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Vertebrate limb regeneration and the origin of limb stem cells

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ABSTRACT The existence of multipotent cells in the adult tissues and organs of those vertebrates that are capable of regeneration has been accepted for decades. Although studies of vertebrate limb regeneration have yet to identify many of the specific molecules involved in regeneration, numerous tissue grafting experiments and studies of cell lineage have contributed significantly to an understanding of the origin, activation, proliferation and cell-cell interactions of these progenitor cells. This has allowed the development of ideas about the regulation of pattern formation to restore the structure and function of lost tissues and organs. An understanding of the molecular mechanisms controlling these processes has lagged behind the dramatic advances achieved with other model organisms. However, given the intense, new research interest in stem cells over the past few years, there is good reason to be encouraged that insights about the biology of mammalian stem cells will accelerate progress in understanding the biology of regeneration in organisms that can regenerate. Advances in regeneration research will then feed back in terms of devising new strategies for therapies to induce regeneration in organisms such as humans that have traditionally been viewed as incapable of regeneration.

KEY WORDS: *regeneration, limbs, wound healing, stem cells, dedifferentiation*

A large number of organisms are able to regenerate body parts; however, they have attracted relatively little research effort over the decades. While regeneration is considered biologically fascinating, the prevalent view that humans are incapable of regeneration has lessened its perceived relevance to human health and medicine. It has been recognized for many years that there are cells in adult mammalian tissues that are involved in maintaining homeostasis in tissues that are constantly replacing their cell populations (e.g. blood, skin, hair and gut epithelium). But the large-scale replacement of tissues, much less entire organs or body parts in mammals has not been considered possible, despite numerous examples of this ability in other organisms. In recent years, this view has begun to change significantly as a result of the discovery of multipotent cells in adult mammalian tissues that can respond to injury and proliferate to give rise to cells which assume a variety of fates. For example, bone marrow derived cells, in addition to serving as blood cell progenitors, are now known to be able to contribute cells to the repair of muscle, brain, liver, heart and blood vessels (Blau *et al.*, 2001).

The presence of multipotent cells in adult tissues that respond to injury to replace damaged or missing parts, has been an accepted feature of regeneration in lower vertebrates for decades. Although studies of vertebrate limb regeneration have yet to identify many of the specific molecules involved in regeneration, numerous tissue grafting experiments and studies of cell lineage have contributed significantly to an understanding of the origin, activation, proliferation, and cell-cell interactions of these progenitor cells. This has allowed the development of concepts about the regulation of pattern formation to restore the structure and function of lost tissues and organs (Bryant *et al.*, 1981; French *et al.*, 1976; Gardiner and Bryant, 1998; Mescher, 1996). An understanding of the molecular mechanisms controlling regenerative processes have lagged behind the dramatic advances in understanding of developmental mechanisms achieved in standard model organisms, none of which regenerate as adults. However, given the intense research interest in stem cells of the past few years, there is good reason to be encouraged that insights about the biology of mammalian stem cells will accelerate progress in understanding the biology of regeneration in organisms that can regenerate. In turn, advances in regeneration research will inform new strategies and therapies to induce regeneration in organisms, even humans, that have traditionally been viewed as incapable of regeneration.

Abbreviations used in this paper: AEC, apical epidermal cap; ECM, extracellular matrix; MMP, matrix metalloproteinase; WE, wound epidermis.

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The Regenerating Limb as a Model System for studying the Origin of Stem Cells

Most vertebrate embryos can regenerate appendages during early stages of limb bud development when the limb cells and tissues are still undifferentiated. In the best analyzed case of "embryonic" regeneration, limb bud regeneration in frog tadpoles, differentiation progresses from proximal to distal, and is paralleled by a decline in regenerative ability (Dent, 1962; Endo *et al.*, 1997; Muneoka *et al.*, 1986b). Pre-differentiative regenerative ability has also been described in mice (Wanek *et al.*, 1989), and chicks (Hayamizu *et al.*, 1994; Kostakopoulou *et al.*, 1996), and it is possible that human digits have regenerated following an intrauterine trauma. Limb regeneration in the embryo is indistinguishable from limb development, and most surgical manipulations of developing limbs have in reality made use of regeneration to draw inferences about the cellular interactions controlling development. Despite this early and often impressive regenerative ability, in all of these examples, regeneration is only possible in regions of the developing limb that have not yet undergone differentiation. One important inference from these observations is that the mechanism of regeneration is intact in mammalian and avian limbs, and likely in other organs, and can be activated provided the cells are undifferentiated. In these non-regenerating animals, adult differentiated cells are unable to re-assume an embryonic phenotype or function (Gardiner and Bryant, 1996).

Appendage regeneration is common among the adults of many non-vertebrate organisms, but among adult vertebrates, it is unique to the urodele amphibians (newts and salamanders). Although regeneration of a large range of tissues and organs is possible in urodeles, it has been most intensely studied in the limb. In contrast to the situation in larval stages when the developing limb bud is composed largely of undifferentiated cells, the adult limb is composed of fully differentiated tissues. Thus in the limb bud, the source of progenitor cells for regeneration is not an issue; whereas, in the adult, this question has attracted considerable research attention. It is known that the progenitor cells for regeneration are of local origin, residing within a few millimeters of the amputation plane (see Wallace, 1981), and that they generate a population of undifferentiated, proliferative cells that accumulate at the distal end of the amputated stump to form the regeneration blastema. Interactions between blastema cells control the subsequent growth and pattern formation that reestablishes the normal pattern of all the differentiated tissues that were removed by amputation. Of particular significance is the fact that the regenerated limb can be re-amputated, and the process of regeneration recapitulated. Thus, limb progenitor cells persist in the regenerated limb, and are by definition self-renewing. Although the definition and concept of the identity and function of stem cells is currently in a state of flux (Blau *et al.*, 2001; Marshak *et al.*, 2001), a generally agreed upon, working definition of a stem cell is "…a cell with the capacity for prolonged or unlimited self-renewal, combined with the capacity to produce at least one type of highly differentiated progeny" (Flake, 2001). Hence, investigations of the cells in adult urodele limbs that give rise to the regeneration blastema, are likely to contribute to the further understanding of stem cell biology, and reciprocally, perhaps will benefit from an understanding of mammalian stem cells.

