REVIEW ARTICLE

Looking Ahead to Engineering Epimorphic Regeneration of a Human Digit or Limb

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Approximately 2 million people have had limb amputations in the United States due to disease or injury, with more than 185,000 new amputations every year. The ability to promote epimorphic regeneration, or the regrowth of a biologically based digit or limb, would radically change the prognosis for amputees. This ambitious goal includes the regrowth of a large number of tissues that need to be properly assembled and patterned to create a fully functional structure. We have yet to even identify, let alone address, all the obstacles along the extended progression that limit epimorphic regeneration in humans. This review aims to present introductory fundamentals in epimorphic regeneration to facilitate design and conduct of research from a tissue engineering and regenerative medicine perspective. We describe the clinical scenario of human digit healing, featuring published reports of regenerative potential. We then broadly delineate the processes of epimorphic regeneration in nonmammalian systems and describe a few mammalian regeneration models. We give particular focus to the murine digit tip, which allows for comparative studies of regeneration-competent and regeneration-incompetent outcomes in the same animal. Finally, we describe a few forward-thinking opportunities for promoting epimorphic regeneration in humans.

Introduction

APPROXIMATELY 2 MILLION PEOPLE have had limb amputations in the United States due to disease or injury,¹ with more than 185,000 new cases every year.² Of those, 45% are due to trauma (including combat-related events³), 38% to diabetes, and 15% to nondiabetic dysvascular disease.¹ It is projected that the number of people with lost limbs will more than double by 2050, largely due to aging and the rising incidence of diabetes.¹

The only current option for amputees that have lost mobility and gross motor function is a prosthetic device. While electro-mechanical devices have made notable advances in the last few decades, restoration of full functionality is still lacking.⁴ For example, hand injuries account for 30% of amputations⁵ and for those patients the disparity is particularly problematic between prosthetic capacity and required complex functions, such as fine motor skills and sensation for tactile input.⁶ Additionally, current lower limb prosthetics lack the range of motion associated with normal gait, dexterity, and movement feedback.^{7,8}

The ideal response after amputation would be epimorphic regeneration, a process by which an entire limb is completely restored in terms of tissue composition, three-dimensional macro- and ultrastructure, and functionality. Epimorphic re-

generation occurs in certain animals, including urodele amphibians, which are able to regenerate limbs,⁹ tails,¹⁰ lenses,¹¹ and jaws.¹² Such regenerative potential is much more limited in mammals, but does occur in both mice^{13–15} and humans^{16,17} at the distal tip of digits.

This review aims to present introductory fundamentals in epimorphic regeneration to facilitate design and conduct of research from a tissue engineering and regenerative medicine perspective. It begins with a description of the clinical scenario of human digit healing, featuring published reports of regenerative potential. For insights into the process of epimorphic regeneration, the steps of limb regrowth in urodeles are delineated. The few mammalian regeneration models are then noted, with particular focus on the murine digit tip, which allows for comparative studies of regeneration-competent and regeneration-incompetent outcomes in the same animal. Finally, a few forward-thinking opportunities are described for promoting epimorphic regeneration in humans.

Clinical Cases of Digit Regrowth

Medical approaches to handle digit or limb amputation focus on wound management for patient safety.¹⁸ Currently, no medical efforts are directed toward limb (arm or leg)

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regeneration after amputation, as there have been no observations of such regrowth in humans. Clinical reports in digits (fingers and toes), however, have documented some rare cases of regenerative potential for distal tips in children and young adults.^{16,19,20} Lack of a fundamental understanding of the processes involved in epimorphic regeneration of digits, however, relegates such regenerative results to stochastic events instead of designed clinical outcomes. Furthermore, as digits and limbs are both complex highly organized multitissue functional structures, a more complete understanding of digit regeneration, including key upstream signaling events, may help to extend similar responses to limb amputations.

In all cases of digit amputation, the primary focus is on wound care. Secondary outcomes are also targeted in certain cases for adults. For example, clinicians may attempt to restore an aesthetic attribute or maintain gross manual dexterity.²¹ As a result, depending on the type of amputation, clinicians may use skin flaps to cover the wound to minimize tissue necrosis,^{22,23} replant the amputated tip,^{24–28} or fit for a prosthetic device.²⁹

For certain cases in children, clinical intervention is often kept minimal with a focus on cleaning and dressing the wound. It is in such scenarios of healing by secondary intention²² that rare cases have been reported of spontaneous digit regrowth for amputations distal to the nail fold in young individuals. For example, in reports of guillotine amputations in children (<8 years old), complete wound closure and subsequent finger lengthening has been observed.¹⁶ Additionally, a case study of a toe injury in a 15-year old boy reported new bone growth.²⁰ Thus, epimorphic regeneration seems possible in distal digit tips in children, who are thought to have a high inherent healing potential; however, it is critical to gain an understanding of how to reliably recreate a regeneration-permissive wound environment immediately after injury to extend such responses for a wider range of injury types and patient ages.

