restoration of the finger contour and fingerprint, restoration of normal sensation, and finger elongation (Douglas, 1972; Illingworth, 1974). This regenerative response is level-specific, and successful regeneration is reported following amputation through the proximal region of the distal phalanx (Illingworth, 1974). Similar results have been reported in juvenile mice that have been amputated under controlled experimental conditions, and studies show that the regenerative response involves the elongation of the distal phalanx as well as partial restoration of the fat pad (Borgens, 1982; Muller et al., 1999). While regeneration of bone tissue is commonplace following bone fracture, regrowth of bone from a free surface, such as the amputated distal phalanx, is a unique regenerative response in mammals. Clinical and experimental studies indicate that this regenerative response does not involve endochondral ossification but involves direct appositional ossification (Vidal and Dickson, 1993, and data not shown).

The digit tip of the mouse includes a ventral fat pad and a dorsally located distal phalangeal bone. The phalangeal bone is surrounded by loose connective tissue and is enclosed within the nail organ. The nail inserts near the distal interphalangeal joint and regenerative ability has been associated with the nail organ (Zhao and Neufeld, 1995). During digit development, a number of genes are specifically expressed in the digit tip and subsequently become associated with the forming nail, the connective tissue surrounding the distal phalanx, or the distal phalanx itself (Reginelli et al., 1995; Nog-Muller and Muneoka, 2000; Han et al., 2003). Msx1 and Msx2 are both expressed in the devel-
opining digit tip mesenchyme with Msx2 expression extending to the distal ectoderm; however, in vivo amputation studies indicate that regenerative potential correlates with the proximal extent of the Msx1 expression domain (Reginelli et al., 1995). Using an in vitro digit tip regeneration model, we have recently shown that the Msx1 mutant embryo displays a regeneration defect, whereas the Msx2 mutant embryo regenerates normally (Han et al., 2003). We have been able to rescue the defective regeneration response in Msx1 mutants in a dose-dependent manner by treating the amputated digits with purified BMP4, and we have been able to inhibit regeneration of wild-type digit tips by treatment with the BMP signaling antagonist Noggin. These studies, carried out on developing digits, establish that a signaling pathway involving Msx1 and BMP4 is essential for digit tip regeneration. Since digit formation in the Msx1 mutant limb is completely normal (Satokata and Maas, 1994), and the formation of the distal phalanx in limbs lacking Bmp4 is normal (Selever et al., 2004), the data implicate the Msx1-BMP4 pathway as required for the injury response phase, rather than the redevelopment phase, leading to regeneration.

There is considerable evidence that Msx1 inhibits differentiation and plays a key role in regeneration. Msx1 is known to be a transcriptional repressor that acts in concert with a linker histone, H1b, to inhibit differentiation-specific gene expression (Lee et al., 2004). Msx1 is expressed during regeneration in a diverse number of systems, including the urodele limb (Koshiba et al., 1998), the anuran limb bud (Yokoyama et al., 2000), the FGF-induced regenerated chick limb bud (Kostakopoulou et al., 1996), the fish fin (Murciano et al., 2002), the anuran tail (Beck et al., 2003), and the mouse digit tip (Reginelli et al., 1995; Han et al., 2003). Limb developmental studies show that Msx1 expression is regulated by the AER during limb outgrowth (Davidson et al., 1991). In vitro studies on cell differentiation provide evidence that Msx1 functions to inhibit the differentiation of a variety of cell types, including myoblasts, condrocytes, osteoblasts, adipocytes, and mammary epithelial cells (Hu et al., 2001). Other studies show that regulated Msx1 expression induces differentiated multinucleated myotubes or myofibers in both urodeles and mammals to undergo fragmentation and cellularization to form uninucleated dividing multipotent cells (Odelberg et al., 2000; Kumar et al., 2004). In combination with our Msx1 mutant studies on digit regeneration, the evidence strongly suggests that Msx1 functions to regulate the differentiated/dedifferentiated status of cells following injury. This conclusion provides a molecular and conceptual framework to begin teasing apart the molecular mechanisms governing the dedifferentiation process of different limb tissues as we move closer to understanding the regeneration process.

**CONCLUSION**

The problem of how to induce a regenerative response in humans was once an unrealistic endeavor, but is now becoming a very reasonable goal. Studies on amphibian regeneration models, along with our detailed understanding of limb development in higher vertebrates, provide clearly defined paths toward this goal. It is clear that the regeneration process involves an intricate interface between the wound-healing response, on the one hand, and a redevelopment process during which patterning and morphogenesis drive limb outgrowth. In amphibian regeneration, the earliest response to amputation injury by the epidermis and by the cells of the stump converts the wound into a site that is permissive for redevelopment. This response involves the dedifferentiation of epidermal cells to establish a signaling apical epithelium, the dedifferentiation of stump fibroblasts that undergo a directed migratory response to form a blastema, and the dedifferentiation of myofibers to form unincellular myoblasts. In contrast, the mammalian wound epithelium is slow to form and, while stump fibroblasts are migratory, their migration appears random and many eventually differentiate into myofibroblasts that contract the wound. Regeneration studies have identified a number of molecular markers that can be used to monitor attempts to modulating the mammalian early injury response toward a more successful regenerative response. At the same time, the mouse digit represents an emerging model for regeneration because of the striking contrast between regeneration-competent digit regions that are literally millimeters away from regions that fail to mount a regeneration response. Numerous lines of evidence, including functional studies of mouse digit regeneration, have identified the homeobox containing gene Msx1 as a critical regulator of the regeneration response in fish, amphibians, birds, and mammals. Progress in our understanding of the molecular basis of the regenerative response is moving rapidly, and the goal of eventually inducing a regenerative response in humans may lie in the not so distant future.

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