Given the amount of effort that has gone into identifying both the source of regeneration progenitor cells and how blastema cells interact to stimulate growth and pattern formation, it is surprising how little is known about how limb cells become blastema cells. As discussed below, a major source of limb stem cells is connective tissue, particularly in the dermis of the skin, although other differentiated cells also participate. Connective tissue cells, or fibroblasts, do not appear to be highly specialized, and are not well characterized, particularly in urodeles, leaving it unclear whether all fibroblasts are capable of becoming blastema cells. It is an open question as to whether the fibroblast population is heterogeneous, with only a subpopulation functioning as blastema cell precursors. Based on almost no data about fibroblast phenotypes and function, it has been assumed that the regeneration blastema arises via a mechanism of "dedifferentiation" of fibroblasts and other cell types. As a consequence of the lack of critical data, the term "dedifferentiation" historically has been used loosely and appears to mean different things to different authors (discussed in (Carlson, 1998). Unfortunately the term is frequently used to refer to the reversal of cell fate of differentiated fibroblasts, as if it is known with certainty that this process occurs, when in reality it is inferred but not proven. Similarly, the observation that a population of undifferentiated, quiescent stem cells has not been identified to date is not evidence that they do not exist, particularly since none of the modern tools of molecular biology have been utilized to look for such a population. If dedifferentiation of fibroblasts does occur during urodele limb regeneration, understanding the signals and responses controlling this process likely will contribute to an understanding of the origin and regulation of fate of mammalian stem cells.

The intent of this review is to provide an overview of what is known about limb regeneration, with a focus on what is known about the origin of limb blastema cells. Much of what is known in general about limb regeneration has been well reviewed in the past (see Carlson, 1998; Gardiner and Bryant, 1998; Mescher, 1996; Wallace, 1981), and an extensive review of the classic literature would be redundant. There are however, some recent additions to the limb regeneration literature that are relevant to the focus of this review. In addition, given the recent explosion in stem cell biology research, we have attempted to emphasize the data from both classical and modern studies of limb regeneration that complement studies in mammals. In particular we focus on evidence for there being at least three phases to regeneration, of which the first two (wound healing and dedifferentiation) are likely to be most critical to devising strategies for inducing regeneration in mammals. Finally, recognizing what we do not know is critical in guiding future research efforts to understand how to control of regeneration, we have attempted to emphasis promising areas for such future research.

There are a least Three Phases of Limb Regeneration

With recent applications of molecular techniques to studies of limb regeneration, the sequence of events that occurs in response to limb amputation is being identified with finer spatial and temporal resolution than in the past. Relatively few studies of regeneration have included analyses of patterns of gene or protein expression, and the majority has been based on relatively gross morphological criteria. By necessity, studies to date have been focused mainly on the later stages of regeneration, and have involved studies of tissue grafting and cellular contribution. Such studies led to the conclusion that regeneration and development are more similar

than they are different (Bryant and Gardiner, 1992). More recently, studies of gene expression have revealed striking differences between these two processes, particularly during the early stages of regeneration prior to blastema formation (Gardiner and Bryant, 1998). Based on these differences, two distinct phases of regeneration have been identified; a preparation phase that is unique to regeneration, and a redevelopment phase that is similar to limb development. Based on recent studies of the induction of accessory limbs from lateral wounds on limbs (discussed below), we now recognize that there are at least three distinct phases to limb regeneration, and as more is learned about specific molecular details, it is likely that even more will be evident.

Taken as a whole, recent molecular analyses of regeneration, in conjunction with classical studies, indicate that the minimum requirements for limb regeneration are: a skin wound, adequate innervation and a positionally diverse blastema. These requirements allow us to separate the process into three distinct phases: wound healing, preparation and redevelopment. The first two phases obviously are essential prerequisites for adult regeneration, because they set the stage for redevelopment. Several of the characteristics of each phase of regeneration are summarized in Table 1.

Phase I - Wound Healing

Within the first hour after amputation, or skin removal, epithelial cells begin to migrate as a sheet to cover the exposed mesenchymal tissues. Wound closure is rapid and is complete within two hours in smaller animals (Carlson *et al*., 1998). By all criteria studied to date, wound closure of an amputation stump is the same as that of a lateral wound on the side of a limb. The rate of closure is equivalent, and the same early genes are expressed. In the case of lateral limb wounds, there is no subsequent outgrowth; instead of proceeding on to Phase II, the skin eventually regenerates without scarring. Little is known about the sequence of events leading to skin regeneration, though presumably in common with the limb regeneration pathway, the cells responsible arise from migrating fibroblasts (Gardiner *et al*., 1986). Induction of expression of some genes (*Msx-2* and *MMP-9*) occurs prior to wound closure, and thus does not depend on wound epidermis (WE). However, the continued expression of those genes, as well as others expressed after epidermal wound closure, is inhibited by a graft of mature skin (Carlson *et al*., 1998; Gardiner *et al*., 1999; Yang *et al*., 1999). Thus the WE appears to be able to maintain, and perhaps induce, the expression of a number of genes. Nerves are not required for either wound healing or for progression to skin regeneration, and may even indirectly delay the latter. Although major nerves are present at the wound surface of an amputated limb, they are absent at the site of a lateral limb wound. Nonetheless, the early stage regeneration genes are expressed in both types of wounds. Although the skin eventually regenerates, the process is delayed until the end of limb regeneration by the interactions between blastema cells and the Apical Epidermal Cap (AEC) that promote limb regeneration. Denervation of the limb inhibits progression to Phase II, and adult skin regenerates in place of the AEC. The inhibition of limb regeneration in this case may be related to the inhibition that can be caused by a graft of mature skin to cover the amputated stump. Lateral limb wounds are not innervated, do not form either an AEC or an outgrowth, and the skin is regenerated.

