

# Memory Encoded Throughout Our Bodies: Molecular and Cellular Basis of Tissue Regeneration

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**ABSTRACT:** When a sheep loses its tail, it cannot regenerate it in the manner of lizards. On the other hand, it is possible to clone mammals from somatic cells, showing that a complete developmental program is intact in a wounded sheep's tail the same way it is in a lizard. Thus, there is a requirement for more than only the presence of the entire genetic code in somatic cells for regenerative abilities. Thoughts like this have motivated us to assemble more than just a factographic synopsis on tissue regeneration. As a model, we review skin wound healing in chronological order, and when possible, we use that overview as a framework to point out possible mechanisms of how damaged tissues can restore their original structure. This article postulates the existence of tissue structural memory as a complex distributed homeostatic mechanism. We support such an idea by referring to an extremely fragmented literature base, trying to synthesize a broad picture of important principles of how tissues and organs may store information about their own structure for the purposes of regeneration. Selected developmental, surgical, and tissue engineering aspects are presented and discussed in the light of recent findings in the field. (*Pediatr Res* 63: 502–512, 2008)

**Quality of healing depends on the capability to recall the structure of lost parts.** After the body is formed *via* morphogenesis and organogenesis, it is not easy to repair, replace, or regenerate most of its structures if they become damaged. Indeed, most injuries to our bodies are healed incompletely, with a loss of structure or function to a variable extent. For example, deep wounds in adult skin heal by granulation tissue formation and reepithelialization, which repairs the integumental defect but does not regenerate the original skin architecture (*e.g.*, hair follicles and other appendages, dermal lines, folds). This is rather puzzling, because it is known that the skin contains all necessary components needed for a complete regeneration, including both mesenchymal and epidermal stem cells (1). On the other hand, nature also provides multiple examples of the body's ability to heal or even completely replace much more complex structures than the skin, such as a lizard tail or a deer's antlers (2).

Comparing all these processes, it appears that they have a principle in common: a certain kind of "memory." Cells, tissues, and organs somehow need to know what to do—and when and how to do it—to regenerate lost or damaged body parts. This ability of "remembering" the body's own structure becomes evident when regenerating structures that no longer exist in the injured body (such as a lost lizard's tail). Furthermore, there must be a mechanism that maintains body shape over the years (or even centuries) of life, except for the changes related to aging. We speculate that the extent and quality of this structural memory of tissues will determine the quality and completeness of the regenerative processes. Whether it is a perfect neof ormation of an entire tail or just healing through bad scarring on a diabetic's leg, the tissues and cells always perform their reparative tasks using a pre-defined set of biologic functions, and under the influence of microenvironmental conditions. Below we review current knowledge on skin repair, and where appropriate, we make broader factual connections from the aspect of postulated structural memory that is encoded within tissues and organs (Fig. 1).

## THE CONCEPT OF STRUCTURAL MEMORY IN TISSUES

**Anatomical and functional organization of the body's structural memory.** Partitioning the body into individual organ systems to be comprehensive in our understanding of anatomy, physiology, and diseases, is always somewhat artificial. And while some systems were quite easily identified centuries ago (cardiovascular, nervous, respiratory, digestive), other systems were difficult to discover—and until now, their definitions remain speculative, based on functional unity rather than anatomical demarcation (*e.g.*, the endocrine system or immune system, whose elements can be scattered in multiple unrelated locations such as bones, intestine, spleen, etc.). When it comes to the definition of mechanisms that maintain the body's shape and restore its structure after injury or damage, we encounter even larger difficulties. First, it is clear that certain healing potentials exist literally everywhere within the organism, thus ranking tissue healing among ubiquitous

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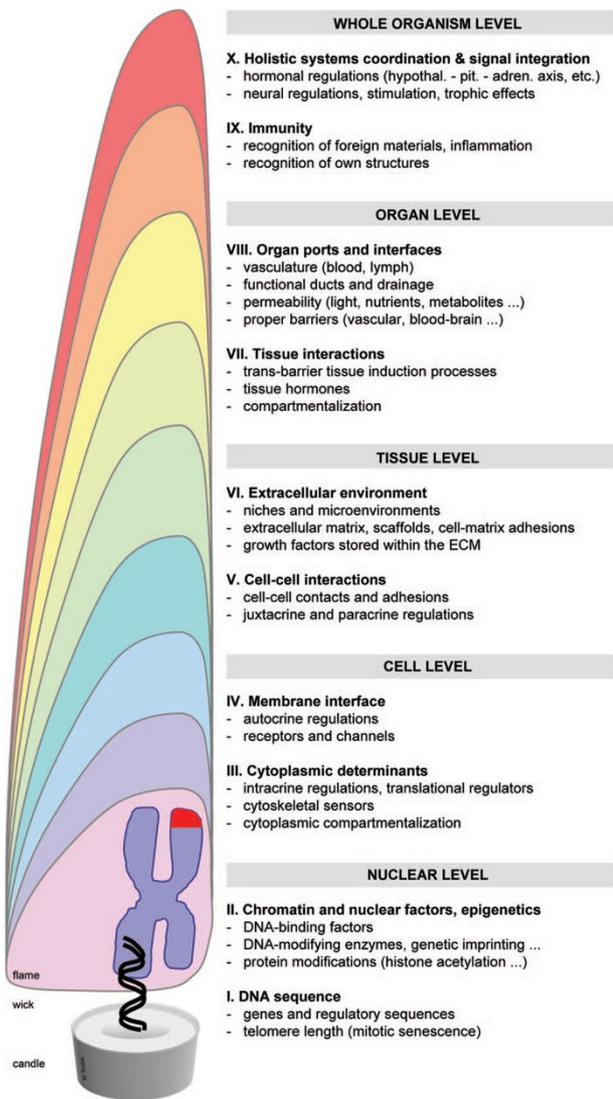
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**Abbreviations:** ECM, extracellular matrix; ESC, embryonic stem cell; FGF, fibroblast growth factor; PMN, polymorphonuclear neutrophils



