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Concise Review: Translating Regenerative Biology into Clinically Relevant Therapies: Are We on the Right Path?

Jennifer Simkin

University of Kentucky, jennifer.simkin@uky.edu

Ashley W. Seifert

University of Kentucky, awseifert@uky.edu

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JENNIFER SIMKIN, ASHLEY W. SEIFERT

Key Words. Animal models • Tissue regeneration • Tissue-specific stem cells • Monocyte

^aDepartment of Biology,
University of Kentucky,
Lexington, Kentucky, USA

Correspondence: Ashley W. Seifert, Ph.D., Department of Biology, University of Kentucky, 675 Rose Street, Lexington, Kentucky 40506, USA.
Telephone: 859-216-2668;
e-mail: awseifert@uky.edu;
or Jennifer Simkin, Ph.D., Department of Biology, University of Kentucky, 675 Rose Street, Lexington, Kentucky 40506, USA.
Telephone: 901-484-3771;
e-mail: jennifer.simkin@uky.edu

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ABSTRACT

Despite approaches in regenerative medicine using stem cells, bio-engineered scaffolds, and targeted drug delivery to enhance human tissue repair, clinicians remain unable to regenerate large-scale, multi-tissue defects *in situ*. The study of regenerative biology using mammalian models of complex tissue regeneration offers an opportunity to discover key factors that stimulate a regenerative rather than fibrotic response to injury. For example, although primates and rodents can regenerate their distal digit tips, they heal more proximal amputations with scar tissue. Rabbits and African spiny mice re-grow tissue to fill large musculoskeletal defects through their ear pinna, while other mammals fail to regenerate identical defects and instead heal ear holes through fibrotic repair. This Review explores the utility of these comparative healing models using the spiny mouse ear pinna and the mouse digit tip to consider how mechanistic insight into reparative regeneration might serve to advance regenerative medicine. Specifically, we consider how inflammation and immunity, extracellular matrix composition, and controlled cell proliferation intersect to establish a pro-regenerative microenvironment in response to injuries. Understanding how some mammals naturally regenerate complex tissue can provide a blueprint for how we might manipulate the injury microenvironment to enhance regenerative abilities in humans. *STEM CELLS TRANSLATIONAL MEDICINE* 2018;7:220–231

SIGNIFICANCE STATEMENT

Continued study using mammalian models of regeneration will undoubtedly deepen our understanding of how the injury microenvironment stimulates blastema formation in lieu of fibrotic repair. The challenge ahead is how to translate basic biological understanding into meaningful clinical advances that manifest into tractable regenerative therapies. A key problem to solve is the extent to which the local injury microenvironment can be manipulated to induce blastema formation and subsequent regeneration.

INTRODUCTION

For most of us, the sight of an amputated digit is a reminder that humans cannot regenerate organs in response to severe trauma and prompts the question, *why not us?* Throughout the animal kingdom, we observe a panoply of animals that can regenerate limbs, spinal cords, hearts and even entire bodies from a small fragment, which begets the question, *how do they do it?* Historically, scientists have approached the *how* through careful description of regenerative phenomena in animals at the genomic, molecular, cellular, and tissue level of organization, and by inhibiting the regenerative process at various stages. Many such studies promoted the idea that understanding the various mechanisms regulating regeneration in animals could provide a pathway toward stimulating regeneration in humans [1].

In an unlucky twist of fate, the ability to genetically and transgenically modify certain organisms to study embryonic development left classic animal models of regeneration on the sidelines. Focus shifted toward stem cell biology and tissue engineering, which ultimately produced the modern field of regenerative medicine. The progression of regenerative medicine coincided with rapid technological advances in genomic sequencing, computational genomics, gene manipulation, cellular re-programming, and the production of tissue scaffolds and bioreactors. The result is that scientists are now able to reprogram adult somatic cells into multipotent and totipotent stem cells [2] and subsequently differentiate these cells into defined cell types [3], build complex tissue scaffolds with three-dimensional printing technology to incorporate stem cells (reviewed in [4]), and construct simplistic organs *ex vivo* for

Reparative (epimorphic) regeneration

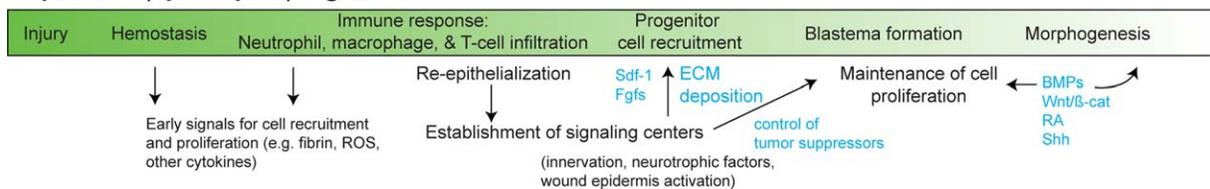


Figure 1. An outline of the events during reparative (epimorphic) regeneration in mammals. Injury initiates hemostasis, an immune response and re-epithelialization. These early events help to establish key signaling centers and a regenerative microenvironment that recruits progenitor cells. Subsequent progenitor cell proliferation forms a blastema, a defining characteristic of epimorphic regeneration. The blastema undergoes morphogenesis to restore the missing tissue, and subsequent growth will ensure a functional replacement. Vertebrate models have shed light on specific growth factors, chemokines, and extracellular matrix proteins that control reparative regeneration and include: SDF1, FGFs, BMPs, Wnt/ β -catenin signaling, RA, and SHH. Abbreviations: BMPs, bone morphogenetic proteins; ECM, extracellular matrix; FGFs, fibroblast growth factors; RA, retinoic acid signaling; SDF1, stromal derived factor 1; SHH, sonic hedgehog signaling; ROS, reactive oxygen species.

transplantation [5]. And yet, despite conceptual and technological advances, we still cannot faithfully induce a digit or other complex organs to naturally regenerate in humans. A reckoning suggests that a path forward for regenerative medicine is to directly re-engage with regenerative biologists to understand how animals regulate the injury environment to create local bioreactors in situ that can organize cells to faithfully replace damaged tissue.