Phase II - Dedifferentiation

Within the first one to two days, the outgrowth response to injury from an amputation is distinct from the response of a lateral limb skin wound, even though both eventually lead to regeneration of the skin, one sooner and the other much later. The end result of Phase II as we presently view it, is the genesis of a population of undifferentiated, proliferating blastema cells that is able to progress to Phase III to undergo the process of redevelopment of the limb. Thus Phase II is the period of limb regeneration when either cells in mature limb tissues dedifferentiate and/or quiescent stem cells are activated to give rise to the cells of the blastema. Several days after amputation, cells derived from connective tissue fibroblasts begin to migrate under the wound epidermis and accumulate at the distal tip of the amputated stump. The onset of the proliferative stage of regeneration is coincident with the onset of cell migration (Gardiner *et al*., 1986), and shortly thereafter the regeneration blastema is formed, and Phase III begins.

The divergence of Phase II limb regeneration from the skin regeneration pathway is first evident from the expression of *Hoxa-9* and *Hoxa-13* in the distal stump tissues of an amputated limb (discussed below), but not in a lateral skin wound (Gardiner *et al*., 1999). Over the next several days, a number of other genes exhibit a similar dichotomy in expression. Presumably Phase II depends on signals generated during Phase I (wound healing), since a mature skin graft inhibits Phase II gene expression and the subsequent events associated with dedifferentiation. It is unclear what signals divert a wound away from skin regeneration and toward Phase II limb regeneration events, but it appears they may be derived from nerves, or at least require the presence of nerves. Denervation of a limb around the time of amputation prevents the progression to Phase II, even though there is tissue histolysis and limited proliferation in denervated stumps. These events presum-

TABLE 1

THE PHASES OF REGENERATION

PHASE I - WOUND HEALING	
Epidermal healing	Epidermal sheet migrates to cover the wound area within 1-2 hrs
Induction of gene expression	Genes common to wound healing and limb regeneration are expressed (e.g. *msx-2* and *MMP-9*)
Nerve dependency	Not dependent on nerves
PHASE II - DEDIFFERENTIATION	
Dedifferentiation	Cells in the stump tissues lose their specialized characteristics and become migratory
Blastema formation	Cells derived from fibroblasts migrate to form the blastema and begin to proliferate
Induction of gene expression	Re-expressed genes show spatial and/or temporal patterns that differ from development
Nerve dependency	No regeneration if nerve supply is interrupted
PHASE III - REDEVELOPMENT	
Growth and pattern formation	Classic responses to grafting are the same as in developing limbs; developing and regenerating limbs can cooperate to form a chimeric limb
Induction of gene expression	Genes show similar expression and function as in developing limbs
Nerve dependency	Continued growth depends on nerves, but differentiation is nerve-independent
Positional dependency	Requirement for a blastema consisting of cells that are positionally diverse in origin

ably are a consequence of the degeneration of injured muscle fibers that have been denervated, but regardless, limb stem cells are not accumulated because no blastema is formed.

The induction of *Hoxa-9* and *Hoxa-13* expression indicates that Phase II begins soon after amputation, at least by 24 hours (Gardiner *et al.*, 1995). Induction of *HoxA* expression is thus far, the earliest reported molecular event specific to regeneration. Expression of several other genes is induced earlier, but they are also expressed in lateral skin wounds, which do not form outgrowths or accessory limbs (see below). Both *Hoxa-9* and *Hoxa-13* are expressed in the distal-most cells of the amputated stump, which several days later will give rise to the early blastema. The early blastema subsequently increases in size as a consequence of continued recruitment of cells from the stump, and cellular proliferation. As the blastema grows, a region of cells expressing *Hoxa-9* but not *Hoxa-13* is generated at the base of the blastema. When the regenerated skeletal elements begin to differentiate, both *Hoxa-9* and *Hoxa-13* are expressed in the autopod, whereas *Hoxa-9*, but not *Hoxa-13*, is expressed in the zeugopod. This final spatial expression pattern is the same as in developing limbs in urodeles as well as other vertebrates. Based on molecular and genetic evidence, specification of the distal-most region of the pattern (autopod) is a consequence of the coexpression of both 3' and 5' members of the *HoxA and HoxD* complexes. The early coexpression *Hoxa-9* and *Hoxa-13* in stump cells indicates that Phase II is initiated by the reestablishment of the distal-most part of the limb pattern, regardless of the level of amputation. The more proximal regions of the pattern arise subsequently during Phase III as a consequence of growth of the blastema and the intercalation of intermediate parts of the pattern (Gardiner and Bryant, 1998). The early establishment of the distal tip of the limb ensures that the regenerated tissues will always be an exact replacement of the portion of the pattern that is removed.

Phase III - Redevelopment

At the beginning of Phase III, the now large undifferentiated blastema appears by a variety of different criteria to behave like a developing limb bud. The most direct demonstration of the similarity of blastemas and limb buds comes from experiments in which grafts were made between developing and regenerating limbs. In those studies, limb bud and blastema cells behave identically, and the patterns of cellular contribution to chimeric limbs is the same as to either developing or regenerating limbs (Muneoka and Bryant, 1982; Muneoka and Bryant, 1984). More recent studies have demonstrated that the spatial and temporal patterns of gene expression during blastema stages of regeneration are in most ways comparable to those during limb development of urodeles as well as other vertebrate embryos (Gardiner and Bryant, 1998).