**Figure 1.** Abstract model of integration of different elements that maintain the body plan. Multiple biologic mechanisms are involved in the storage or production of the information necessary for preservation of the body's architecture. Although these individual mechanisms obey the known principles of physiologic regulation and can be categorized into groups of increasing complexity, their mutual interactions do not appear as a plain hierarchical relationship. Rather, the simpler (lower) levels form a foundation for, and are contained within, the more complex (upper) levels, and complex feedback loops exist as well. Thus, for illustration purposes, individual mechanisms are assigned different colors in a flame diagram depicting their recursive containment within each other and their mutual dependence. The functional complexity starts on the bottom with the DNA in the nuclei of individual cells, and concludes on the top with complex regulatory systems that synchronize the entire organism. This diagram was assembled as an aid or a "checklist" for analytical considerations in the fields of wound healing and tissue engineering. Extremely complex interactions between all structure-maintaining systems are simplified in the following analogy with a candle flame: All regulatory (instructional, structure-memorizing) levels are parts of the same "flame." As such, they are mutually more or less interconnected, and changes in any level may influence the entire system. The genomic DNA sequence, when complete and correct, is a fundamental piece (a "wick" in the picture). Without it, the "flame" dies (*e.g.*, fatal mutations, chromosomal loss, *etc.*). However, a candlewick on its own never makes a flame, while a flame cannot be without a wick and it can only be ignited with another flame. And so it is with DNA and life in tissue engineering: We do not understand the basis of life to create living structures, but we can use preexisting small functional pieces—live cells, tissues and gene constructs—to repair damaged body parts. The more that the basic (lower) regulatory levels are maintained in our

processes such as metabolism or ion homeostasis and making any anatomical demarcation impossible. Second, the involvement of immunocytes and stem cells migrating into the wounded area from remote locations clearly demonstrates a coordinated action of the body, which is further underlined by the eventual involvement of the most complex regulators, such as nervous or endocrine stimuli in certain healing locations. Third, our understanding of how the body is formed during embryonic development is still insufficient, and so is our understanding of how structures once created are physiologically maintained during the lifespan, implying that our current insight into tissue repair may be rudimentary at best. And finally, the explosion of data on complex tissue- and organ-specific healing processes makes it impossible to review all the current knowledge within a journal article like this one. Therefore, we use a substantial level of abstraction and conceptual simplification, together with references from the literature for details, to delineate a generally applicable sketch of how tissues remember and preserve their own structure.

**Explaining the term "memory" used in this article.** The mere repopulation of a damaged site by neighboring cells may be viewed as a basic healing mechanism and may eventually lead to perfect regeneration in some locations. However, healing may also involve sophisticated processes such as neural stimuli (*e.g.*, in muscles) or complex differentiation events that recapitulate parts of embryonic development (*e.g.*, lizard tails). Thus, we use the term structural "memory" in the broadest, all-inclusive sense. In other words, for purposes of this review, the structural memory of the body is understood as a set of multiple factors that includes biomolecules, sub-cellular and extracellular structures, and signaling networks, together with all related biologic mechanisms, that instruct cells to maintain or recreate complex structures of tissues and organs. This set may include a broad range of elements: from the chemistry of DNA sequences, through cell surface determinants (antigens, receptors, channels, *etc.*), latent growth factors silently deposited within the extracellular matrix (ECM), mechanical pressure and temperature changes, up to neural regulation and behavioral changes.

One important aspect of this definition is that we do not find any evidence of centralized storage of the body's structural information, so there is no analogy with computer memory or our mental cognition. Rather than this, tissue structural memory is distributed throughout all body structures and encoded directly within them, starting with DNA as the primary in-

engineered graft, and the more that the upper regulatory levels are compatible with it, then the better our tissue constructs will be. And similarly as we block the top of a burning flame with a candelisuffer, in analogy it is possible to remove the upper levels of regulation in living systems. By doing this, we lose the top levels of body integration, but the lower regulatory levels persist with a reduced functionality (*e.g.*, organs, tissues or cells dissected from live animals can temporarily survive *in vitro* in culture, slowly losing their original biologic properties because of being deprived of nerve impulses, hormonal peaks, blood stream, *etc.*). No diagram and no analogy is perfect, but we believe that this scheme may at least serve as a good starting point for young colleagues. It represents a broad concept of principles involved in organ regeneration, and their mutual interconnections during decades-long, day-by-day maintenance of the body's fine structure in health and disease.

structions for building all higher-order, more or less self-remembering systems. Immunologic memory is an example of generally accepted mechanisms of cell/tissue-encoded memory. Another important aspect is that there is very little real knowledge of how the body maintains its structural organization. The majority of facts remain to be discovered and this article cannot aim higher than to be a fair attempt at extrapolating to a bigger picture based on the current fragmented experimental data.

Some of the elements of structural memory may not exhibit any apparent roles under physiological conditions *in vivo*, and perhaps may be dispensable under *in vitro* culture conditions. For example, certain cell surface antigens are never exposed to the immune system, or certain growth factors are never released from the ECM unless the tissue is wounded—and unwounded tissues are maintained perfectly fine in their absence. Thus, genes of unknown functions or genes showing no phenotypical defect after knocking them out may fall under this category. The elements of structural memory also may be separated from each other by distance and multiple barriers (such as the multipotential bone marrow stem cells that reside far from lungs, or the presence of fibrinogen that usually does not occur within intact organs). However, under specific conditions—either natural, accidental, or medicinally induced—some or all of these memory elements may be brought together, start mutual interactions and thus acquire a new sense that directs somatic cells to regrow lost body parts or reactivates the original imprinting present during development.