Insights from Nonmammalian Systems

Urodele and anuran amphibians are the most commonly used research models to study epimorphic regeneration in adult tetrapod vertebrates.^{30,31} Only urodeles have the ability to regenerate missing body structures throughout life.³² As a reference to the process of epimorphic regeneration, below is a brief description of three phases: wound healing, blastema formation, and differentiation/tissue regrowth (Fig. 1).

Wound healing

The initial biologic response to amputation is wound healing, composed of processes that temporally overlap, but are generally termed independently as (1) coagulation and hemostasis, (2) inflammation, (3) cell proliferation, and (4) wound remodeling (reviewed in Yokoyama³³). The first two stages are initiated almost immediately and are a necessary part of survival. Hemostasis is accomplished by vasoconstriction and the formation of a fibrin clot, which covers the wound and provides a substrate for migration of epidermal cells.³⁴ Immune cells promptly infiltrate the wound tissue, secreting important cytokines and proteases, such as matrix metalloproteinases (MMPs).³⁵ This process directly regulates leukocyte infiltration, cellular proliferation, remodeling of damaged tissue, and wound closure.³⁶

A wound epithelium (WE) forms within 12 h from cells that have migrated from the surrounding tissue of the amputation

plane.³⁷ This WE isolates and protects the wound from further external insults (reviewed in Han *et al.*³⁰), as well as encapsulates a permissive regenerative microenvironment (reviewed in Murawala *et al.*³⁸). After ~7–8 days, the WE thickens to form the apical ectodermal cap (AEC),³⁹ a transient³⁰ secretory epithelium (reviewed in Murawala *et al.*³⁸ and Campbell and Crews⁴⁰) that coordinates cellular processes involved in the regeneration of the lost structure. The AEC is analogous to the apical ectodermal ridge, the limb bud signaling center during development.^{38,41,42}

Proper histolysis, or extracellular matrix (ECM) remodeling, is critical for successful regeneration.⁴³ This process begins a few days after amputation⁴⁴ and is regulated in part by MMPs, a group of proteases secreted by macrophages, osteoclasts, basal wound epidermis, and chondrocytes.⁴⁵ MMP degradation of the ECM is instrumental in the release, migration, and proliferation of cells from stump tissues (reviewed in Bellayr *et al.*³⁶), as well as presentation of growth factors that affect cellular activity.^{46,47} Levels of MMP activity are highly correlated with a regenerative outcome, with lower levels observed in nonregenerating amphibian limbs.⁴⁷ The effect of lower MMP activity, however, seems not to be limited to decreased degradation of existing ECM, as it is also correlated with increased ECM deposition observed in the form of a basal lamina below the WE⁴⁸ and associated stiff scar tissue,^{46,49} processes that stunt regenerative potential. Thus, regeneration is not only dependent on ECM deposition, but also on its proper remodeling.

Blastema formation

One key event in limb regeneration in nonmammalian species is the formation of a blastema, a heterogeneous population (cell mass) of lineage-restricted progenitor cells derived from the cells in the stump tissue,⁵⁰ which occurs between days 6 and 20 in the urodele.³³ Cells from these tissues lose their differentiated properties and accumulate beneath the wound epidermis to form the blastema. Macrophage-regulated immune signaling has been found to be fundamental in providing a regenerative-permissive environment for the blastema, where systemic macrophage depletion inhibited limb regeneration in salamanders.³⁵ The relative contribution of cells within the blastema, however, does not reflect the composition of the adjacent tissues. Dermal cells over-contribute relative to skeletal cells, composing 43% of the cells in the blastema versus 2%.⁵¹ This disproportionate contribution of cell phenotypes may be related to the function of these cells in the blastema, where the dermal cells play an important role in tissue patterning. Thus, the heterogeneity of the blastema is not merely a reflection of the available surrounding cells, but instead likely relates to its function in regeneration.

The blastema is composed of lineage-restricted progenitor cells that retain the inherent information from their tissue of origin. Cells will dedifferentiate during formation of the blastema, but are then predominantly limited to the synthesis of tissues of the same lineage or shared embryonic origin (reviewed in Nacu and Tanaka⁹). For example, it has been shown⁵⁰ that after dedifferentiation, muscle cells only gave rise to muscle, Schwann cells only gave rise to Schwann cells, and chondrocytes only gave rise to cartilage tissue. Dermal cells, however, seemed to have increased plasticity, since they participate in the formation of not only dermal tissue, but also other connective tissues such as tendon and cartilage.