Being mindful of a species sampling bias and confounding traits such as age, size, and life-stage [6], regenerative ability appears to be unevenly distributed among adult vertebrates (reviewed in [7]). Generally speaking, fishes exhibit extensive regenerative ability [8, 9] and among tetrapods, Urodele amphibians stand as outliers given the extent of their regenerative abilities [10]. Beyond these species, some frogs, lizards, and mammals show enhanced regenerative capacity of complex tissues as adults suggesting either, regenerative ability is broadly suppressed in vertebrates and has re-emerged in some species, or regenerative ability has been broadly lost and subsequently re-evolved in some instances. In spite of the interesting evolutionary questions these comparisons raise, scientists have tended to focus on those vertebrates with the most extensive powers of regeneration. Using a few key species, the hope was that discovering the underlying mechanisms in these models might stimulate new approaches or insight into developing regenerative therapies for humans [1, 11].

In particular vertebrates, appendage amputation triggers cellular reactions—activated cell-cycling, developmental signaling, morphogenesis, and differentiation—and studies in these animal models provide a basic blueprint for how tissues can naturally regenerate (Fig. 1). While studies in fish and salamanders continue to provide resolution at the molecular level for *how* vertebrate regeneration occurs, lack of closely related nonregenerating species makes it difficult to disentangle the mechanisms differentially driving a regenerative or fibrotic response to injury [12]. Important genomic, cellular, and physiological differences exist between vertebrates necessitating a broader expansion of regenerative animal models. In this light, adult mammalian models of regeneration are poised to make a unique contribution to regenerative medicine. Adult mammals more closely mimic the human condition with respect to genomic architecture, metabolic rate, immunity, and homeothermy. Moreover, mammalian models of regeneration can provide a comparative system to study regeneration and scar formation between species (e.g., ear holes, skin, etc.) or in the same tissue (e.g., distal digit tip vs. middle phalanx), and thus studies can uncover the switches regulating a fibrotic or regenerative response to injury. A similar paradigm has been

exploited to compare embryonic scar-free healing to adult fibrotic repair [13, 14]. While this body of work has contributed much to our understanding of skin healing and regeneration, the confounding factors of developmental stage (e.g., incomplete state of tissue development, cellular differentiation, immune system maturation, etc.) make it difficult to determine the extent to which embryonic scar-free healing mimics instances of naturally occurring adult regeneration. Thus, the focus of this review is directed toward complex tissue regeneration in adult mammals.

EPIMORPHIC REGENERATION AND FIBROTIC HEALING

Definitions of regeneration create an important foundation for studying regenerative phenomena. One can investigate regeneration in the context of homeostatic regeneration (i.e., regular and repeated turnover of a single cell type or multiple cell types within a tissue), or in the context of reparative regeneration (i.e., replacement of complex tissue in response to injury). Although these two broad classes of regeneration are distinct, in many cases, homeostatic regeneration and reparative regeneration can occur within the same tissue. For example, the mammalian epidermis undergoes continuous replacement during the majority of a normal lifetime (reviewed in [15]). Thus, epidermal cells exhibit homeostatic regeneration. However, if a large portion of epidermis is lost along with the underlying dermis, as in full-thickness skin injuries, although keratinocytes migrate and proliferate to re-epithelialize the wound and replace the lost section of tissue, the epidermally-derived hair follicles fail to regenerate. The distinction between the two types of regeneration is important because it underscores the idea that regenerating organs require the coordinated activity of multiple cell types and that regenerative ability at the single cell level does not necessarily translate into tissue-level regeneration. On the other hand, the fact that all animals exhibit homeostatic regeneration of some tissues is promising from a clinical perspective because it suggests there is some regenerative ability intrinsic to all vertebrates, including humans. In our mind, studying the mechanisms that control reparative regeneration is the key to understanding how to stimulate regeneration.

When studying tissue regeneration in vertebrates, reparative regeneration is defined as epimorphic regeneration (i.e., regenerative morphogenesis involving cell proliferation) [11, 16] (Fig. 1). Epimorphosis is initiated by amputation or direct injury and leads to a hemostatic response including the recruitment of neutrophils, macrophages, osteoclasts, lymphocytes, and other hematopoietic cells [17–22]. Re-epithelialization is initiated in damaged epidermis

and occurs when keratinocytes migrate to cover the injured tissue. Meanwhile, enzymatic activity from resident cells contributes to tissue histolysis in the damaged area [23–28]. During these initial events, inflammatory cells set the stage for local cell proliferation [18, 20, 21, 29] and in cases of appendage regeneration, resident cells transition to form a blastema, a recruited heterogeneous mass of cells that undergo morphogenesis to replace the missing tissue. Blastemal cells respond to signals from regenerating axons, Schwann cells, and the wound epidermis to remain in a proliferative state (reviewed in [30]). Ultimately, blastema cells will differentiate into a functional replacement of the lost organ, and regeneration of many vertebrate tissues (i.e., fin, limb, digit, ear pinna, etc.) occurs through a blastema intermediate. Some complex tissues can regenerate without the formation of a blastema (e.g., heart, skin, bone fracture, etc.) [19, 31, 32] and in these cases resident cells that accumulated in response to injury differentiate directly into the replacement tissue.

Notably, epimorphic regeneration involves several processes that also occur during scar formation [19]. Specifically, both epimorphic regeneration and fibrotic repair are necessarily triggered by injury and a hemostatic event [10, 33]. Leukocytes infiltrate the damaged tissue and produce chemotactic signals for fibroblasts, endothelial cells, and axons, while also releasing proliferative signals for local fibroblasts (reviewed in [34]). Re-epithelialization seals the wounded area from the environment and new matrix is laid down to support cell infiltration in both injury responses [19, 35–37]. Beyond similarities, important differences exist in the response to injury between fibrotic healing and tissue regeneration [38–41] and ultimately, a regenerative microenvironment gives rise to a regeneration blastema, which can self-regulate in size, pattern, and tissue complexity [42]. With this in mind, a major goal of regenerative medicine should be to stimulate formation of a regeneration blastema [12]. If the local microenvironment can be manipulated to shift the injury response, the self-sustaining nature of a blastema should be capable of morphogenesis (Fig. 2A).