Figure 1 shows the key components of the structural memory combined in a hierarchical scheme, being represented as elements of one integral, interconnected, multilevel body maintenance system. Inherently, the “upper levels” usually contain and use all “lower levels” as their functional components. For example, immunity (level IX) is based on genes, cell signaling, matrix interactions, functional vasculature, *etc.*, although it does not depend on—but can be controlled by—the level X elements. Figure 1 is used as a framework for our review. For brevity, we explain the natural chronological order of wound healing processes, while referring to corresponding memory elements from Figure 1. For simplicity, skin healing is used as a major model system throughout the article, with reference to selected interesting details from other organs when appropriate. We will also make connection between what we learned from studying wound repair to its current and potential applications in the field of tissue bioengineering.

### SIMILARITIES BETWEEN HEALING AND EMBRYONIC DEVELOPMENT

**Scarless epithelial closures as an archetype of wound healing.** Inside a developing embryo, multiple organs and structures are formed by mutual fusion of primitive anlagen (such as pharyngeal arches, neural lips, palatal shelves, endocardial cushions, *etc.*). Embryonic tissues usually respond to new contacts—when established in the right place and at the right time—by removal of the epithelium from the contact area. Then, confluence of the underlying layers concludes the

tissue union. Many of these processes are essentially akin to the embryo wounding itself (by removing the epithelium), and then healing the defect generated. Interestingly, all these essential morphogenetic processes are accomplished without notable traces or scars, similar to healing of wounds during the fetal period (3–5). The neural tube and the palate are among the best-studied epithelial closures occurring during embryonic development. Although the primary cellular mechanism and timing of epithelial disappearance from the surface of apposing palatal shelves remains controversial (6–9), it is known that these mucosal cells deposit transforming growth factor beta 3 (TGF- $\beta$ 3) into the ECM before they disappear (10). Although this growth factor pool may be used by the depositing cells themselves (10,11), it is possible that it also serves as a message for subsequent developmental processes governing the epithelial closure. Intriguingly, TGF- $\beta$ /BMP is the same signaling pathway associated with scar formation and scarless wound healing (12–14), and TGF- $\beta$ 3 was demonstrated to mediate antiscarring effects. Important implications of TGF- $\beta$  signaling (see Ref. 15 for a detailed review of all ligands and receptors) for healing of injured epithelial surfaces of the body such as skin, mucosa, or endothelium, are demonstrated by the decades-long promises in developing antiscarring agents (16).

**Practical utilization of developmental knowledge.** The epithelial closures mentioned above serve as a limited example of how developmental biology helps tissue engineers understand the processes involved in organ formation. In general, the continuing elucidation of additional developmental mechanisms involved in tissue morphogenesis comes with the expectation that they could be repeated or mimicked i) to prevent postnatal manifestation of prenatal defects (17), or ii) to be used in engineering of tissues and organs. It is not even partially possible to mention here all the parallels between embryogenesis and organ regeneration. We have to refer here to a rapidly growing body of knowledge in the standalone field of developmental biology. For the readers' interest, the organs with the most understood morphogenesis with potential implications for tissue engineering or regeneration include the heart and cardiovascular system (18–25), teeth (26), craniofacial bones and tissues (27–36), the neural-crest-derived structures (37,38), bone marrow (39), skeleton (40), and skin (41–43).

### THE INITIAL PHASE OF HEALING

**Players in regeneration: cells, matrix, and soluble mediators.** When tissues are injured or damaged, organic debris has to be removed from the site of injury, and new components must be delivered and/or recreated, there. Everything must happen in an orchestrated manner and involves mutual interactions of cells, ECM and soluble factors (44). Not surprisingly, the same basic components are used in tissue engineering (usually referred to as cells or stem cells, scaffolds, and inducing compounds). Any imbalances between these key players or other elements of structural memory may result in improper repair, such as insufficient healing (chronic ulcers, recurrent hernia, unhealed fractures), overhealing (hypertro-

phic scars, keloids, fibrosis), or other aberrations (ectopic joints, teratomas). Whereas tissue engineering procedures are artificial and still highly experimental, natural wound healing generally involves three regular stages that overlap in space and time (45,46): i) hemostasis and (optional) inflammation, ii) a proliferative phase of granulation and tissue (neo)formation, and iii) tissue remodeling including scar maturation. Initially, hemostasis and platelet aggregation are followed by wound infiltration with lymphocytes, phagocytes, and cells involved in later proliferative phases. In the tissue formation phase, processes named granulation, angiogenesis and epithelialization begin to fill and cover the wounded area, accompanied by wound contraction (47,48). During tissue remodeling, there is increased repatterning and turnover (synthesis, deposition, remodeling, and degradation) of the ECM molecules (49,50), and loss of surplus cell types by apoptosis. Cell-cell and cell-matrix interactions, together with a correct balance of soluble molecular mediators, are vital for appropriate wound healing in all three phases.