FIG. 1. The process of amphibian epimorphic regeneration. An intact limb consists of tissues of various types, including dermal, skeletal, neural, and vascular. After amputation, the wound heals to form an epidermal layer, the underlying tissues undergo matrix remodeling, and cells in the region secrete soluble factors. A heterogeneous cell mass, or blastema, forms from the proliferation and migration of cells from the adjacent tissues. The blastema then gives rise to the various new tissues that are spatially patterned to reconstruct the original limb structure. Color images available online at www.liebertpub.com/teb

Thus, despite dedifferentiation to form the blastema, participation during tissue regrowth is related to the original cell phenotype.

Despite blastema heterogeneity, a variety of genes have been proposed as potential markers of a regenerating blastema. This cell population expresses both transcription factors involved in maintaining an undifferentiated phenotype (*Msx1* and *Msx2*^{46,52,53}) and markers of mesenchymal cells in developing limbs (*Prrx1*^{46,54}). In addition, genes expressed include those involved in developing limb patterning (*tbx5*⁵⁵, *hoxa-9*, and *-13*^{54,56}) and key factors for initiation, outgrowth, and patterning during limb regeneration (*fgfs*^{57,58}). Thus, the heterogeneity of the cell population and the complexity of the process of regeneration result in a blastema with a complicated gene profile.

Within the blastema are ECM proteins such as tenascin, hyaluronic acid, fibronectin, and low levels of collagen.^{59–61} This protein assembly is compliant and provides directional guidance (reviewed in Christensen and Tassava⁴¹ and Thornton⁶²) and cues that allows proliferation and cell migration.^{63,64} Other microenvironmental factors, such as fibroblast growth factor (FGF), BDGF, and GGF2, arise from interdependent signaling between the AEC and neuronal structures.³⁹ The resulting physiochemical environment regulates the processes of blastema formation,⁴⁸ proliferation, and dedifferentiation.^{39,65,66} Early nerve signaling also induces the WE to become a functional AEC⁶⁷ that expresses mitogenic factors.^{57,58} The cells in and around the blastema together regulate the processes necessary for the formation of a stable cell mass and subsequent tissue outgrowth.

Tissue regrowth

Positional and cell lineage memory of blastema cells make it a self-organizing structure (reviewed in Stocum and

Cameron⁶⁸) with the necessary information to regrow a missing structure. Additional signaling from the regenerating microenvironment, however, can reprogram this positional identity to regulate the appropriate growth and pattern of the regenerating structure (reviewed in Yakushiji *et al.*⁶⁹).

The inherent positional identity of certain blastema cells determines the proximal–distal patterning of the regenerate. In the axolotl limb, distal and proximal cells in the blastema do not mix even after 4 days of proliferation. Such spatial separation is extended to tissue regrowth, where proximal–distal positioning is retained in the structures of the regenerated limb.⁷⁰ Relative position to the amputation plane is also preserved in that only those structures distal to the transection are reformed.⁹ Connective tissue cells,^{71,72} seem to play a critical role in regenerating the appropriate structures by participating only in the formation of structures that are more distal to its given positional memory. This regenerative instruction is preserved, however, even when repositioned to another location. Upon transplantation of a hand blastema to the amputation plane of the upper arm, the connective tissue cells of the hand were only found in the hand tissues of the regenerated limb.⁷¹ Thus, certain cells store information regarding their originating amputation site during tissue regrowth.

The intrinsic positional information of blastema cells can be respecified by soluble factors. In a dose-dependent manner, a concentration gradient of retinoic acid (RA) can induce molecular and cellular changes during limb regrowth.⁷³ RA can confound inherent proximal–distal identity to modulate the differentiation and patterning processes⁶⁹ by affecting the expression of developmental genes, such as *hoxA13*,⁵⁶ *meis*,⁷⁴ and *shh*.⁷⁵ Additionally, RA regulates the expression of surface proteins, such as *prod-1*, modulating cell–cell interaction and, therefore, proximal–distal positional identity among blastema cells.⁷⁶ Thus, presentation of exogenous molecules can be used

to mitigate positional identity of cells in the blastema and alter patterns of tissue regrowth.

Signals from nerves are a central aspect of the natural progression of epimorphic regeneration. A nerve diverted to a skin wound in the axolotl (a urodele amphibian) will induce the formation of an ectopic blastema,^{48,54} while denervated limbs fail to regenerate after amputation.³⁹ After denervation, however, the regenerative process can be rescued by the exogenous delivery of FGF and bone morphogenetic protein (BMP) cytokines.⁵³ Nerves also secrete other growth factors that are relevant for regeneration: for example, keratinocyte growth factor upregulates the expression of the epidermal embryonic marker sp9 in the AEC,³⁸ FGF2 promotes MMP expression in surrounding fibroblasts and keratinocytes,⁶⁵ and newt anterior gradient has been suggested to be involved in the proximal-distal patterning process during the regrowth stage.⁷⁷ Thus, together the blastema and the surrounding tissues interact to generate a permissive microenvironment that ultimately allows for tissue regrowth.