ADULT MAMMALIAN MODELS OF EPIMORPHIC REGENERATION

Bonafide examples of epimorphic regeneration in adult mammals do exist, and these can occur with or without formation of a blastema. Regeneration of the costal cartilage in mice and humans [44] and the annual replacement of deer antlers occur without a blastema intermediate, relying instead on dedicated stem cells from the perichondrium and periosteum respectively [45, 46]. Likewise, full-thickness skin regeneration occurs through direct differentiation and remodeling of granulation tissue similar to heart regeneration in zebrafish [9, 47, 48]. In contrast, regeneration of ear pinna holes and distal digit tips proceed through a blastema before replacing the missing tissue [37, 39, 47, 49–53]. An additional strength of mammalian regeneration models lies in the ability to directly compare the differential response to injury that leads to fibrotic repair or regeneration (Fig. 2A).

A biopsy punch through the external ear pinnae removes cartilage, connective tissue dermis, muscle, adipose tissue, epidermis, and hair follicles with associated sebaceous glands. Using this ear punch assay, researchers have determined that rabbits [50, 52, 54] and African spiny mice [39, 47, 53] are capable of epimorphic regeneration. In contrast, an identical injury undergoes fibrotic repair and scar formation, remaining as an open hole in other rodent species including *Rattus norvegicus*, *Myomyscus*

brockmani, outbred *Mus musculus*, and inbred *Mus* such as the MRL/MpJ strain [1, 39, 47, 53, 55] (Figure 2B). Interestingly, anecdotal reports of ear pinna regeneration in cats, pikas, and hares suggest that additional mammalian species may exhibit enhanced regenerative ability including others to be discovered [1]. It should be noted that the size of the hole in the ear punch assay matters in so far as holes 2mm or smaller can be closed with scar tissue. Thus, a ≥ 4 -mm ear punch assay provides a model to compare epimorphic regeneration and fibrotic repair in the same tissue across closely related species.

Amputation through the distal digit tip (third phalanx—P3) removes bone, bone marrow, connective tissue, dermis, epidermis, and nail (Fig. 2C). Regeneration of the distal digit tip has been documented in young humans [56–58], primates [59], neonatal mice [51] and adult mice [38, 49]. Digit tip regeneration, however, is amputation level dependent. A distal amputation through the P3 element results in complete structural and functional replacement, whereas an amputation through the second phalangeal element (P2) results in scar formation and loss of original structure [37, 38, 49] (Fig. 2C). Comparing regeneration competent (P3) and incompetent (P2) amputations provides a model for studying fibrotic repair and regeneration within the same tissue, and thus the same individual.

HOW DO MAMMALS ESTABLISH A REGENERATIVE MICROENVIRONMENT?

Comparative models of epimorphic regeneration and fibrotic repair highlight a major question for regenerative medicine: is a regenerative response to injury driven by intrinsic cellular differences (i.e., response competency) or variation in extrinsic signals (i.e., signal variability)? While not new, this question has been addressed almost exclusively during cellular regeneration using skeletal muscle in the context of aging. Transplantation experiments with rodent skeletal muscle have addressed potential differences in intrinsic and extrinsic controls of regeneration [60, 61]. For instance, muscle satellite cells in older rodents show a reduced ability to self-renew when compared with those in young animals, and thus older animals lose muscle mass over time [62]. This impaired self-renewal in aged cells has been linked to over-activation of the p38 α / β MAPK pathway and this defect is sustained in older cells when they are transplanted into young muscle fibers [60]. These and other studies suggest that a progressive decline in the capacity for satellite cell self-renewal has an intrinsic component. However, when older satellite cells are cultured in vitro with serum from young mice, self-renewal capacity seen in younger muscle is restored [63] suggesting that extrinsic inputs are capable of inducing cellular regeneration. Furthermore, transplantation of whole muscle fibers, which normally lose muscle mass, from old animals into young animals restores the ability of the older transplanted muscles to gain mass over time [61] supporting extrinsic regulation of the stem cell niche. Together, these studies suggest that intrinsic and extrinsic factors are required to regulate progenitor contribution to tissue regeneration. Do intrinsic or extrinsic differences in cellular phenotype equally contribute to the differential response to injury when comparing epimorphic regeneration and fibrotic repair?

Resident Cells: Strict Maintenance of Cell Proliferation

Evidence from newts and salamanders suggests that blastema cells are derived from within 500 μ m of the initial wound site [64,