**First reaction—leukocytes and inflammation.** When the injury is large and deep enough to disrupt mesenchymal layers, vasoconstriction, blood clotting and aseptic inflammation are the body's first responses (Fig. 1, all levels involved). Following such a deep injury, platelets and other blood cells become entrapped and aggregate into a blood clot. Different cell types have different temporal involvements in wound closure, but in general, the platelets, polymorphonuclear neutrophils (51), as well as the endothelial cells lining the capillaries at the wound site, release chemotactic factors. In later phases of healing, PMNs must transmigrate across the endothelial cells of the capillaries and other nearby blood vessels. For this to occur, the mesenchymal endothelial cells must become activated by proinflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ . Proinflammatory cytokines prime the endothelial cells for leukocyte adhesion and diapedesis by inducing the expression of P- and E-selectins, as well as the intracellular adhesion molecule 1 and 2. These adhesins interact with the corresponding integrins present at the cell surface of PMNs, including CD11a/CD18 (LFA-1), CD11b/CD18 (MAC-1), CD11c/CD18 (gp150, 95), and CD11d/CD18 (49). Multiple proinflammatory cytokines also recruit and activate fibroblasts and epithelial cells. Another important player in wound healing are macrophages, which replace the PMNs after the first couple of days. Migration of blood monocytes (*i.e.*, future macrophages) into the wound is regulated by the interaction of  $\alpha 4\beta 1$  integrin and endothelial vascular cell adhesion molecule 1. At the wound site, monocytes respond to growth factors, bind the ECM, and mature to become macrophages producing additional signaling molecules—TGF- $\beta$ , TGF- $\alpha$ , basic fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor. These growth factors stimulate the production of adequate or excessive amounts of the ECM, as well as induce increasing cell proliferation and reepithelialization to replace damaged cells. Macrophage immunity aids wound healing, and in experiments targeted at the reduction of scarring, which inhibited macrophage invasion into the wound, the

efforts were not successful and there was a resulting delay in healing (46,52).

**Immunologic memory—the extent of inflammation affects healing outcomes.** In general, the amount of inflammation determines the quality of tissue repair, and inflammation is required for scar formation (49,53). The immunology and physiology of inflammation (Fig. 1, levels I–IX) is so complex that it is beyond the scope of this review. From the aspect of body structure maintenance, specific immune responses that involve creation of memory cells is not the only memorizing action characteristic of the immune system. Also, multiple mechanisms of nonspecific immunity, including inflammation, can be viewed as a part of the body's structural memory. Although specific immunity is more plastic and highly adjustable to novel antigens, nonspecific immunity provides a quite rigid set of defense tools against generally expected and commonly occurring agents (bacteria, foreign particles, dead tissue, *etc.*). Despite this functional difference, both types of immunity, in principle, carry the information needed to distinguish self from foreign and healthy from sick, thus serving as complementary body-structure-maintaining subsystems, and both play distinct roles in wound healing. Using the aspect of memory mechanisms in healing, we can distinguish two important impacts of immunologic defense: 1) Recognition of self *versus* foreign antigens, which is encoded in the immunologic memory (both specific and nonspecific), is crucial in determining which structures are to be destroyed within the wound and which will be saved; and 2) The extent of immunologic destruction affects the amount and types of other memory units (differentiated cells, acellular matrices/scaffolds . . . ) left in the wound to serve as a starting material for healing.

In the ideal situation, only the debris and devitalized tissue are removed from the wound, and a maximum of viable cells and ECM is saved and nurtured. Such a situation may occur in very small injuries, but in general, it is unlikely in most real wounds. Therefore, cell signaling and the production of various bioactive proteins during the inflammatory phase are actively investigated with the hope of uncovering therapeutic options for treating wounds associated with the up- or down-regulation of inflammation (such as chronic ulceration, wounds in elderly and diabetic patients, and possibly certain types of fibrosis). Both the polymorphonuclear neutrophils and macrophages produce high levels of destructive enzymes and oxygen radicals that may cause tissue damage (54). In contrast to postnatal wound healing, healing in the early fetal skin exhibits scarless regeneration of the dermal architecture. Among the numerous differences between fetal and postnatal skin is the major hallmark of fetal repair, which is the lack of a typical inflammatory response (46,49).

## WRECKED TISSUE REMNANTS, AND BUILDING NEW SCAFFOLDS

**Scaffold memory and growth factors storage—the extracellular matrix.** It is known that if an epithelial surface or a small part of the parenchyma is lost, many organs may regenerate quite well, providing that the mesenchymal structures remain preserved (Fig. 1, level VI). For example, small

skin abrasions or liver damage may demonstrate scarless healing. However, if the mesenchyme is damaged or lost, the tissue or organ may become mutilated. This is particularly evident in tissues with extremely complex architecture, such as the kidney, which represents a great challenge for artificial matrix microfabrication (55). It is apparent that the ECM serves as a destination area for cells, with the ability to instruct the attracted cells to perform certain functions and/or differentiation processes. In certain locations, ECM itself may play extremely specific roles that require unique physical properties, such as transparency in the eye (56). Furthermore, the intact mesenchymal stroma defines the shape of the healing structure, thus representing a kind of “tissue memory” (Fig. 1, level VI) that works even in certain cases of total cell loss (e.g., dermabrasion).

Accordingly, from the bioengineering point of view, a scaffold is prepared as the first structure during construction of artificial tissues or organs. So how is its function achieved? The function of providing the shape and size of a regenerating organ is clear—ECM serves as a mechanical support for cells in the same fashion as the shape of a cultivation flask demarcates the cell growth area. In addition to this mechanical role, it is known that molecules stored in the ECM can direct cell proliferation, migration, and differentiation (57–59). For example, growth factors released to and/or stored within the ECM (PDGF, TGF- $\beta$ ) and fibronectin encourage the proliferation and migration of cells to the wound bed, and production of ECM molecules by fibroblasts. Cells bound to the ECM often respond differently to growth factors and other stimuli when compared with unattached cells. In the absence of signals arising from attachment to the ECM, many cells will commit apoptosis (60). Recent studies have shown that double P- and E-selectin deficient mice and intracellular adhesion molecule 1 deficient mice all demonstrate dramatic delays in wound closure that is primarily associated with a lack of infiltration of neutrophils and macrophages into the wound site (49). Another recent study demonstrates that mechanical strain may significantly alter the storage behavior of the ECM, leading to different rates of growth factor release with direct effects on adjacent cells (61). Thus, fabrication of matrices capable of a simultaneous release of multiple growth factors in desired concentrations (62) under various mechanical conditions represents a major challenge. Future development of tissue engineering may include utilization of microdevices or advanced intelligent biomaterials for precisely controlled protein release. Design of temporary biodegradable matrices, such as fibrin for the delivery of cells, and research on 3D matrix properties are other active frontiers of current research (63–68).