Bone regrowth is a key defining indicator of the process of epimorphic regeneration. Formation of new bone happens through endochondral ossification,⁷⁸ which is a recapitulation of the developmental process in which cartilage is initially formed and then replaced with bone tissue.⁷⁹ Bone regrowth is critically dependent on growth factors such as BMP2 for chondrogenesis⁸⁰ and Gdf5 for joint formation.⁸¹ Furthermore, apoptosis⁷⁸ is necessary for the replacement of cartilage by mineralized bone.⁸² Thus, factors that modulate cellular processes or the microenvironment can regulate bone regrowth after amputation.

Epimorphic regeneration is the result of tightly regulated cellular and microenvironmental cues that modulate wound healing, blastema formation, and tissue regrowth to restore a lost limb after amputation. These processes are well studied in amphibians, as briefly reviewed above. While these non-mammalian systems provide critical insights into the key events for regenerating amputated structures, specific signals and mechanisms are not all conserved in mammals.⁸³ Thus, examination of mammalian models is central for the translation of epimorphic regeneration to the clinical scenario.

Mammalian Animal Models

Epimorphic regeneration has long been observed in nonmammalian systems, but more recent studies have also included a few mammalian models of regeneration. Organized tissue regrowth has been observed in deer antlers and mouse ears, providing insights into elements of mammalian regeneration.⁵

Deer antlers are periodically lost and fully regenerated without scarring (thoroughly reviewed by others in Kierdorf and Kierdorf⁸⁴). Comparable to the amphibian model, antler regeneration includes signaling from wound healing and neural factors, as well as bone regrowth through endochondral ossification. In this system, however, a blastema-like structure does not form and regeneration is instead stem cell dependent. Another mammalian model is the immunocompromised MRL/MpJ mouse, which is considered to have a greater regenerative capacity compared to most other strains⁸⁵ and in particular regenerates ear tissue without scarring after a hole punch.⁸⁶ The regenerative process of the ear mimics elements of amphibian epimorphic regeneration, including ECM histolysis,⁸⁷ forma-

tion of a blastema-like structure,⁸⁸ and regrowth of multiple tissue structures.⁸⁹ In this case, however, it is not necessary to have bone regrowth, which is one of the most critical elements of epimorphic regeneration in digits or limbs.

New bone is a primary outcome in recent mammalian studies focused on epimorphic regeneration of mouse digit tips.⁹⁰ In this system, regeneration is level dependent, occurring for amputations at the distal portion of the terminal phalanx bone and not the more proximal bone.^{15,91} This unique model allows for comparative studies between regeneration-competent and regeneration-incompetent regions for the careful characterization of processes and mechanisms in mammalian epimorphic regeneration. Due to its particular value in translating epimorphic regeneration to the clinical scenario, below is a deeper discussion of the murine digit tip model.

Mouse Digit Tip Regeneration

The digit tip in CD1 mice is capable of regrowing multiple structures, including bone, after amputation.⁹⁰ As a mammalian model system, this serves as a useful basis for translation of epimorphic regeneration to amputations in human digits or limbs (Fig. 2). Furthermore, this response in the mouse is level-specific in that amputation at the distal end of the third phalanx bone (P3) leads to regeneration (regenerative-competent region), while a more proximal injury or through the second phalanx bone (P2) leads to scar formation (regenerative-incompetent region).^{15,91} These similar, but regeneratively distinct regions within the digit provide for a comparative model to study processes in scar formation versus epimorphic regeneration. It then becomes possible to carefully characterize the wound site and injury response in terms of the microenvironment, cell populations, and cell processes. In addition, it allows for mechanistic studies of possible treatment modalities, including the delivery of cells, factors and/or matrix, as well as control of physiochemical cues. To further examine this murine model of regeneration, below is a description of the responses to amputation of the regeneration-competent and regeneration-incompetent regions of the mouse digit.

Wound healing

Immediately after injury, the initial response is similar in both regeneration-competent and regeneration-incompetent regions. The wound healing phase includes formation of a blood clot, infiltration of inflammatory cells, and wound closing ~12 h post amputation (HPA) for fetal mice,⁹² 4–8 days post amputation (DPA) for the neonatal model,^{91,93} and 9–10 DPA for the adult model.¹⁵ The divergence between scar formation (a nonregenerative response) and epimorphic regeneration might be modulated by the histolysis phase. In the regenerative case, ECM remodeling includes intensive bone erosion to expose the bone marrow cavity to the wound site,¹⁵ where much less remodeling and no marrow exposure occurs in the absence of regeneration. Also, in the more commonly occurring nonregenerative response, the wound epidermis covers the injury ~4–5 DPA in the neonatal model⁹³ and 8–13 DPA in the adult model.⁹⁰ This is followed by collagen deposition between the epidermal layer and the transected bone, which impedes communication between the surface layer and most underlying tissues,^{90,93} leading to scar formation and