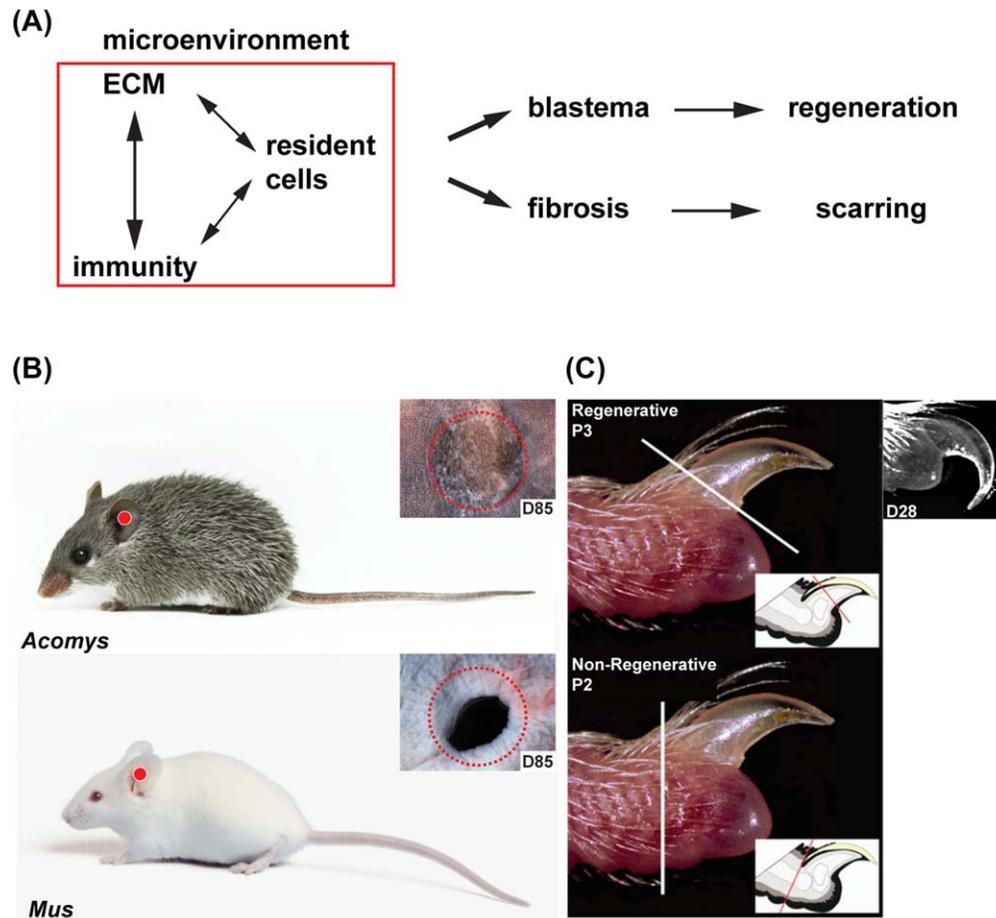


Figure 2. The injury microenvironment differentially regulates healing outcomes in mammalian models of regeneration and fibrotic repair. **(A):** The composition of the local microenvironment is determined by crosstalk among the ECM, cells of the immune system, and resident tissue progenitor cells. Comparative studies suggest a pro-regenerative microenvironment stimulates blastema formation in lieu of fibrosis. Combinatorial therapies that target these three facets could dictate the future of regenerative medicine. Comparative models of natural regeneration and fibrotic repair include a 4-mm ear punch assay **(B)** and digit tip amputations **(C)**. These models inform how the local tissue environment functions during both tissue repair processes. **(B)** A 4-mm biopsy punch through the external ear pinna of the African spiny mouse (*Acomys*) results in epimorphic regeneration, whereas the same injury through the ear pinna of outbred lab mice (*Mus*) results in a scar. **(C)** Amputation of the third phalangeal element (P3) results in epimorphic regeneration, whereas amputation through the second phalangeal element (P2) results in scar formation. Image in **(C)** adapted with permission from [43]. Abbreviation: ECM, extracellular matrix.

65] supporting local cells as essential building blocks to re-create lost tissue. In mammalian digits, lineage tracing studies have shown that blastema cells are derived from lineage-restricted cells insofar as lineages can be accurately labeled [66]. What is less clear is the extent to which blastema cells represent a population of quiescent tissue-specific progenitor cells or local cells that have de-differentiated from mature tissue. Tissue-specific progenitor cells have been discovered throughout many mammalian tissues [67], and these cells are essential for repairing damaged muscle [68–70], cartilage [44], and bone [71, 72]. On the other hand, stem cells with varying plasticity have been isolated from a wide array of adult tissues [73, 74] suggesting that inherently plastic cells like connective tissue fibroblasts may contribute a majority of the starting material for tissue regeneration [75]. The tricky problem is identifying stable markers for inherently plastic cells [66, 76, 77]. Nonetheless, the available evidence supports the idea that multiple lineage-restricted cell types contribute to the blastema during complex tissue regeneration rather than formation of a

pluripotent cell mass [75, 78–82]. Coupled with existing abilities to reprogram cells, perhaps the more pertinent question is not one of origin, but figuring out how resident cells are activated to proliferate and how proliferation is controlled.

The ability for cells to re-enter the cell cycle and commit to cell proliferation is paramount for blastema formation. Early thymidine tracing experiments in newt limbs showed that the only actively dividing cells prior to amputation were found in the epidermis and blood, and neither population contributed to the actively dividing cells of the blastema [64]. Surveying adult tissues from mammalian regeneration models demonstrates that the number of actively dividing cells is negligible prior to injury, specifically when compared with tissues with high rates of cell turnover (e.g., small intestine) in the same animal (Fig. 3). Interestingly, actively dividing cells are found throughout uninjured adult tissues in the highly regenerative axolotl (Fig. 3). These data suggest quiescent cell populations in adult mammalian tissues are re-activated to undergo cell division after injury and make up the