**Provisional ECM during wound healing.** The provisional ECM, present in healing wounds, is different in composition from the ECM found in normal tissue. It includes fibronectin, a mechanically weak type III collagen, elastin, glycosaminoglycans, glycoproteins, and proteoglycans. Impaired production of normal collagen in Ehlers-Danlos syndrome causes delayed wound healing (69). For years, it was believed that decorin deficiencies (a TGF- $\beta$  binding factor) lead to weak connective tissues. However, it is interesting to note that a

recent study demonstrates the opposite: improved cellularity and tensile strength is seen in collagen matrices engineered with decorin-deficient fibroblasts, and a similar effect can be achieved by stimulation of normal fibroblasts with TGF- $\beta$ 1 (70). This brings new promise for matrix engineering. The provisional ECM has an increased amount of hyaluronan and fibronectin, which creates an extremely hydrated matrix that provides for additional cell migration. There is also a steady increase in the amount of collagenase and other enzymes that are responsible for collagen degradation. Specifically designed compositions of the ECM are critical in the bioengineering of multiple tissues and organs, including bone and cartilage (71–74), cleft palate repair (4,75), brain injury repair (76), heart valves (77,78), and the skin, which is the most advanced living artificial organ at the present time (42). The elastic component of the ECM, characterized by molecules of elastin that crosslink with one another, confers a resilience to recoil after stretching. Thus, it is extremely important in the bioengineering of heart valves and large blood vessels. The fibronectin component of the ECM that binds with numerous molecules and interacts with collagen, fibrin, heparin, and specific membrane receptors on responsive cells, is consistent with its role as a part of the instructional memory operating during wound healing. The cell-binding domain, made up of amino acids Arg-Gly-Asp (RGD), functions in helping cells adhere to and migrate within the ECM (54). The RGD component has been a recent focus of study for scientists investigating the targeting of cancer cells that upregulate RGD (79), with possible implications for healing of cancer lesions *via* ECM manipulation.

## DEVELOPMENTAL PROGRAMS IN CELLS

**Memory within the wound neighborhood.** The neighborhood of a wounded tissue may serve as an additional type of “tissue memory” or a “material pool” needed for repair (Fig. 1, levels I–VIII). In the simplest situation, fully differentiated cells or alternatively, progenitor cells, just simply migrate into the wound from surrounding unwounded areas (epithelial cells, macrophages, endothelial cells, fibroblasts, parenchymal cells, *etc.*). The regenerative potential of neighboring cells may go far beyond this repopulating capability. For example, differentiated corneal keratocytes not only heal eye scratches extremely efficiently, but they also retain progenitor cell properties when injected into a developing embryo, with the ability to migrate together with the neural crest and differentiate to multiple tissues (80). However, in the majority of epithelial cell types, the developmental program has already been executed once and its outcomes are permanently embossed in the differentiated state of these cells, which are now ready to simply repopulate the damaged site. This situation is analogous to the recovery of a burnt forest: The soil is there, the shape of hills is preserved (“remembered”), and the only requirement is to add some seedlings there, nurture them, and then wait. In clinical practice, reepithelialization of skin abrasions or liver regeneration are typical examples of this healing mechanism that requires a certain degree of mesenchymal stromal integrity. In tissue engineering, this mechanism is exercised when more or less differentiated cells are seeded

into scaffold matrices of future implants *in vitro* (81) or delivered straight to the target tissue *in vivo* (82).

**Developmental memory recalled from local or circulating stem cells.** However, if the natural stroma is totally lost, or if an artificial scaffold cannot be manufactured, or if the structural or functional interconnections of cells are too complex (e.g., neural tissues or muscles), simple cell replacement cannot warrant full regeneration. The only hope is tissue engineering with the use of stem cells including embryonic stem cells (ESCs; Fig. 1, level IV). Stem cells possess the developmental plasticity (83) enabling them to recapitulate certain developmental pathways. In naturally healing tissues, there are three major sources of new materials: i) differentiated cells and stem cells remaining in the wounded area, ii) the same cell types plus blood and lymphatic vessels in the wound neighborhood, and iii) cells coming from remote locations, usually *via* the bloodstream, such as fibrocytes and circulating stem cells. Whereas tissue engineering can wisely use all of these natural resources, its main mission is to find alternative solutions in cases where any of the natural healing mechanisms are missing or insufficient. This may turn out to be an extremely complex task, since the stem cells used in experiments, including ESCs, often show unexpected affinities to adult tissues together with surprising differentiation capabilities—e.g., adipose or pancreatic stem cells can form neurons or glial and other cell types (84,85), and amniocytes can form kidney structures (86). Some of the natural and artificial cell repopulation processes will be discussed in the paragraphs below.

