Employing the Biology of Successful Fracture Repair to Heal Critical Size Bone Defects

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Abstract Bone has the natural ability to remodel and repair. Fractures and small noncritical size bone defects undergo regenerative healing via coordinated concurrent development of skeletal and vascular elements in a soft cartilage callus environment. Within this environment bone regeneration recapitulates many of the same cellular and molecular mechanisms that form embryonic bone. Angiogenesis is intimately involved with embryonic bone formation and with both endochondral and intramembranous bone formation in differentiated bone. During bone regeneration osteogenic cells are first associated with vascular tissue in the adjacent periosteal space or the adjacent injured marrow cavity that houses endosteal blood vessels. Critical size bone defects cannot heal without the assistance of therapeutic aids or materials designed to encourage bone regeneration. We discuss the prospects for using synthetic hydrogels in a bioengineering approach to repair critical size bone defects. Hydrogel scaffolds can be designed and fabricated to potentially trigger the same bone morphogenetic cascade that heals bone fractures and noncritical size defects naturally. Lastly, we introduce adult *Xenopus laevis* hind limb as a novel small animal model system for bone regeneration research. *Xenopus*

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hind limbs have been used successfully to screen promising scaffolds designed to heal critical size bone defects.

Abbreviations

BMPs	Bone morphogenetic proteins
Cbfa1	Core binding factor 1
CDMP-1	Cartilage-derived morphogenetic protein-1
CSD	Critical size defect
CXCR-4	Receptor for SDF-1
ECM	Extracellular matrix
FDA	Food and Drug Administration
FGFs	Fibroblast growth factors
GDF-5	Growth/differentiation factor 5
GF-11	Skeletal growth factor
HA	Hydroxyapatite
HDDA	1,6 Hexanediol diacrylate
HIFα	Hypoxia-induced factor alpha
IGF	Insulin-like growth factor
IHH	Indian hedgehog
IL	Interleukin
M-CSF	Macrophage colony stimulating factor
MMP	Metalloproteinase
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
OPG	Osteoprotegerin
PDGF	Platelet-derived growth factor
PTHrP	Parathyroid hormone related peptide
RANKL	Receptor activator of nuclear factor kappa-B ligand
SDF-1	Stromal cell-derived factor-1
SHH	Sonic hedgehog
TCP	Tricalcium phosphate
TGFβ	Transforming growth factor beta
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

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1 Successful Bone Regeneration Resembles Bone Development

Bone is a tissue that can repair by regeneration very effectively. This ability is enhanced by the postnatal characteristic mechanism of bone maintenance, whereby processes of resorption and formation balance continuous remodeling. Although there are distinct aspects of both processes, successful regeneration such as fracture healing occurs by a cellular cascade that resembles skeletal development and regeneration. The regenerative cascade requires coordinated cellular events of cell migration, cell differentiation, and proliferation of multiple cell types (Willie et al. 2010; Mehta et al. 2012). Understanding bone morphogenesis and the regenerative process of fracture repair form the basis for creating therapeutic tools designed on principles of bone tissue engineering.

1.1 Endochondral Bone Development

Skeletal precursor cells aggregate, proliferate, and form a mesenchymal condensation that becomes a temporary cartilaginous template for the future skeletal element (Hall and Miyake 2000). Embryonic long bone formation begins when skeletal precursor mesenchymal cells (MCs) form aggregations associated with capillaries (Fig. 1a). Sonic hedgehog (SHH) and bone morphogenetic proteins (BMPs) are important mediators of the correct location and patterning of undifferentiated skeletal mesenchyme in limb bud. Sox9 is expressed in cells undergoing aggregation before they condense and form cartilage. Sox9 represses Runx2 and β-catenin and regulates collagen type II expression. Core binding factor 1 (Cbfa1) is a transcription factor expressed in condensation as well (Ferguson et al. 1999).