The transition from Phase II and Phase III is not distinct either spatially or temporally. There is a period of time when dedifferentiation of stump tissues (Phase II) is continuing proximally while undifferentiated blastema cells are proliferating distally and the blastema is growing (Phase III). The requirement for transition to Phase III is a population of blastema cells that are positionally diverse in their origins (Bryant *et al.*, 1981; Maden and Holder, 1984). Without that, the blastema will regress (see below). Nerves do not exert as critical an influence over the outcome at this Phase as they do as for Phase II. Denervation of a Phase I or II limb regenerate results in a cessation of growth and absence of new limb structures. Denervation in Phase III of a slightly later stage blastema similarly results in a cessation of growth, however, some limb structures do differentiate. It appears that nerves provide signals that are required for blastema growth, regardless of the stage of regeneration. Since nerves are also required for Phase II, it is reasonable to suggest that denervation blocks continued dedifferentiation of stump cells during early blastema stages of regeneration. This would prevent the formation of dedifferentiated cells at the proximal boundary of the blastema, and hence prevent intercalation between the *Hoxa-9/Hoxa-13* expressing cells and more proximal cells, thus inhibiting the subsequent replacement of the missing limb structures. Denervation of early blastemas has a similar effect as prolonged exposure to retinoids at similar stages, which also is proposed, for completely different reasons, to inhibit the proximal-distal interactions necessary for regeneration, and which results in truncated limbs (Bryant and Gardiner, 1992).

From the point of view of there being multiple phases in regeneration, it appears that once the process progresses to Phase III, it may continue unassisted as it did during embryogenesis. If this proves to be the case, then the focus of efforts to stimulate regeneration in humans needs to be on Phases I and II, with the goal of providing an environment that induces the genesis of a population of cells that can redevelop a limb.

Contribution of Limb Stump Tissues to the Regeneration Blastema

Perhaps the most critical issues in limb regeneration research are the identification of the sources and the development of an understanding of the factors controlling the genesis of blastema cells. Much of the research pertaining to this issue has been recently reviewed (Carlson, 1998; Gardiner and Bryant, 1998; Mescher, 1996), and it appears that all tissues of the mature limb contribute cells to some extent. In addition, at this time there are no data indicating extensive metaplasia or transdifferentiation during normal limb regeneration. The principle experimental strategy has been to graft marked tissues into regenerating limbs and then follow the fate of grafted cells into the regenerated tissues. This approach is limited by the fact that most tissue grafts are composed of multiple cell types. In particular, most tissues have connective tissue fibroblasts associated with them, and thus it is not possible to know with certainty which cell type(s) provided the progenitor cell(s) for any particular regenerated tissue. Early anatomical studies led to the conclusion that regeneration is tissue-specific, with different tissues contributing to the regenerate in proportion to their abundance in the stump (Chalkley, 1954; Hay and Fischman, 1961). Subsequent lineage studies have demonstrated this is not the case (Muneoka *et al.*, 1986a). The finding that some cells, specifically dermal fibroblasts, over-contribute to the blastema and the regenerate indicates that those cells are multipotential, and are a likely source of limb stem cells that contribute to several tissues during regeneration.

Just as the view of cells in regenerating limbs has tended to downplay multipotentiality, until recently stem cells in adult mammals have been considered to be restricted in their developmental potential to the tissues in which they reside. This is now recognized not to be the case, and it is clear that the presence of multipotent stem cells in adult tissues is the rule rather than the exception (see Blau *et al.*, 2001).

Nerves and Blood Vessels

A regenerating limb contains both nerves and blood vessels that are continuous with structures at more proximal levels in the stump, and these structures appear to contribute to the regenerated limb. However, they do not appear to contribute cells to the population of undifferentiated, proliferating blastema cells. This appears particularly to be the case with blood vessels since the early blastema is poorly vascularized. Several histological studies have led to the conclusion that dedifferentiating stump tissues and the early population of blastema cells derived from those tissues (corresponding to Phase II of regeneration) are avascular (Mescher, 1996), and that blood vessels eventually grow into later stage blastemas from preexisting vessels arising at more proximal levels of the limb. However, a recent study has challenged this view (Rageh *et al.*, 2002).

Axons from nerves in the limb stump proximal to the amputation levels grow rapidly into the dedifferentiating region of the stump, the early blastema and the overlying epidermis. The presence of nerves is required for regeneration to progress to the later stages of regeneration (as discussed above). In response to injury, the axons initially regress proximally, leaving behind the connective tissue sheath (fibroblasts and Schwann cells). These cells appear to proliferate and contribute to the blastema (Chalkley, 1954; Hay and Fischman, 1961), and subsequently appear to reassociate with the regenerating axon to form the nerve-associated connective tissue and nerve sheaths.

A dependence on nerves is one characteristic that distinguishes an early stage blastema from a developing limb bud, which is not nerve dependent (see Fekete and Brockes, 1987). The independence from nerves of late stage blastemas is a characteristic that makes late stage regeneration more similar to limb development. It has not been suggested that nerve dependency is related to a contribution of cells from nerves, since nerve-associated connective tissue cells are present in the stump of both innervated and denervated limbs. Rather, the evidence is for the contribution of a factor(s) from regenerating axons that is required for the early events of regeneration. The phenomenon of nerve-dependency has been extensively studied, and several candidate molecules have been hypothesized to function as the elusive "neurotrophic factor" that functions as either a growth inducing or growth permitting factor (Mescher, 1996; Mullen *et al.*, 1996; Muneoka *et al.*, 1989). Because proliferation of blastema cells is affected by denervation, the "neurotrphic factor" is considered to function solely as a growth inducing or growth promoting factor (Mescher, 1996). Because tissue degradation occurs in denervated limbs, nerves have not been considered to influence the process of dedifferentiation, even though that process is not understood. It is clear that muscle fibers degenerate when injured and denervated, but the relationship between that process and the dedifferentiation of other tissues in the stump is unclear. In contrast, studies of accessory limbs induced from lateral limb wounds (discussed below) lead to the conclusion that nerves are in fact required for dedifferentiation (Phase II of regeneration). This conclusion is consistent with findings from recent studies of mammalian stem cells demonstrating the importance of secreted signaling molecules such as growth factors in the activation and proliferation of stem cells. It seems worth considering that nerves are a source of such factors that would be required for dedifferentiation and recruitment of limb stem cells. Advances in identifying such signaling molecules and understanding their mode of action have been limited by lack of bioassays that are appropriate for the use of modern techniques. At the end of this review, we focus on three assays that might be applicable to such analyses.