**Age, mitotic memory, and acquired mutations.** The developmental potential, *i.e.*, a differentiated state *versus* multi- or totipotency, is not the only memory carried by cells used in tissue engineering. Nonstem cells also remember their relative senescence (*i.e.*, their “mitotic age” causing the Hayflick limit in cell culture; this essential vitality indicator has a loose correlation with the overall age of the organism) through telomere-independent as well as telomere-shortening-dependent mechanisms (Fig. 1, level I) (87,88). Thus, senescence assays may become an important tool in tissue engineering to avoid premature ageing and loss of engineered implants. Moreover, mutation and chromosomal analysis of cells used for organ construction is an adequate prerequisite to prevent implant failure or malignant transformation. This is particularly important when ESCs are used. Even well-developing early embryos are known to carry *de novo* originated chromosomal aberrations, and frequent remarkable genetic mosaicism has been well documented. Thus, single-cell ESC genetic diagnosis becomes a frontier in assisted reproduction and bioengineering (89–91), fulfilling one of the most important roles—providing a source of genetically healthy cells.

## ORGAN LEVEL: INTEGRATION OF ALL MEMORY MECHANISMS

**Granulation.** Possibly one of the best examples of the fact that wound healing is a predefined, uniformly programmed process, is granulation. Centuries ago it was noticed that

healing wounds, regardless of their location, acquire a very similar and quite uniform appearance characterized by soft, moist gelatinous tissue with a bumpy surface comprised of small red nodules (Lat. *granulationes*) formed by new capillary loops (46). Granulation tissue always consists of i) revascularization components: new blood vessels and endothelial cells, ii) immune components consisting of inflammatory cells—mostly macrophages and neutrophils, but also other leukocytes in lesser amounts, iii) structural cells, that is fibroblasts and myofibroblasts, and iv) enzymes and components of a provisional ECM produced by fibroblasts. At the end of the granulation period, ECM production levels in the wound no longer exceed the amount of its degradation; granulation ceases and the excess fibroblasts commit apoptosis, which converts the granulation tissue from a cell rich environment into one that consists mostly of collagen (92,93).

**Fibroplasia—The beginning of tissue neof ormation.** After the inflammatory period, cells within the wound begin to organize a new, primitive tissue (Fig. 1, levels V–VI). Tissue formation and remodeling begins about 2–3 days after a wound occurs, overlapping with the inflammatory phase. This requires selective apoptosis of certain cell types such as the PMNs and macrophages, as well as selective proliferation of mesenchymal cells such as endothelial cells and fibroblasts (93). Fibroblasts are a part of the mesenchymal stroma, and their migration into a wound is referred to as fibroplasia. By the end of the first week, fibroblasts are the predominant cells within the wound site. Factors such as fibronectin, PDGF, FGF, TGF- $\alpha$  and - $\beta$ , and C5a, facilitate fibroblast migration and proliferation. TGF- $\beta$  has been suggested as having a dual role: as a down-regulator of inflammation, as well as being the key growth factor for inducing fibrosis. To get to the wound site, fibroblasts migrate utilizing the fibrin/fibronectin wound clot, adhering to fibronectin. After migration and proliferation, fibroblasts begin to lay down ground substance and collagen. The collagen laid down early in wound healing, type III, is extremely important for the maintenance of wound strength. Fibroblasts also secrete growth factors that attract epithelial cells to the wound site.

**Fibrocytes—Fibroblast-like progenitor cells.** The peripheral blood circulation contains a distinct population of bone marrow-derived fibroblast-like progenitor cells, referred to as fibrocytes, that retain the plasticity to differentiate along the mesenchymal lineages. Fibrocytes stimulated with profibrotic cytokines and growth factors *in vitro* produce large quantities of ECM components and further differentiate into cells identical to contractile myofibroblasts and fibroblasts observed to emerge at healing (94). Fibrocytes compose approximately 0.1–0.5% of circulating nonerythrocytic mononuclear cells. Fibrocytes express the MHC class I and II and costimulatory molecules CD80 and CD86, which allows them to exhibit antigen-presenting activity and activate both the CD4+ and CD8+ T lymphocytes. Therefore, it is hypothesized that undifferentiated circulating fibrocytes may possess monocyte-like characteristics, and that human fibrocytes may be present in the peripheral blood as mononuclear CD14+ precursors. As nonstimulated immature mesenchymal cells, fibrocytes do not produce large amounts of collagenous proteins or other ECM

components until they differentiate into mature myofibroblasts or fibroblasts. Instead, fibrocytes are a vital source of matrix metalloproteinase 9, an ECM-degrading enzyme that facilitates wound remodeling (94). Fibrocytes can differentiate into several mesenchymal lineages: fibroblasts, myofibroblasts, and adipocytes (95), thus having a potential to functionally populate bioengineered scaffolds.

**Fibroblasts and their role in wounds.** Once fibrocytes have completed their maturation from CD14<sup>+</sup> mononuclear cells, differentiation into cells ultrastructurally and phenotypically similar to myofibroblasts and fibroblasts is promoted by stimulation with TGF- $\beta$ . Fibroblasts are mesenchymal cells with a major role in building and remodelling the ECM. About a week after wounding, contraction of the wound takes place. Fibroblasts differentiate into myofibroblasts, which contain actin similar in structure to the actin found in smooth muscle, and function in wound contraction (96). Myofibroblasts attach to one another and the ECM at the wound edges, and pull on the ECM when they contract, thus reducing the wound size. This may be critical in healing of vitally important organs, such as the heart after myocardial infarction (97). The contraction period ends when myofibroblasts commit apoptosis (93,98). Contraction of a wound can last for several weeks and may continue, even after the wound is completely reepithelialized. It can lead to disfigurement and loss of function if excessively prolonged. Tensile strength is enhanced primarily by the degradation of type III collagen and the reorganization and production of type I collagen found in scar tissue. Tensile strength is also increased by the enzyme lysyl-oxidase, which covalently cross-links the collagen molecules. Over several months or more, the tensile strength of scar tissue can only achieve a maximum of about 80% of normal tissue strength (54). Recent findings indicate the involvement of TGF- $\beta$ 3 and PAI-1 in scar contraction (3), and specific fibroblast contractile and migratory properties may underlie scarless wound healing in the fetal period (99).