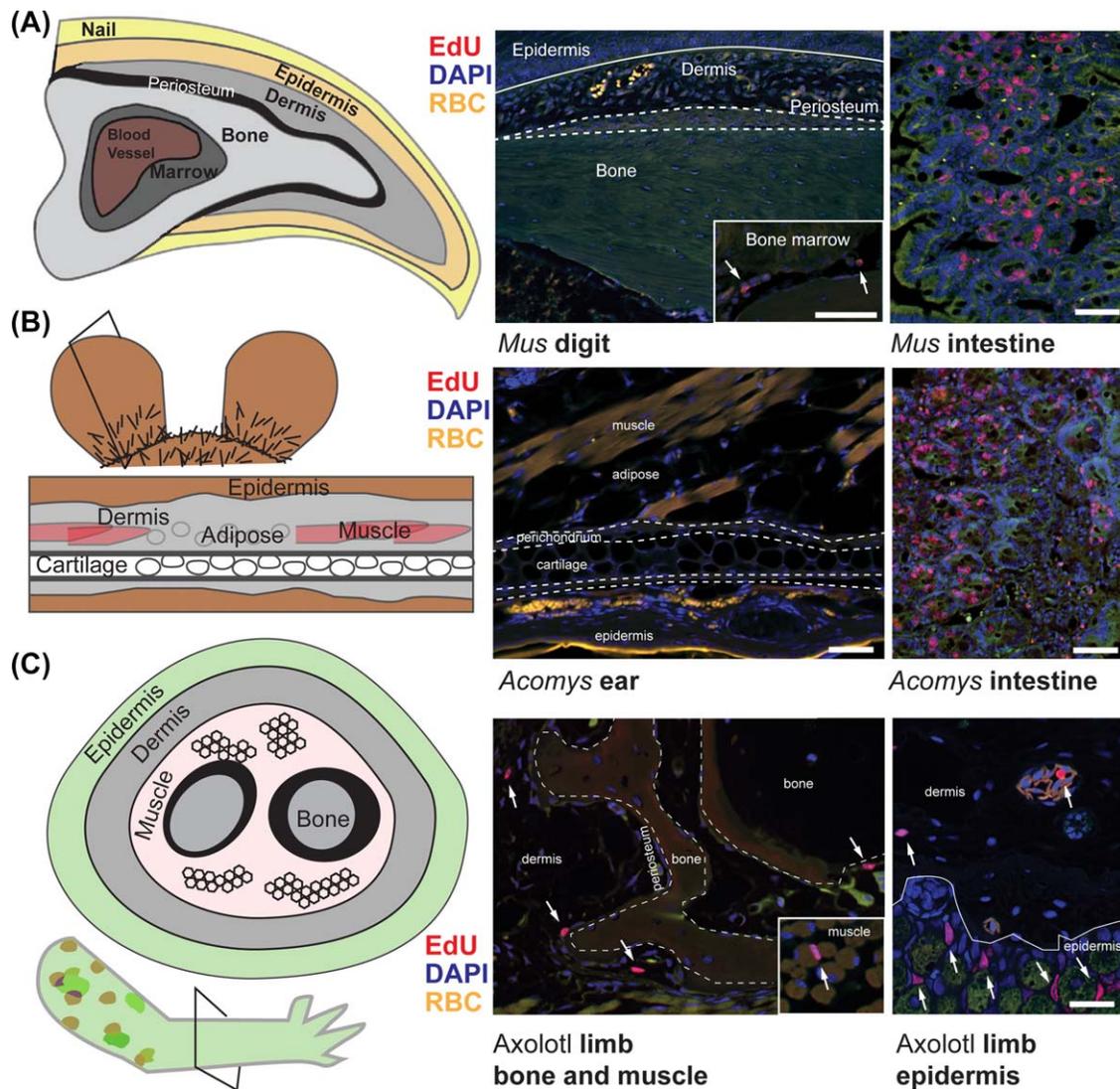


Figure 3. Tissue analysis of the S-phase marker EdU in uninjured mouse digit tip, spiny mouse ear pinna and salamander limb. **(A):** Proximal to distal (longitudinal) section of the third phalangeal element (P3) from an adult *Mus* shows very few EdU+ (red) cells which are restricted to the bone marrow (inset, white arrows). EdU staining of adult *Mus* small intestine is displayed for comparison. **(B):** Proximal to distal (longitudinal section) section through an adult spiny mouse ear pinna shows very few EdU+ (red) cells. EdU staining of adult spiny mouse small intestine is displayed for comparison. **(C):** In contrast to mammalian regeneration models, cross-section of an uninjured 1-year old axolotl limb shows many EdU+ cells (white arrows) in the dermis, periosteum, connective tissue surrounding muscle (inset), epidermis, and blood vessels. Scale bars = 50 μ m. EdU+ cells = red; DAPI (4',6'-diamidino-2-phenylindole) nuclear stain = blue; autofluorescent RBC = yellow. Abbreviation: RBC, red blood cells.

bulk of the blastema rather than existing as a permanent slow-cycling population.

An outstanding hypothesis concerns the degree to which resident cells from regenerating species may be *primed* and possess a lower barrier to cell-cycle re-entry or whether differential regulation of tumor suppressor proteins regulates activation of progenitor cells [83]. Experiments with newt and mammalian myotubes in culture showed that newt, but not mammalian myotubes, could re-enter the cell cycle in response to serum [84]. Compared to the newt muscle cells in culture, mammalian primary and established myoblast cultures displayed a differential regulation of several tumor suppressor genes and differential control of certain tumor suppressor proteins, including retinoblastoma protein (pRb) and p53, to initiate cell cycle arrest [84–87]. Inactivating *pRb* alone in mammalian C2C12 myotubes failed to initiate cell cycle re-entry

[84]. However, combined inactivation of both *pRb* and the Ink4a isoform *Arf* was able to drive C2C12 de-differentiation, and inactivation of *p53* was able to induce cell-cycle re-entry in primary myotubes [86, 88]. Additionally, tumor suppressors such as *Arf* are specific to mammalian cells [89] and expression of human *Arf* in regenerating axolotl or zebrafish tissues inhibits regeneration [84, 90, 91].

Mammalian models of epimorphic regeneration shed further light on cell cycle control in vivo, and suggest differential regulation of tumor suppressor pathways during regenerative or fibrotic healing [39]. In ear punches from regenerating and nonregenerating mammals, injury appears to similarly activate resident cells to re-enter the cell cycle [39]. In the early stages of regeneration and fibrotic repair, perichondrial cells and dermal fibroblasts are positive for cell cycle proteins Ki67, pRb, pHH3, and they incorporate

EdU. However, during fibrotic repair in *Mus*, resident cells lose these cell proliferation markers coincident with nuclear localization of the tumor suppressors, p21 and p27 [39]. In contrast, during regeneration in *Acomys*, resident cells continue to proliferate as a blastema forms to produce new tissue to close ear holes. Importantly, nuclear localization of p21 and p27 did not occur in blastema cells, although it was observed in cells proximal to the injury. This local control of cell proliferation appears key to ensuring new tissue is appropriately produced and does not lead to unregulated growth. In line with observations from the spiny mouse ear, differential regulation of tumor suppressor pathways also occurs when comparing digit cells from regenerative and non-regenerative amputations. When cells were cultured from the nonregenerative P2 region of the *Mus* digit they divided more slowly and upregulated *Cdkn2a* (p16) and *Cdkn2b* (p15) when compared with P3 cells on plastic [92]. Together, these studies support regulation of tumor suppressor pathway activation, whether extrinsically or intrinsically controlled, as a key feature of cell proliferation during epimorphic regeneration.