Skeletal Tissues

Although there is histolysis of bone and cartilage in response to injury, descriptive studies have led to the conclusion that new skeletal tissues arise from cells of the periosteum or perichondrium (Chalkley, 1954; Hay and Fischman, 1961). Since adherent connective tissue and muscle can be removed from skeletal tissues, they can be grafted to experimentally determine their contribution to the blastema (Muneoka *et al.*, 1986a). Such studies have demonstrated that cells from differentiated skeletal tissue and associated perichondrium contribute to the blastema at lower frequency than their representation in normal tissues. Such results lead to the conclusion that cells from tissues other than skeletal tissues contribute to the normal regeneration of the skeleton. Consistent with this conclusion are results from several experiments (discussed below with respect to connective tissues) demonstrating that a normal limb skeleton can be regenerated by cells derived solely from connective tissue fibroblasts. If the humerus is removed prior to amputation, the regenerated limb still forms a normal skeleton distal to amputation even though the missing skeletal elements are not reformed in the stump proximal to the amputation plane (Goss, 1956). Limbs in which contribution from all tissues other than dermal fibroblasts has been prevented by x-irradiation, will still regenerate skeletal tissues of normal and complete pattern (Dunis and Namenwirth, 1977; Holder, 1989; Lheureux, 1983).

Muscle

One of the more studied issues in regeneration research has been that of the contribution of differentiated muscle fibers to the regenerated limb. This issue is complicated in large part by the fact that muscle is a complex tissue composed of multiple cells types, including differentiated myotubes/myofibers, nerve and vascular tissues, connective tissue fibroblasts, and muscle stem cells (satellite cells/post-satellite cells). This issue has attracted attention recently because of several studies of the behavior of cells from a newt muscle cell line (Brockes, 1998; Kumar *et al.*, 2000; Lo *et al.*, 1993; Tanaka *et al.*, 1997). In particular, it has been demonstrated that newt A1 cells can form myotubes *in vitro* that can be induced to reenter the cell cycle and fragment to give rise to mononucleated cells. These mononucleated cells then contribute to regenerated limbs *in vivo*. These studies represent the most direct evidence to date that differentiated muscle fibers can dedifferentiate during limb regeneration.

The origin of the myogenic cells in the newt A1 cell line has not been identified, but as is the case with the well-characterized mouse C2C12 myogenic cell line, these cells could be derived from satellite cells (post-satellite cells in adult urodeles, see below) associated with mature muscle fibers (Lattanzi *et al.*, 2000). Myogenic newt A1 cells can be induced to form mytoubes *in vitro* that will then respond to serum stimulation by entering S phase. *In vitro* these cells arrest in the cell cycle prior to mitosis; however, when implanted into blastemas, they give rise to mononucleated cells by an unknown mechanism that appears not to require cytokinesis. (Velloso *et al.*, 2000). It has also been reported that a

few labeled cells from induced implanted myotubes become incorporated into regenerated cartilage during regeneration, suggesting that transdifferentiation has occurred (Lo *et al*., 1993). An essential caveat to the observation of infrequent transdifferentiation or stem cell conversion is that the biological relevance of such observations is uncertain (Blau *et al*., 2001). Regardless, it does appear that newt AI myotubes can be induced to undergo dedifferentiation *in vivo*, and thus they constitute one source of progenitor cells for muscle regeneration.

In large part, uncertainty regarding the origin of muscle progenitor cells for regenerated muscle in adult newts originates with the brief report of the lack of satellite cells in this tissue (Hay and Doyle, 1973). Satellite cells are myogenic stem cells present beneath the external lamina of skeletal muscle fibers in larval urodeles and in other vertebrates (see Cameron *et al*., 1986). Satellite cells in mammals have been well studied and are known to be the sole source of myogenic stem cells in adult muscle (Li *et al*., 2000; Pastoret and Partridge, 1998), and are also the source of cells for the mouse myogenic C2C12 cell line (Lattanzi *et al*., 2000). Since adult urodele muscle lacks satellite cells, it has been assumed that muscle regeneration in these animals is unique among vertebrates in that the major source of myogenic cells arises from fragmentation of myofibers (Echeverri *et al*., 2001; Hay and Fischman, 1961). Although adult urodele muscle does not contain satellite cells, there is instead a unique cell type, the post-satellite cell, enveloped in its own external lamina, adjacent to the external lamina of the myofiber (see Cameron *et al*., 1986). It appears that post-satellite cells are derived from larval satellite cells during metamorphosis, and that they are functionally equivalent to the satellite cells of other adult vertebrates. Post-satellite cells respond to injury by incorporating 3H-thymidine, proliferating in culture and fusing to form new myotubes. Myotubes derived from post-satellite cells express both blastema cell and myoblast specific antigens, which is comparable to what is observed *in vivo*. It seems likely that as in other vertebrates, a major source of regenerated muscle is a population of stem cells that are intimately associated with differentiated myofibers.

Regardless of the source of cells, it is evident that regenerated muscle arises from cells present in preexisting muscle tissues. The conclusion that muscle arises from a discrete lineage that is separate from other limb tissues is consistent with the origin of muscle during limb development. During development, limb myoblasts originate in the somites and migrate into the developing limb bud after it is relatively well formed. Experimental treatments that inhibit the migration of muscle progenitor cells result in the development of muscle-less limbs (Kieny and Chevallier, 1979), equivalent to the regeneration of muscle-less limbs (Dunis and Namenwirth, 1977; Holder, 1989; Lheureux, 1983). Limb regeneration can be inhibited by x-irradiation that blocks cellular contribution from stump tissues. Grafts of skin from unirradiated limbs will rescue regeneration; however, the limbs that form lack muscle, even though they have a normal skeletal pattern and contain tendons, connective tissues, nerves and blood vessels. Such experiments demonstrate that muscle is derived from muscle-specific progenitor cells that are not involved in the control of growth and pattern formation during limb regeneration.