**Fibroblast identity, regulation, and utilization in tissue engineering.** As a part of the body's architectural memory, the original mesenchymal fibroblasts that normally constitute tissues and organs have very specific gene expression patterns, defining their anatomical position within an axial coordinate system (Fig. 1, level II) (100). On the other hand, fibroblasts immigrate to wounded tissues, similar to immune cells involved in regeneration, carry the developmental memory of a cell type designed to execute a predefined set of functions during wound healing, regardless of the wound location. Unfortunately, there is poor understanding of whether these immigrated fibroblasts permanently carry a bone marrow positional imprinting or whether they will be reprogrammed to match the destination tissues. How this reprogramming could be regulated and how cells from remote locations can reconstruct the architecture of damaged organs remains to be shown in future research. Current data suggest that fibroblasts are subject to a broad and quite nonspecific external regulation and modification that can be used not only to enhance healing (101), but also may result in pathology (102–104). For example, invasive tumors can take over the regulation and start to use fibroblasts for the production of cell-invasion-favorable

ECM, a situation referred to as normal wound healing gone awry (105). Recently, identical stereotyped transcriptional signatures of fibroblasts have been described in wounds and tumors (48). This is a major step forward, and a complete understanding of fibroblast regulation may become a powerful tool for tissue engineers, especially after it has been demonstrated that genetic engineering can revert fibroblasts back to the embryonic stem cell level with all the ESC-related developmental memory (106). Additional current studies support a hypothesis that cells within tissues sense environmental changes and adjust their reactions to the usual *versus* injurious stimuli (107). Differences in the wound healing of burns *versus* mechanical wounds, together with intriguing findings that fibroblast can memorize former exposure to specific biologic and mechanical stimuli, including wound coverage and outside pressure (103,108–111), demonstrate that fibroblasts are effective biosensors that can carry acquired memories over a certain period of time. Therefore, not only the source and age of fibroblasts, but also the methods of handling, culture media used, mechanical irritation such as tension, stretching or centrifugal forces, or 2D- *versus* 3D-culture, may substantially alter the experimental or therapeutic outcomes (66). Interestingly, similar properties have been discovered in mesenchymal stem cells (112). Subsequently, due to a need to repeat the same experiment with just subtle changes in fibroblast treatment and/or handling, the fibroblast plasticity and memory may make many bioengineering studies incredibly long and cumbersome. Precise standardization of all bioengineering procedures and their detailed description in publications emerges as a must in the current tissue engineering field.

**Epithelization.** Epithelial plasticity plays an important role in development, wound healing, and cancer (113–115). Depending on the wound size, epithelialization occurs within hours to days after the injury of skin or mucosal membranes (Fig. 1, level VII). Epithelial cells migrate from the neighborhood to the surface of the granulation tissue. The cells undergo morphologic changes that enable them to detach from the neighboring cells and basement membrane without undergoing apoptosis, thus facilitating migration. This morphologic change includes degradation of desmosomes and hemidesmosomes. Epithelial cells also function in phagocytosis of the scab tissue and foreign matter, and are able to dissolve or dissect the clot, debris, and the ECM utilizing plasminogen activators, collagenases, and matrix metalloproteinases to help degrade the granulation tissue below. The epithelial cells continue to migrate until cells from either side meet in the middle. At this point, contact inhibition stimulates the epithelial cells to revert to their normal morphology and resume cell–cell and cell–matrix interaction. The basal cells continue to divide and proliferate until the normal epithelial structure is attained (46).

**Full-thickness healing of epithelial structures.** Deep wounds in the adult skin severely impair the regenerative capability of hair follicles and skin glands to the extent that they may be completely lost, despite a presence of fully competent stem cells. The latest findings support the idea that these stem cells do not just get a proper developmental signal during natural wound healing, but they may be primed to

recapitulate the skin morphogenesis under artificially induced Wnt signaling stimuli (1). This is in agreement with the latest study demonstrating that such regeneration may occur, although rarely, even without artificial stimulation, triggered *via* the same signaling pathway (116). Altogether, these exciting findings may accelerate development of new treatments for wounds, hair loss and other degenerative skin disorders. From the aspect of tissue memory, keratinocytes seem to be less sensitive to external stimuli than fibroblasts, and a successful fully functional replacement of urethral epithelium with epidermal cells (117) demonstrates that maintaining the body's original architecture is not always necessary for achieving good therapeutic results. Moreover, although certain changes in gene expression may occur in cells taken from individuals with metabolic disorders, recent findings suggest that keratinocytes from diabetic patients may be equally good for wound coverage as normal keratinocytes. This allows for the use of immunologically less problematic autografts for reepithelialization of chronic ulcers in diabetic patients (118). However, it is the denuded matrix of the wound surface that carries the memory of what epithelial type should reside on it, and in wound healing, the matrix determines whether normal epithelial cells would or would not be prone to populate the wound area. Therefore, considerable effort is made in engineering instructive signals into substrates that would enhance epithelialization, using the methods ranging from soaking matrices with growth factors, through chemical matrix surface activation, up to the use of whole organisms to make changes in wounded tissues, such as medicinal maggot application (119,120). Cell memory and sensitivity to mechanical stimuli has been recently used in promising studies of acute and chronic wound epithelialization with the use of ultrasound and shock waves, and are now entering phase III clinical evaluation (121). From a scientific point of view, intravital observation of wound healing processes is currently becoming a seminal source of information on natural cell behavior that could also be used in tissue engineering and assessment of biologic functionality of bioengineered tissues (122–124). The effects of temporary wound coverage, antibacterial protection, and other external conditions on the healing process are also an integral part of current research (125–128).