Extrinsic Inputs and Developmental Signals

Although injury promotes formation of a new bone callus in non-regenerative P2 amputations, and new cartilage nodules in the nonregenerative *Mus* ear pinna, in both cases these new tissues are usually disorganized and mispatterned [39, 47, 53, 71]. With respect to bone and cartilage, these observations support direct differentiation of periosteal and perichondrial cells into new tissue, but underscores the failure to organize a multicellular response typical of epimorphic regeneration. A failure to simulate epimorphosis in these mammalian tissues may result from different signaling cues produced in response to injury. Fibroblasts isolated from the P3 and P2 region of mouse digits display distinct differences in their ability to signal to the epidermis; P3 fibroblasts induce formation of a mucosal-type epidermis while P2 cells induce a stratified epidermis in keratinocyte cocultures [93]. These findings support fibroblast identity as a key component of regeneration, similar to how fibroblasts from different dermal layers or anatomical regions differentially contribute to wound healing [66, 76]. Similarly, in most mammals the ability to regenerate hair follicles in full-thickness skin wounds is lost during ontogeny and can be traced to changes in fibroblast signaling patterns, specifically a downregulation of Wnt signals and an upregulation of inflammatory cytokines in mature fibroblast populations [94]. However, studies using the *Mus* digit tip model suggest that fibroblasts from normally nonregenerative amputation levels maintain the ability to respond to appropriate patterning signals as both P2 and P3 digit cells show similar expression of BMP receptors and SMAD activation [95], and addition of exogenous BMP2 and BMP7 in vivo is able to induce skeletal patterning in nonregenerative amputations of neonatal and adult mice [40, 96–98]. While these studies suggest patterning can be rescued if the proper signaling environment is restored, there appears to be a temporal component as well. Treatment with BMP2 induces skeletal patterning of adult P2 only at specific time points after amputation [98]. BMP2 treatment 9 days after adult P2 injury induces segmental regeneration and patterned bone formation, but BMP2 treatment 24 days after injury fails to induce a cellular response. A re-injury to the P2 bone 24 days after injury rescues the ability of cells to respond to BMP2 and create new patterned bone [98]. Together, these examples support specific and appropriately timed

molecular signals within the regenerative microenvironment as important extrinsic cues regulating the injury response (Fig. 2A).

Inflammation and Immunity

Prior to blastema formation, cellular interactions with the immune environment partially determine if a blastema will form [18, 20, 21, 29, 99–101]. In adult tissues, the immediate response to injury includes the infiltration of a diverse subset of leukocytes including neutrophils, macrophages, and T cells. While intense interest has focused on macrophages and T-lymphocytes during mammalian wound healing [102–105], their importance in blastema-based regeneration is just beginning to be understood [18, 20, 21, 29]. Macrophages are essential to the mammalian regeneration process and depletion studies in the spiny mouse ear show that loss of early macrophage populations delays regeneration until macrophages re-populate the tissue to stimulate blastema formation [18]. Similarly, depletion studies in the mouse digit tip show loss of early macrophage populations inhibits re-epithelialization, tissue histolysis, blastema formation and differentiation [20]. Rescuing re-epithelialization can promote blastema initiation, but without macrophages the blastema regresses and fails to differentiate into new tissue [20]. Together with studies in salamanders [21] and zebrafish [29, 106], these studies show that macrophages are a key initiator of blastema-based regeneration in adult vertebrates. Identifying specific macrophage signaling products will be essential for determining how local immunity regulates the tissue microenvironment to promote regeneration.

While the critical macrophage signaling pathways are not yet known, timed depletions studies show that early infiltrating macrophage populations are essential for regeneration [20, 21, 29]. The timing of macrophage infiltration is closely linked to their phenotype and activity. Studies during skeletal muscle repair [102], liver repair [107], spinal cord injury [108], myocardial infarction [109], and skin healing [110, 111] document changes in macrophage phenotype with time. In these wound healing models, the initial macrophage populations are dominated by pro-inflammatory (classically activated M1) phenotypes while later repair stages are dominated by anti-inflammatory (alternatively activated M2) resolving phenotypes [111]. Characteristic differences include cytokine expression and metabolic activity between these macrophage subtypes [112, 113]. These observations of changing macrophage phenotypes in wound healing models combined with the timed depletion studies in regeneration models would lead one to hypothesize that initial pro-inflammatory signals from macrophages are required for mammalian tissue regeneration.

Comparing the early microenvironments of spiny mice and laboratory mice ear injuries has revealed important similarities and differences in the initial immune environment. First, the initial pro-inflammatory responses are distinct: early stages of regeneration are dominated by high NADPH-oxidase derived reactive oxygen species (ROS) production and low myeloperoxidase activity, whereas early stages proceeding fibrotic repair exhibit low NADPH-oxidase derived ROS production and high myeloperoxidase activity [18]. Second, compared with fibrotic repair in lab mice, the local inflammatory cytokine milieu is unique during regeneration in the spiny mouse (unpublished data) and digit tip studies provide evidence that specific cytokines, for example, oncostatin M, tumor necrosis factor alpha (TNF α), and stimulation of lymphotoxin beta receptor, support blastema formation in vivo and in vitro [114, 115]. These cytokines are produced by macrophages, Schwann cells [114], the epidermis [116], and potentially

T cells (unpublished data). Last, macrophages exhibit a distinct spatial distribution in vivo. CD206+ cells (M2) infiltrate scar-forming and regenerating wounds early after injury whereas CD86+ cells (M1) appeared to primarily infiltrate the injury micro-environment in scar-forming wounds [18]. Together, these observations demonstrate distinct inflammatory environments are present prior to a regenerative or fibrotic healing outcome and point toward the need for a more detailed phenotyping to determine the functional requirement for specific macrophages and cytokine pathways during regeneration.