Connective Tissues

Cells that form the connective tissue of the dermis and that surround muscles, nerves and blood vessels are collectively referred to as fibroblasts. Of all cells types, limb fibroblasts have the most significant influence on regeneration both in terms of contribution to the blastema and the control of growth and pattern formation in the regenerating limb (see Mescher, 1996). In contrast to cells from other limb tissues, dermal fibroblasts contribute to the blastema at a frequency that is about twice their occurrence in mature limb dermis. This population of cells gives rise to nearly 50% of the blastema cells on average, and as much as 78% of the cells at the maximum (Muneoka *et al*., 1986a). In contrast, less than 20% of all cells in the stump are dermal fibroblasts. Since half of all limb fibroblasts are located in the dermis (Tank and Holder, 1979), it is likely that the other 50% of the limb fibroblasts give rise to the other 50% of the blastema cells, assuming that stump fibroblasts respond to dedifferentiation signals in the same fashion as dermal fibroblasts.

In addition to over contributing to the blastema, fibroblast-containing tissues are the only mature limb tissues that influence growth and pattern formation during regeneration. It also appears that among these tissues, the dermis has a particularly dominant effect. Supernumerary outgrowths can be induced by grafts of skin (Bryant *et al*., 1987), and the pattern of the final regenerate is determined by the orientation of the grafted skin, rather than of the stump (see Muneoka *et al*., 1986a). Finally, as discussed above, all limb tissues other than muscle can be regenerated from grafts of skin as the sole source of progenitor cells for the blastema.
The recognition of fibroblasts, and dermal fibroblasts in particular, as the cell type from which the majority of the regenerated limb tissues are derived is of considerable consequence to the design of future experiments to study the mechanisms controlling regeneration. If there is a quiescent stem cell population for limb regeneration, it likely can be isolated from the dermis. Likewise, if dedifferentiation is the mechanism for induction of limb progenitor cells, then connective tissue fibroblasts must be responsive to the growth and differentiation factors induced by amputation of the limb.

Signals controlling the Initiation of Regeneration

Although many classical studies clearly indicate that intercellular signaling is critical for the induction and progression of regeneration, such studies have not led to the specific identification of any of these signals. A likely reason for this limitation is that such studies have been inherently descriptive and have not been amenable to the isolation and identification of specific molecules. It is obvious that important facts about regeneration can be learned from studying regenerating systems, such as urodele limbs. However, since urodele limbs regenerate when amputated, it is not possible to test if a molecule can stimulate regeneration. As a consequence of this dilemma, an experiment needs to either inhibit regeneration (e.g. by denervation of the limb) or test the ability of a candidate molecule to rescue an inhibited limb. Interpretation of negative results in such experiments is challenging at best. We think that recent studies have suggested at least three model systems that have the potential to allow for the isolation and identification of signaling molecules controlling the genesis of limb blastema cells.

Regeneration-Specific Gene Expression

Though expression of several important genes has been studied during limb regeneration, with one exception, all are also

expressed during limb development. In many cases, the spatial and temporal patterns of expression differ, but the function appears to be conserved (Gardiner *et al.*, 1998). To date, only *Hoxc-10* has been demonstrated to exhibit regeneration-specific expression (Carlson *et al.*, 2001). Genes within the *HoxC* complex are involved in specification of positional identity along the rostral-caudal axis of vertebrate embryos. *Hoxc-10* is expressed in developing hindlimbs and tails, but not in the forelimbs of either urodele larvae or of other vertebrate embryos (Carlson *et al.*, 2001; Peterson *et al.*, 1994). *Hoxc-10* is however expressed at high levels in response to forelimb amputation in axolotls. Thus *Hoxc-10* expression in regenerating forelimbs indicates the presence of regeneration-specific signals. Presumably there are elements in the promoter region of the axolotl *Hoxc-10* gene that are response to these signals, and provide an opportunity to isolate and identify those signals.

Fragmentation of Mouse Myotubes In Vitro *in response to Blastema Extracts*

It has recently been demonstrated that the differentiation of myotubes *in vitro* can be reversed by expression of the transcription factor, *msx-1* (Odelberg *et al.*, 2000). These studies take advantage of the mouse C2C12 cell line that was originally derived from skeletal muscle satellite cells. C2C12 is a pluripotent mesenchymal precursor cell line that can be induced to undergo myogenesis, adipogenesis, chondrogeneses or osteogeneses depending on a variety of well characterized growth and differentiation factors. Of particular significance to understanding regeneration is the discovery that *msx-1* expression induces multinucleated C2C12 myotubes to give rise to proliferating, mononucleated cells that subsequently can be induced to differentiate into multiple cells types as did the original C2C12 cells. This ability of myotubes to revert to a mononuclear phenotype is similar to that reported for newt A1 cells (discussed above). However, the behavior of the two cell types differ, in that newt cells do not give rise to proliferating mononucleated cells *in vitro*. In addition, newt A1 myotubes are stimulated to enter S phase of the cell cycle by serum exposure; whereas, C2C12-derived myotubes are not. Regardless, the *msx-1* induced "dedifferentiation" of C2C12 myotubes is significant as a demonstration of the developmental plasticity of committed or differentiated cells, and how they can be induced to give rise to cells with more stem cell-like properties (see Blau *et al.*, 2001).