***Vasculature, blood supply, and oxygen during wound healing.*** Tissue healing increases metabolic turnover in tissues and is highly dependent on nutrition (129), energetic supplies, oxygen (130), and prompt waste removal. For example, fibroblasts, the main cells that deposit granulation tissue, depend on oxygen to proliferate and lay down the new ECM. Too little oxygen may inhibit their growth and deposition of ECM components, and can lead to excessive fibrotic scarring. Thus, healing processes would fail without effective blood and lymph circulation (46,131).

Blood vessels are often interrupted in wounds. Thus, new vessels must grow into the wounded tissue from the neighborhood (Fig. 1, level VIII), and not surprisingly, most active healing processes occur in the front line of revascularization. Endothelial cells are attracted to the wound area by fibronectin found in the fibrin scab and by growth factors released by other cells. Endothelial growth and proliferation is also stim-

ulated by hypoxia and the presence of lactic acid in the wound. In a low-oxygen environment, macrophages and platelets produce angiogenic factors which attract endothelial cells chemotactically, such as macrophage-derived angiogenic factor (93). The IL-8, FGF, and vascular endothelial growth factor secreted by macrophages are also important for angiogenesis. New capillaries bud from existing capillaries and progress forward, binding the fibrin within the wound site. To migrate, endothelial cells need collagenases and plasminogen activator to degrade the clot and part of the ECM. Zinc-dependent metalloproteinases digest basement membranes and ECM to allow for cell proliferation and angiogenesis (54). When macrophages and other growth-factor-producing cells are no longer in a hypoxic, lactic-acid-rich environment, they stop producing angiogenic factors; when tissue is adequately perfused, migration and proliferation of endothelial cells is reduced. Eventually, blood vessels that are no longer needed die by apoptosis (132). All these events demonstrate that “memory” for proper healing does not always need to be something explicitly physical (like an ECM scaffold), but it also can be encoded indirectly, *e.g.*, as the reactivity of cells to external stimuli.

***Practical problems with vasculature in healing, regeneration, and bioengineering.*** A widespread problem encountered in everyday life of the elderly is decreased natural angiogenesis in chronically photodamaged skin, while acute UV irradiation increases angiogenesis (133). Resolving this puzzle seems to be one of the keys to understanding and the treatment of skin aging and photodamage, as well as to development of the best practices for suntanning. In clinical practice, unavoidable vascular damage is often created during actinic, cytostatic or surgical therapy of tumors, and thus, development of gentle and specific tumoricidal therapies like targeted photodynamic approaches may help spare the normal tissue of oncology patients (134). Although in some other oncological strategies there has been a considerable effort made toward inhibition and/or destruction of blood vessels to devitalize tumor tissues (135), the opposite effort is usually exercised in tissue engineering and wound healing research (136,137) to keep regenerating or engineered tissues alive and functional. Three major areas of vessel engineering can be distinguished: i) reconstruction of large vessels using transplants and prostheses, ii) creation or induction of vessels in bioengineered constructs (*i.e.*, imitation of embryonic vasculogenesis), and iii) enhancement of vessel invasion into the engineered material from the neighboring tissues (angiogenesis). Promising approaches, such as novel chemical compounds (138), modified protein matrices (139), genetically engineered stem cells (140), innovative application systems (141), and recombinant biomolecules (142), have demonstrated the potential to enhance angiogenesis and protect against ischemia in exciting experiments with wounded organs, including the skin and heart. However, attempts to engineer blood vessels may face severe difficulties due to a local tissue memory that imprints organ-specific phenotypes to endothelial cells (143). This phenotypic change will be extremely difficult to reproduce in engineered organs (Fig. 1, level VII), because the plasticity, reprogrammability, natural

developmental timing and physiologic consequences of endothelial functional heterogeneity are not fully understood. Recent findings support another intriguing possibility related to phenotypic endothelial variations: Endothelial cells may be involved in scarless wound healing *via* regulation of leukocyte transmigration (144). Overall, vascular problems currently represent a major source of problems in organ engineering. Certain wound types, such as chronic ulcers (145,146), are difficult to revascularize, and alternative oxygen delivery techniques *via* diffusion are a viable temporary solution (147). Because of the complex spatiotemporal dynamics of the vasculature, computer modeling is gaining increasing involvement in planning and assessing vascular replacements and other operations (135,148).

## CONCLUSIONS

Although we have tried to capture a simplified image of the current knowledge of regenerative mechanisms in their broad complexity and mutual interconnections within a living organism, this article does not cover the maintenance of the body integrity at the highest levels such as neurohumoral regulation circuits, psychological influences on tissue physiology, or the concepts of life and its maintenance *per se* (149–151). We have concentrated on mechanisms that are known in molecular detail and have a direct influence on current biotechnologies. Whether it is wound healing or bioengineering, cells always need guidance to create proper structures. It is a goal of bioengineers to construct the best possible tissue scaffolds and to generate or mimic as many natural instructive signals as possible. Giving a complete list of these signals would be a task comparable to explaining the entire body of developmental biology. Thus, this article has rather attempted to provide an overview of the topic. By summarizing known principles of body structure maintenance in a systematic manner, we aim to make it easier to think about the body plan in a categorical and structured fashion. Hierarchization of the key principles of tissue memory is important for making decisions on the dispensability *vs.* indispensability of the individual components used in the fabrication of artificial organs, and may help with troubleshooting when our construct does not function in an expected manner. Furthermore, it is a general expectation that computational biology will bring a substantial insight into systems biology and will help to understand the self-preserving dynamics of body organization. We hope that our article will contribute to establishing a simplified, easily understandable framework effective in communicating the biologic events of wound healing not only among biomedical researchers, but also between biologists and computer scientists.

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