Extracellular Matrix Composition

In addition to developmental signaling pathways and the immune environment, blastema organization, maintenance and morphogenesis is governed by the extracellular matrix (ECM). Far from a passive support structure, the ECM is capable of providing cues for cell migration, proliferation and differentiation (reviewed in [117, 118]). A surge in bioengineered scaffolds has capitalized on the instructive abilities of the ECM and de-cellularized scaffolds derived from natural tissues can promote tissue regeneration in bone, cartilage, muscle, and skin (reviewed in [119]). Synthetic and natural scaffolds have been modified to mimic conditions observed during embryonic development using developmental matrix molecules to guide cell differentiation. For instance, scaffolds rich in the ECM fiber tenascin-C have been shown to promote chondrocyte differentiation in vitro [120, 121]. While this can be beneficial in certain contexts, other studies have shown that treating cartilage explants with tenascin-C can increase the loss of proteoglycans and exacerbate tissue histolysis [122], suggesting that trying to direct cellular activity by enriching for single matrix components can have antagonistic outcomes as well. These studies underscore the need for understanding the composition and dynamic nature of naturally occurring regenerative matrices.

The mammalian regenerative matrix environment appears to superficially resemble the ECM present during embryonic development which is composed of matrix proteins that stimulate proliferation (tenascin-C), cell migration (fibronectin) and are more easily remodeled (collagen type 3) [28, 39, 47, 115, 123–126]. Low resolution comparison of the regenerative ECM suggests this matrix environment may be conserved across regenerative species [39, 47, 123, 124, 127, 128]. In adult tissues, tenascin-C and fibronectin contribute to the periosteum, perichondrium, tendon, and muscle where they surround progenitor cells [39, 124, 127], and perhaps play a role in supporting the progenitor cell niche [129, 130]. In vitro studies have demonstrated a direct positive effect of tenascin-C and fibronectin on salamander and mouse myotube proliferation [124]. Despite parity across regeneration models, comparative studies in mammalian models of regeneration and scarring redirect our understanding of regenerative matrices. Healing Ear punches in *Acomys* and *Mus* revealed similar levels of tenascin-C during the first fifteen days post amputation suggesting that traditional components of a regenerative ECM are deposited during fibrotic repair as well [39]. Despite early expression and deposition of tenascin-C during fibrotic healing in *Mus*, proportionally higher expression of *Col1a1*, *Col3a1* and chondroitin sulfate proteoglycans occurred prior to, and during scar formation. In contrast, persistently high levels of tenascin-C were maintained throughout the regeneration process in *Acomys*, and proportionately higher levels of matrix fibers involved in nerve guidance preceded blastema formation [39]. These comparisons

indicate that relative composition *and* timing of ECM deposition is critical toward healing outcomes.

Far from a static structure, the ECM is dynamic, undergoing active remodeling as some components are digested and others deposited. Functional studies in newts have shown that active remodeling of the ECM by matrix remodeling enzymes is required for blastema formation [27]. Although remodeling enzymes such as matrix metalloproteinases 9 and 13 (MMP9 and MMP13) are upregulated in response to injury regardless of the outcome, timing of expression is, again, a prominent difference between regeneration and scarring. In regenerating systems, these enzymes remain significantly upregulated during blastema formation and morphogenesis when compared with fibrotic repair [25, 27, 39, 124, 131]. While the exact role of remodeling enzymes during regeneration remains obscure, a few studies suggest that degraded ECM components, as well as growth factors and chemokines released by histolysis, are able to promote cell migration [132, 133]. As we continue to discover the extent to which the ECM facilitates regeneration, a more comprehensive understanding of its temporal composition will guide attempts to design next-generation bio-reactive scaffolds for clinical applications in vivo and ex vivo.

CURRENT REGENERATIVE APPROACHES TO COMPLEX TISSUE INJURY

The clinical challenge of regenerating organs and complex, multi-tissue injuries is immense. In cases of severe organ damage or dysfunction, the gold standard remains whole organ transplantation from a matched donor [134, 135] and in cases of severe tissue damage, autologous tissue transplant. While the long-term prognosis for transplant patients can be quite good, especially for certain procedures, the availability of suitable organs for transplant is relatively low and even when a match is made, there is still a need for lifetime immunosuppressive therapy [135]. It is no surprise then that there is a ravenous appetite from the patient and clinical communities to see basic scientific advances transformed into useful regenerative therapies. Unfortunately, although advances in regenerative biology continue to be made, very few regenerative technologies have either proved their efficacy in humans or significantly outperformed surgical alternatives [134, 136]. The failure to create new, useful, and tractable regenerative therapies for complex tissue trauma or loss can be traced to inherent design problems with ex vivo constructs (i.e., vascular integration, innervation, mechanical properties, etc.), an incomplete understanding of how tissues naturally develop and regenerate, and a poor understanding of how the immune system positively/negatively interacts with local cells to regulate tissue morphogenesis during natural instances of tissue regeneration.