FIG. 2. Regeneration in the murine digit model. Epimorphic regeneration in the murine digit is level-specific and provides an opportunity for comparative studies of mammalian epimorphic regeneration. Transection through the P2 element results in the frequent outcome of fibrotic scar tissue formation. Transection through the more distal P3 element instead results in the regeneration of missing tissue. Color images available online at www.liebertpub.com/teb

inhibition of regeneration. In the regenerative case, however, the epidermal layer migrates between the degrading bone surfaces, undercutting the bone stump of the digit tip at a more proximal level¹⁵ and in closer proximity to the nascent blastema-like structure.

Formation of a blastema-like structure

Concurrent with wound healing,⁹¹ during epimorphic regeneration, is the formation of a stable mass of cells from which the tissue of the new structure arises. This blastema-like structure is located at the edge of the eroding bone stump¹⁵ and the timing of formation correlates with wound closure. Formation of this cell mass is more rapid in fetal and younger mice, occurring at 12 HPA in fetal mice⁹² and 4–6 DPA in neonatal mice⁹¹ versus 10–12 DPA in adult mice.¹⁵

The cells that amass at the injury site are derived from the local adjacent tissues and not from distant sources, such as stem cells from the vascular system.^{94,95} These cells are lineage-restricted progenitors that contribute to forming only the tissues of their originating germ lineage.^{95,96} In those studies, it was shown that ectodermal cells participate in the formation of the epidermis, nail plate, and sweat glands, but not the mesodermal tissues of the bone marrow cavity, blood vessels, tendons, nail bed, dermis, or surrounding mesenchyme of the digit. The converse was also found to be true in that mesodermal cells do not contribute to ectodermal tissues. In some cases, the potential of the cells is even more limited as the bone precursor cells only participate in bone formation, tendon precursor cells only form tendons, and endothelial cells participate in blood cell and blood vessel formation. Thus, this blastema-like struc-

ture is composed of a heterogeneous population of cells that retain inherent limitations from their parental tissue or embryonic origin.

A high proliferative capacity is likely an important characteristic in the formation of a blastema-like structure. Amputations through regenerative regions result in a greater number of proliferative cells compared to nonregenerative regions in mouse digits.^{13,15,91} Connective tissue fibroblasts isolated⁹⁷ from regeneration-competent regions also had higher proliferation rates than those isolated from regeneration-incompetent regions upon *in vitro* culture under multiple two-dimensional and three-dimensional culture conditions (adherent, suspension, and collagen gels).⁹⁸ Proliferative differences in the *in vitro* expanded cells were retained even after reimplantation into amputated joints.⁹⁷ Thus, those cells that participate in the formation of a blastema-like structure seem to have a greater inherent proliferative capacity compared to analogous cells from nonregenerative regions.

No single marker is known to be expressed in all the different cell phenotypes in the blastema-like structure; however, a wide range of gene expression has been described in this heterogeneous population. Some cells express stem cell markers, such as those for pluripotent cells (Sox 2 and Rex1⁹⁹), bone marrow mesenchymal stem cells (Sca1 and CD90⁹⁴), endothelial progenitor cells (SCA-1 and CD31¹⁵), and macrophages (CD11b and F4/80⁹⁴). In addition, these cells have been found to express a general mesodermal marker (vimentin¹⁵), an antiangiogenic factor (Pdgfr-1¹⁰⁰), BMPs and their receptors,^{91,93} as well as developmental genes (*Msx1* and *Msx2*^{13,91,92}). Thus, the lack of a definitive biomarker means that it is difficult to define spatially *in situ* the cells that constitute the heterogeneous population of any blastema-like structure.

Tissue regrowth

During the regrowth stage, cells of a blastema-like structure form all the tissues of the newly growing amputated structure.⁹⁵ Deposition of soft connective tissue, however, is also a part of the nonregenerative scar formation outcome. Thus, in the murine digit tip research model, formation of new bone is considered the definitive indicator of a regenerative response.^{5,101}

Formation of bone during the regenerative response after amputation in neonates and adult mice is similar,¹⁰² occurring through direct (or intramembranous) ossification.^{15,91} This is distinct from endochondral ossification during limb development in embryos or epimorphic regeneration in non-mammalian systems, where formation of bone occurs by replacing hypertrophic cartilage. Bone remodeling includes erosion of the existing bone at the stump, which then exposes the marrow and its associated cells before formation of new bone growth at the distal tip.¹⁵ During regeneration in neonatal mice, it has been shown that bone deposition begins in the second week after amputation with complete mineralization by the third week.⁹¹ In the adult mouse, bone erosion was observed during the first 2 weeks with ossification and complete restoration of the P3 bone ~3–4 weeks after amputation.^{15,103} Initially, the new bone has a trabecular structure that increases in density with time, but is still different from the cortical structure of the bone stump.¹⁵ Thus, formation of bone after amputation does not occur through a developmentally preserved process, but rather a distinct regenerative response.