Recently it has been discovered that a protein extract from regenerating newt limb blastemas can induce the genesis of proliferating mononucleate cells from C2C12 myoblasts (McGann *et al.*, 2001). This extract can also induce S phase DNA synthesis in newt A1 myotubes, as can serum as demonstrated in earlier studies. Both serum stimulated and blastema-extract stimulated A1 mytobes enter, but arrest in the cell cycle. The response of C2C12 myotubes to blastema extract is significant in demonstrating the presence of a factor(s) that is involved in the control of growth and differentiation. Given that *msx-1* is already known to be involved in controlling this response, it is possible that the important signals are operating upstream of the *msx* transcription factors. Several other studies have suggested an important role for *msx* in the regulation of regeneration in urodeles (Carlson *et al.*, 1998; Crews *et al.*, 1995; Koshiba *et al.*, 1998; Simon *et al.*, 1995) as well as mammals (Reginelli *et al.*, 1995; Wang and Sassoon, 1995). In addition, several signaling molecules have already been identified as regulators of *msx* expression. These include members of the FGF and Wnt signaling pathways (Bang *et al.*, 1999; Bushdid *et al.*, 2001; Yokoyama *et al.*, 2001), and both *msx-1* and *msx-2* are known indicators of BMP signaling (Lu *et al.*, 1999; Pizette *et al.*, 2001; Scaal *et al.*, 2002; Vainio *et al.*, 1993). We anticipate that this assay has the potential to identify several important signaling molecules expressed by regenerating limb cells that are involved in the control of dedifferentiation, growth and differentiation.

Induction of Lateral Limbs

As discussed above, an experimental dilemma exists in the study of animals that normally regenerate perfectly. Although there is obvious utility to studying regeneration in an organism that can regenerate, it is difficult to design experiments to test hypotheses

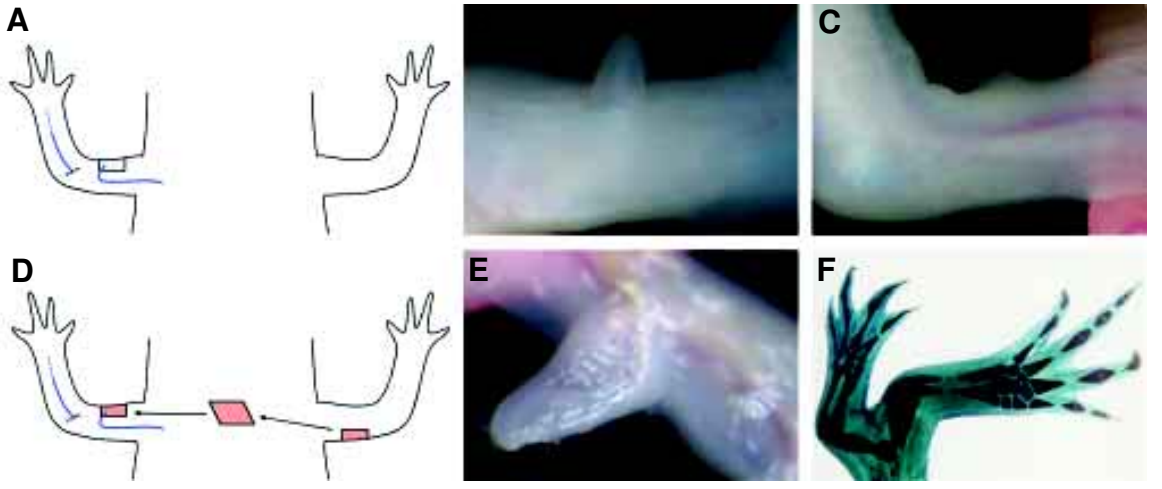

Fig. 1. The induction of lateral limb outgrowths by nerve deviation without (A-C) and with a skin graft (D-F). *A nerve [blue lines in* **(A)** *and* **(D)***] is cut around the elbow level and deviated to the lateral limb wound in both experiments. Additionally, a positional disparity is made by a skin graft in the latter experiment (see D). The outgrowth without a skin graft is symmetrical* **(B)** *and regresses later* **(C)**. *The outgrowth with a skin graft is asymmetrical* **(E)** *and finally forms an extra limb* **(F)**.

about regulatory mechanisms. Since regeneration will always occur without experimental or therapeutic intervention, it is not possible to test the ability to induce a regenerative response. It would be ideal to study regeneration in an animal that is known to have the ability to regenerate a limb, yet dissect the phenomenon in a model system in which regeneration could be induced when it would not normally occur. The induction of accessory limbs from lateral limb wounds is just such a model system. The seminal experiments involving the induction of accessory limbs were performed by Bodemer in the mid-20th Century (Bodemer, 1958; Bodemer, 1959), and expanded upon in recent years (Maden and Holder, 1984; Reynolds *et al.*, 1983). As an assay for the signals that control dedifferentiation, growth and pattern formation, this model system offers the important advantage of testing for a positive response in an organism in which all the necessary components for limb regeneration are know to be present. This model system is also important in that it allows for the experimental distinction of each of the three phases of regeneration as defined above (Fig. 1).

Phase I

If a piece of skin (epidermis and underlying dermis) is removed carefully so as to not induce damage to the underlying muscle and connective tissue, a wound epidermis forms and the skin is regenerated. Several genes that are expressed early in response to limb amputation are also expressed in a superficial lateral wound, and these genes are part of the common early part of the pathway (Fig. 2). Studies of the epidermis that is formed over superficial lateral wounds have not been conducted, but since expression of genes characteristic of Phase I (e.g. *msx-2* and *Mmp-9*) is inhibited by grafts of mature skin to cover an amputated limb stump, the epidermis that heals over a lateral wound is comparable to the WE in its ability to induce or allow expression of these genes.

Although lateral wounds do not give rise to outgrowths independently (see below), it is significant to note that they do regenerate the missing skin, including skin appendages such as glands. These wounds do not form scar tissue as in the case of equivalent wounds in most adult vertebrates. The source of the cells for skin regeneration is not known, however, we presume they are derived from migration of peripheral dermal cells in a fashion similar to what occurs during limb amputation (Gardiner *et al.*, 1986). In this regard, skin regeneration in adult urodeles is comparable to the scar-less wound healing observed in embryonic vertebrates, including mammals (Martin, 1997). Identification of the factors that regulate urodele skin regeneration in lateral limb wounds likely will be useful in guiding efforts to induce skin regeneration or engineer a skin replacement. Since skin regeneration at the site of a lateral wound does not require a nerve (see below), we conclude that Phase I of regeneration is nerve independent.