Current approaches in regenerative medicine attempt to leverage cell-based therapies, scaffolds (natural or synthetic) or a combination of cells and scaffolds to stimulate tissue repair in vivo or build tissues ex vivo for implantation. Targeted drug delivery is a third approach, typified by the application of demineralized bone matrix (DBM) or isolated bioactive molecules to regenerate skeletal injuries [137]. Where damage or dysfunction can be traced to a single cell type or simple tissue (e.g., epidermis, corneal epithelium) available stem-cell therapies have shown encouraging success in humans. For instance, hematopoietic stem cell transplants (HSCT) have been used to successfully treat a range of leukemias and lymphoproliferative disorders by regenerating a

patient's blood system following chemotherapy [138], although like whole organ transplantation, HSCT does carry long-term complications [139]. Limbal stem cell transplants have proven successful in regenerating the corneal epithelium in humans [140] and a recent report has demonstrated proof-of-principle success using transgenically altered autologous keratinocytes to treat junctional epidermolysis bullosa through re-population of a significant portion of the patient's epidermis [141, 142]. In the case of critical size defects in the skeleton (craniofacial, axial or appendicular), autogenous bone grafts are the gold standard and most widely used clinical treatment [137]. However, the osteoinductive properties of demineralized bone matrix (DBM) [143, 144] which have been used clinically for over thirty years, have made DBM products, alone or in combination with autogenous grafts, a viable option to stimulate the intrinsic regenerative properties of bone [137]. Importantly, attempts to bridge a critically-sized fibular defect in humans using the osteoinductive molecule BMP7 (OP1) were not as effective as DBM after one year [145]. This and other attempts to stimulate regeneration using single bioactive molecules reinforces the importance of designing therapies with the complexity of natural tissue development in mind where a multitude of signaling pathways act synergistically and antagonistically to precisely control tissue morphogenesis.

As injuries increase in complexity involving multiple cell types and tissue compartments, regenerative therapies have seen far less clinical success. Replacement of so-called *hollow organs* (e.g., bladder, blood vessels, urethra, etc.) with *ex vivo* constructs from scaffolds alone or cell-seeded scaffolds have achieved a measure of reported success [146, 147], but these approaches are used infrequently and present short and long-term complications [134, 148]. Among complex tissues, the skin is injured more frequently than any other organ. The primary approach to treating acute wounds (small or large) and burns remains the split-thickness autograft [149]. In an attempt to create an artificial skin substitute that could stimulate natural tissue regeneration, Integra was developed as a composite of naturally derived collagen, chondroitin 6-sulphate and Silastic [150]. Integra is used in two stages. First, when placed in a wound or burn, the collagen compartment encourages cell infiltration and neo-dermis production as it is slowly degraded during healing. Subsequently, the Silastic is removed and replaced by a meshed epidermal autograft which restores the epidermal skin compartment [150]. Integra represents one of the true successes for regenerative engineering and has inspired many competing products to facilitate skin regeneration and repair, including de-cellularized extracellular matrices from human and animal dermis [119, 149, 151]. Despite the success of artificial skin and natural scaffolds in treating burns and full-thickness skin wounds, comparative human studies show a long-term clinical outcome no different from split-thickness skin grafts [152, 153]. As outlined above, the available human data supports the need to refine (or reconstruct) current regenerative therapies in light of our expanding knowledge of natural regeneration in animal models. Appreciating the dynamic interaction of cells, the ECM and immune system in the context of adult tissue should provide guidance on optimizing new approaches to regenerative medicine.

FUTURE CLINICAL PRACTICES: BALANCING IMMUNITY, CELL PROLIFERATION, AND FIBROSIS

Continued study using comparative mammalian models of regeneration will undoubtedly deepen our understanding of how the

injury microenvironment stimulates blastema formation in lieu of fibrotic repair. The challenge is how to translate basic biological understanding into meaningful clinical advances that manifest into tractable regenerative therapies. In our view, a key problem to solve in the treatment of complex tissue injuries is the extent to which the local microenvironment can be altered to induce blastema formation. If injured tissue can transition to a blastema then the self-sustaining nature of the blastema will facilitate morphogenesis and regeneration. This same principle can be applied to tissue injuries where a blastema is not required (e.g., skin, heart, lung, etc.). In these cases, the local microenvironment still controls the cellular response to injury and as such, can polarize the healing response.

Current tissue engineering techniques modulate the tissue microenvironment and promote endogenous repair through the creation of scaffolds (reviewed in [119]), and through the modulation of the immune response (reviewed in [154]). It is notable that these current therapies are designed to target individual tissues, that is, collagen-glycosaminoglycan scaffolds for skin (Integra) [150, 155], silk scaffolds for ligament [156, 157], organic scaffolds for bone [158, 159] or hyaluronic acid infused scaffolds for cartilage repair [160]. Similarly, immune-modulatory therapies are designed to target single specific arms of the immune cell response; that is, targeted dampening of interleukin 1 beta pro-inflammatory cascades to improve healing in skin [161–163], calvarial bone defects [164], and arthritic cartilage [165]. Yet the ultimate goal of regenerative medicine is to stimulate integrated multi-tissue regeneration, and the extent to which current scaffold designs and immune targeting drugs induce complex tissue regeneration is unknown. Is it possible to create scaffolds that target multiple tissues with a therapy that stimulates evolution of the immune cell response across healing phases? What would these therapies look like? We can benefit from comparative analyses of regeneration and scarring to quickly guide new therapy designs and help answer these questions. For example, we have learned from comparative analysis in mammals that regenerating systems deposit ECM fibers for nerve guidance [39], that BMP treatment is more successful when combined with factors that mobilize periosteal cells [98], and that early inflammatory events promoting high NADPH-oxidase derived ROS production and lower H₂O₂ stimulate blastema formation [39]. As engineering techniques become more sophisticated enabling dual growth factor delivery [166], guided nerve growth [167], and cytokine capture [168], it should be possible to design dynamic scaffolds to simultaneously incorporate early control of the immune system, guidance of nerve growth, and timed release of growth factors to promote blastema formation.

CONCLUSION

Although many yearn for a single factor or scaffold that will magically jumpstart a regenerative response, it has yet to be discovered, and is more likely not to exist at all. Instead, the complexity of establishing and maintaining a controlled regenerative response in adult tissue will require combinatorial therapies delivered in a progressive timeline to manipulate the local tissue microenvironment. Future goals for designing new approaches in regenerative medicine should embrace an understanding of how to integrate

immune modulation, scaffold design, and controlled cell cycle activation to enhance intrinsic regenerative abilities in humans. In our view, a productive path to discovering new regenerative therapies will arise from comparative studies of regeneration and fibrotic repair, and active engagement between biologists, engineers, and clinician-scientists.

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AUTHOR CONTRIBUTIONS

J.S. and A.S.: wrote and approved the final manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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