Formation of a joint is not necessary during regrowth of the P3 distal tip in the murine model, although this process is conceptually important if epimorphic regeneration is promoted after a more proximal amputation. Little is even known, however, about the molecular mechanisms of formation and patterning of complex structures like joints during normal developmental processes. Factors like FGF have been shown to play an important role in determining the number of phalanges in the developing digits.¹⁰⁴ Additionally, the participation of BMP family members play essential roles in skeletal segmentation processes.¹⁰⁵ The activation of the Wnt/ β -catenin signaling pathway has been demonstrated to be involved in digit regeneration,¹⁰¹ but has also been proven to be necessary to induce early steps of synovial joint formation in vertebrates.¹⁰⁶ Molecular mechanisms of joint formation studied at the developmental level will likely provide important insights to promote future tissue segmentation during induction of limb regeneration.

Regulation of digit tip regeneration

Cellular processes involved in the progression from wound healing to tissue regrowth are tightly regulated by microenvironmental cues that provide the correct spatiotemporal presentation of necessary signals. Macroscopic environmental conditions, such as oxygen concentration, ECM remodeling, and the presence of the nail, alter cellular factors such as BMP and Wnt signaling. As many of the mechanisms that govern wound healing are thoroughly reviewed elsewhere,^{107,108} some of the physical and chemical factors that alter the blastema-like structure and new bone growth are discussed below.

Oxygen concentration, a known regulator of many distinct cellular processes, has distinct effects on different stages of

the regenerative progression. It has been found that there is a complex spatiotemporal pattern of oxygen concentration in the exposed marrow and the blastema-like structure.¹⁰³ Manipulating the oxygen levels can then be used to induce changes in bone erosion and the size of the blastema-like structure.¹⁰⁹ Thus, oxygen concentration can differentially regulate the regenerative outcome by modulating the early responses after amputation.

Direct manipulation of the ECM can also modulate the regenerative outcome by affecting the cellular processes that contribute to the formation of a blastema-like structure. Injecting solubilized ECM products at the amputation plane in regeneration-incompetent regions induced cell migration,⁹⁵ potentially by providing chemotactic cues to local proliferating progenitor cells.⁹⁴ Similarly, promoting degradation of the ECM by the exogenous addition of MMPs increased the recruitment of multipotent cells to the plane of amputation.¹¹⁰ Hence, it is clear that the accessibility of ECM products is critical for the cell recruitment toward the formation of a blastema-like structure, although the specific ECM factors are still unidentified.

BMP is one soluble factor in the ECM that modulates the regenerative process, transcripts of which are present in the proliferating blastema-like structure and adjacent tissues.^{13,93} The exogenous delivery of BMPs in the regeneration-incompetent region induces regeneration, while the BMP antagonist Noggin inhibits the spontaneous response in the P3 region.⁹³ Implantation of BMP-coated beads after amputation to a regeneration-incompetent region promoted formation of a blastema-like structure by increasing migration¹¹¹ and proliferation.⁹³ Additionally, BMPs promoted endochondral ossification for new bone growth, a similar outcome, but distinct mechanism from the direct ossification of naturally occurring regeneration.^{93,112}

The source of BMP in the *in vivo* scenario has not been fully identified, although *in vitro* coculture studies have shown that the possible release of BMPs from fibroblasts differentially affects proliferation and migration of P2 and P3 stromal cells.¹¹³ The BMP effects on cellular processes *in vivo* may be through the SDF-1 α /CXCR4 pathway. After amputation, some cells in the regenerative region secrete SDF-1 α while select cells of the blastema-like structure express its receptor CXCR4. Furthermore, exogenous addition of BMP to regeneration-incompetent regions was able to activate this signaling pathway.¹¹¹ BMP modulation of the regenerative process includes promoting cellular processes involved in the formation of the blastema-like structure.

BMP may also function to promote regeneration by recapitulating relevant embryonic programs during limb and digit development, as evidenced by an upregulation of the developmental genes *Msx1* and *Msx2*.⁹³ The expression level of these genes is correlated to regenerative potential in the level-dependent outcome observed in murine^{13,91,92} and human digits.¹¹⁴ BMP exogenously added to a nonregenerative region can then induce *MSX1* expression and a blastema-like structure formation,⁹³ as well as rescue the impaired regenerative response in *Msx1* mutant mice.⁹¹ Thus, BMP signaling may play multiple roles in promoting and regulating formation of a blastema-like structure and bone growth during regeneration by reactivating developmental pathways.