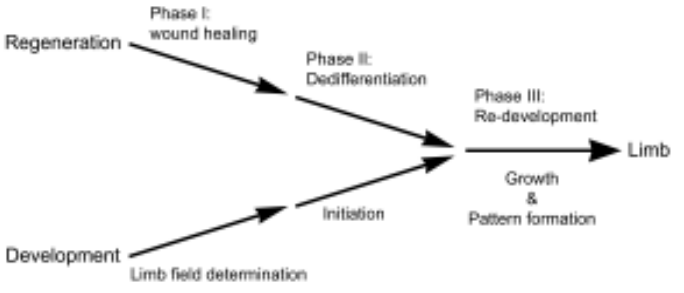

Fig. 2. The converging pathways of limb regeneration and development. *A schematic representation.*

Phase II

If a nerve is surgically deviated to the site of a lateral limb wound, an outgrowth is induced. Outgrowths can also be induced by causing significant damage to the underlying limb tissues without a nerve deviation (Bodemer, 1958; Bodemer, 1959). Such deep tissue damage is presumed to damage nerves and be equivalent to a nerve deviation. In contrast, when the wounds are made without deep tissue damage, outgrowth is dependent on the deviation of a nerve to the wound site. Consequently, a lateral wound with a nerve deviation is an appropriate experimental model for Phase II of limb regeneration, during which a population of undifferentiated cells is generated at the wound site, a process that is nerve dependent. We do not yet know which genes are expressed in lateral wound outgrowths, nor do we know which cells of the mature limb contribute to the outgrowths. Assuming that nerve-induced outgrowths are equivalent to the early, nerve-dependent blastema, it is likely that fibroblasts from the dermis surrounding the wound contribute a majority of the cells.

These outgrowths persist for a few weeks, but eventually regress. Although the function of epidermis that covers the Phase II outgrowths has not yet been studied, we assume that regression occurs because the epidermis does not progress from a wound epidermis to the specialized, thickened apical epidermal cap (AEC) that is required for limb outgrowth during limb regeneration.

Phase III

If in addition to deviating a nerve, a piece of skin is grafted from the opposite side of the limb, an accessory limb is induced to form at the site of the wound (Maden and Holder, 1984; Reynolds *et al.*, 1983). These outgrowths express several genes characteristic of Phase III regeneration, including *Dlx-3* and *Hoxd-11* (Torok *et al.*, 1998). It is known that the interaction of cells from disparate positions within the limb is required in order to get normal outgrowth and pattern formation during regeneration (Bryant *et al.*, 1981; French *et al.*, 1976). Although nerves are required to get an outgrowth from lateral limb wounds (Phase II), formation of an entire limb requires the diversity of positional information that is provided by skin grafting. In the absence of a skin graft, the cells that form the initial outgrowth are all derived from a limited region of the limb and have limited positional information. Surgically created limbs that are symmetrical, and thus are limited in their diversity of positional information, form symmetrical outgrowths similar to Phase II outgrowths from lateral limb wounds (Bryant *et al.*, 1981). Since Phase III outgrowths continue to grow and form normally patterned limbs, the interactions between blastema cells presumably stimulate the epidermis to form an AEC that is permissive for the continued proliferation of blastema cells beyond that observed for Phase II outgrowths.

Relevance of Regenerating Limbs to understanding the Origin of Mammalian Stem Cells

The question of the origin and developmental potential of adult cells that can respond to injury to repair and replace damaged tissues is an area of intense research effort. Assumptions about the

presence and nature of adult stem cells are being challenged, and it is clear that adult tissues are much more plastic and capable of regeneration than has been appreciated at any time in the past. Results from comparative studies from a diversity of animals likely will further an understanding of the mechanisms regulating the behavior and fate of stem cells. In particular, animals that can regenerate perfectly, such as urodele amphibians, remind us that regeneration is an ancient and fundamental biological process, and challenge our creative and scientific abilities to discover how to unlock the regenerative potential within us.

One key area of convergence between studies of urodele regeneration and mammalian stem cell biology concerns the mechanisms whereby the population of progenitor cells is generated. The prevailing view in mammals has been and continues to be that there are small populations of quiescent stem cells, which have only recently been recognized to have significantly broader developmental capabilities than previously thought. In contrast the view from studies of urodeles has been that regeneration cells arise via dedifferentiation of adult cells, even though the distinction between dedifferentiation of differentiated cells and the activation of stem cells has never been determined for urodeles. By extension, we raise the possibility that regenerative cells in adult mammalian tissues may also arise through the process "dedifferentiation", and that the culturing of adult tissues in the presence of mixtures of growth and differentiation factors provides the stimulus for dedifferentiation to occur in a fashion comparable to what is thought to occur in urodeles. From this point of view, the techniques that have been developed empirically for the culturing of mammalian stem cells potentially can provide critical insights into the signals at work in controlling the state of differentiation or dedifferentiation of urodele cells *in vivo*.

Finally, new experimental techniques afford new opportunities to understanding the origin of blastema cells in animals that can regenerate. These techniques include large scale screening of arrayed cDNA libraries and the ability to test the function of candidate genes identified from those screens (Gardiner *et al.*, 1999). Such techniques likely can be applied to studies of regenerating urodele limbs to identify and determine the function of signaling molecules and pathways involved in wound healing, skin regeneration and the genesis of blastema cells (Phases I and II of regeneration). It likely will prove to the be the case that the critical breakthroughs in regeneration research will come from understanding the mechanisms controlling these early phases of regeneration, and the key to inducing regeneration will be in stimulating limb cells to progress to the point of convergence of the development and regeneration pathways.

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