The nail organ has been strongly implicated in regenerative potential as the distal amputation conserves the structure,

while more proximal transections do not. Empirical studies have shown that bone growth was truncated when the nail was artificially removed from distal amputations while bone regrowth was present when the nail was surgically reattached in proximal amputations.¹¹⁵ In more recent studies, it was found that Wnt signaling, strongly associated with the nail stem cells, is activated in the regenerating nail epithelium after amputation and that inhibition of this pathway leads to regenerative failure of the nail and finger.¹⁰¹ While the importance of the nail bed as a signaling center for regeneration seems clear, the mechanisms involved have yet to be elucidated.

Relatively recent use of the murine digit tip model has been extremely helpful in elucidating the steps of epimorphic regeneration in a mammalian system. Ongoing efforts using this comparative model are beginning to identify the microenvironmental factors necessary for complex spatiotemporal progression of regeneration. Deeper understanding of the key mechanisms of epimorphic regeneration in this model will facilitate translation to the clinical scenario.

Translating Epimorphic Regeneration

The process of epimorphic regeneration consists of an extended progression of tightly regulated cellular and molecular events that leads to digit or limb restoration after amputation. Long established observations in non-mammalian species revealed the major steps of epimorphic regeneration. More recent studies in murine models have identified many of the biological limitations of this type of regeneration in mammalian systems. In the clinical scenario, epimorphic regeneration is not the default response, but elements of the process have been observed in children and young adults. Thus, there is the potential to engineer select steps of epimorphic regeneration to promote the partial or complete restoration of a biological digit or limb after amputation in humans (Fig. 3).

Aspects of the epimorphic regeneration processes observed in other species will likely need to be mimicked to promote regrowth of a digit or limb after amputation in the clinical case.

In other species, the AEC forms a protective layer covering the injury site. More importantly, it acts as a signaling center, both receiving and emitting signals, that promotes cell migration and proliferation for the formation of a blastema-like structure. As the traits of the AEC are further elucidated, it may be possible to suture an engineered epithelial layer, much like the present skin grafts,^{116–118} across the injury site. In-depth understanding of the proper soluble factor communication necessary, however, could lead to a more direct approach of delivering growth factors to the region, leveraging drug delivery paradigms that create spatiotemporal gradients.¹¹⁹ These interventions are intended to mimic the signals that induce a stable cell mass that functions as a blastema.

A blastema is formed from cells of adjacent tissues after amputation. Initial studies in mice have already shown the promise of introducing solubilized ECM,^{94,99,120} BMPs,^{13,93,111,112} and MMPs¹¹⁰ in promoting recruitment/mobilization of endogenous cells to proliferate at the transected bone front. Furthermore, the injury response may also be influenced with external bioreactors, as tested with the Biodome¹²¹ that can control parameters, such as hydration, pH, oxygen concentration, and electrical stimulation. Thus, controlling the microenvironment may help induce the proliferation, migration, and dedifferentiation necessary for the formation of a blastema-like structure. Control of the microenvironment is extensively used in tissue engineering and regenerative medicine, so leveraging knowledge from those fields may help regulate *in situ* formation of a blastema-like structure after amputation.

It is possible that manipulation of microenvironmental factors alone is not sufficient to overcome limitations in proliferation and migration for the formation of a blastema-like structure. Another approach is to supplement the cell population at the amputation plane by isolation of select cells from the injury site or elsewhere, expansion of those subpopulations *ex vivo*, and then redelivery of the proper phenotypes for regeneration, a paradigm similar to that used in other regenerative applications.¹²² Additional cell sources for exogenous transplantation may involve stem cells. Cells during epimorphic

FIG. 3. Opportunities for engineering epimorphic regeneration. Epimorphic regeneration has been observed in distal finger tips of children and young adults. Converting such stochastic events into designed clinical outcomes will require altering the default postamputation progression. Engineering the epimorphic regeneration process may include the transplantation of cells, scaffolds, and/or soluble factors, as well as controlling microenvironmental aspects, such as oxygen concentration, tissue hydration, mechanical, and electrical cues. Color images available online at www.liebertpub.com/teb

regeneration control the regenerative process by secreting factors, differentiating, and self-assembling into tissues, all functions similar to those of stem or progenitor cells. Candidate sources include mesenchymal stem cells, which often act as a trigger of endogenous cellular activity, and pluripotent stem cells, which have a tremendous capacity to form self-assembled tissues. The use of adult stem cells or induced pluripotent stem cells then allows for autologous transplantations, avoiding issues related to immunocompatibility. Additionally, the introduction of cells provides an opportunity to tailor cell attributes using *in vitro* manipulation. For example, transplanted cells may be preformed into cell clusters or embedded in hydrogels to drive the three-dimensional cell–cell interactions associated with blastemas. Furthermore, the cells may be modified or reprogrammed (genetically, chemically, or mechanically) before transplantation to tune their functionality for the injury site. Thus, transplantation of exogenous cells may be one approach to overcome the limitations in the formation of a blastema-like structure in the clinical scenario.

Epimorphic regeneration requires the regrowth of tissues of various types, including osteochondral, vascular, and neural. This requires cells of the blastema to differentiate simultaneously to a wide range of different phenotypes within a single complex environment with multiple gradients of diffusible soluble factors. A major part of stem cell research focuses on differentiation to various phenotypes in specific and independent environments. For example, BMP2,¹²³ VEGF,¹²⁴ and FGF¹²⁵ have been found to be potent signals in stem cells to promote differentiation to the osteo, endothelial, and neural phenotypes, respectively. The goal of those studies, however, is to promote a single target phenotype. Instead, in the case of blastema cell differentiation, a cohesive single cell mass must be differentiated to simultaneously give rise to the multiple distinct phenotypes (in the correct proportion) needed to reconstitute the tissues of the digit or limb.

Functional tissues require that synthesized proteins be properly assembled and patterned in the ECM. Disparate, but adjacent tissues arise from tightly orchestrated spatiotemporal cues during development. The process of functional tissue reconstruction, however, may be distinct and not recapitulate the original developmental progression, as in the case of direct versus endochondral ossification. These tissue replacement processes may utilize classic tissue engineering approaches, using biodegradable polymers¹²⁶ or hydrogels,¹²⁷ to provide a scaffold for cell ingrowth. These biomaterials may not only help promote cell differentiation and matrix synthesis, but may facilitate spatial patterning of tissues. For example, degradable scaffolds may create temporary physical barriers as tissues, such as bone and ligament, which develop along adjacent but separate trajectories. In addition, growth factor-tethered hydrogels may create soluble gradients that allow for the blending of tissue types, such as at the muscle–tendon interface (or myotendinous junction).

For complete epimorphic regeneration, the new tissues must have the structural, mechanical, and physiological properties of those in a normal digit or limb. For example, the bone tissue stiffness must allow normal loading without failure, the muscle should be able to generate force for movement, and the neural system must provide motor input and sensory feedback for motion control and sensation. The functions of these different tissues must integrate among themselves, such as muscle contraction leading to bone and

joint movement, to form a complete structure. For long-term viability, the new structure must also be fully integrated with the proximal tissues. For example, the vascular system of the new digit or limb needs to include proper blood flow to allow for thermal regulation and nutrient/waste transport. Thus function and structural organization, within and across tissues, is an important aspect of successful epimorphic regeneration.

Some have speculated that the limited regenerative capacity in species with a complex immune system is due in part to an intense inflammatory response to injury.⁶⁸ This is corroborated by an increased regenerative potential in immunocompromised mice,¹²⁸ as well as a decreased potential in post-metamorphic anurans that have acquired a more developed immune system.^{129–131} Similarly, complete regeneration can occur in mammals *in utero* (before the time when an immune system is fully developed) in tissues, such as skin¹³² and cartilage,¹³³ which normally form fibrotic scars after injury in adult tissues. While it seems that the immune system plays a critical and perhaps regulating role in regeneration, this topic is outside the scope of this review; however, this is an important topic for further investigation to elucidate the characteristics of a regenerative-permissive immune response.

This review focuses on the primary biological processes that regulate epimorphic regeneration. Even after successful epimorphic regeneration of a digit tip, obstacles remain in terms of a broader impact for amputee patients. Extending the response from the tip of a finger to an entire limb will include more complex challenges, such as diffusional limitations within tissues and patterning along the various anatomical axes (proximal–distal, ventral–dorsal, and medial–lateral). Even once tissue growth is initiated, a considerable challenge to address in the future will be to engineer the tissue patterning for joint formation that allows articulation over a full range of motion. Other long-term physiological challenges include regulating distal growth after regeneration and maintaining tissue homeostasis over the lifetime of the organism. Thus, there are abundant research and translational opportunities as the field of epimorphic regeneration advances.

Conclusion

The ability to promote epimorphic regeneration, to regrow a biological digit or limb, would radically change the prognosis and quality of life for amputees. This ambitious goal includes the regrowth of complex tissues that must be properly patterned to create a fully functional structure. We have yet to even identify, let alone address, all the obstacles along the extended progression of biological processes that limit epimorphic regeneration in humans. Insights from nonmammalian and fetal mammalian systems, executing controlled mechanistic studies in the murine digit model, and the expanding knowledge base in tissue engineering and regenerative medicine, will together help develop strategies to translate elements of epimorphic regeneration to the clinical setting.

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Disclosure Statement

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