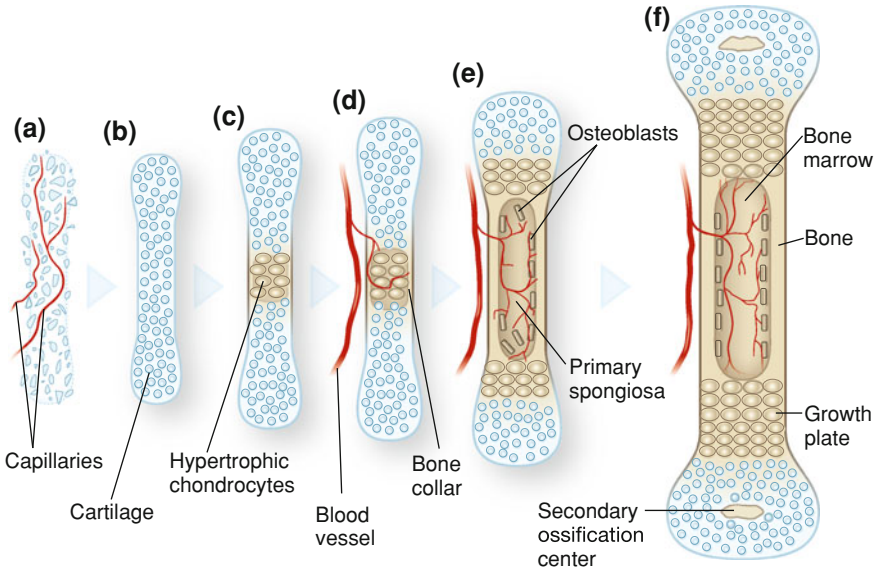

Fig. 1 Schematic of endochondral bone formation. (**a**) Mesenchymal cells aggregate near capillaries and (**b**) differentiate into chondrocytes forming an avascular cartilage model of the future bone. (**c**) At the center of the condensation the chondrocytes cease proliferating and become hypertrophic. (**d**) Perichondral cells adjacent to the hypertrophic chondrocytes differentiate into osteoblasts forming a bone collar. The hypertrophic cartilage regulates formation of mineralized matrix and release of angiogenic factors to attract blood vessels. Eventually hypertrophic chondrocytes undergo apoptosis. (**e**) The coordination of osteoblasts and vascular invasion forms the primary spongiosa. At each end of the diaphysis chondrocytes continue to proliferate with concomitant vascularization resulting in a coordinated process that lengthens the bone. Osteoblasts in the bone collar will form cortical bone, while osteoprogenitor cells in the primary spongiosa will eventually form trabecular bone. (**f**) Secondary ossification centers develop through cycles of chondrocyte hypertrophy, vascular invasion, and osteoblast activity. Columns of proliferating chondrocytes form the growth plate beneath the secondary ossification center. Finally, expansion of stromal and hematopoietic stem cells starts to take place in the marrow space. This figure is based on Kanczler and Oreffo (2008) with permission

MCs in the condensation differentiate into a cartilage model of the future bone (Fig. 1b). Proliferating cells shift from laying down a mesenchymal matrix of collagen types III and I to collagen types II, IX, XI and aggrecan characteristic of the cartilage condensation. Parathyroid hormone-related peptide (PTHrP) stimulates cartilage cell proliferation and represses cartilage differentiation. Cells in the center of the condensation produce Indian hedgehog (IHH), which couples chondrocyte maturation and osteoblast differentiation (Hartmann 2009; Karsenty et al. 2009).

In the central interior of the cartilage model maturing chondrocytes exit the cell cycle, become hypertrophic, the extracellular matrix (ECM) becomes calcified, and the hypertrophic cells eventually undergo apoptosis (Fig. 1c–e). The mature calcified ECM and type X collagen favors vascular endothelial growth factor

(VEGF) dependent vascular invasion from the adjacent blood vessels. Developing bone marrow, the primary spongiosa, is trabecular bone that forms mainly from osteoprogenitors associated with the invading blood vessels. The primary center of ossification is established near the periphery of the cartilage model midway along the length. The bone collar differentiates from external perichondrium cells that express Runx2, the master gene for osteoblast differentiation. Osteoprogenitors resident in the developing bone collar and those associated with invading blood vessels differentiate into osteoblasts and lay down bone matrix. As the collar becomes more vascularized it establishes the first cortical bone and the periosteum of the developing bone (Ferguson et al. 1999; Hartmann 2009; Karsenty et al. 2009). For a comprehensive review of the genetic and molecular control of bone formation see Hartman (2009) and Karsenty et al. (2009).

1.2 Long Bone Regenerative Repair

Bone can heal successfully without forming a fibrous scar. In response to injury a complex series of regeneration promoting cascades is triggered within the resulting fragments of bone. If the bone fragments are positioned optimally, a specific pattern of cellular and molecular events that recapitulates bone development at certain stages during the process occurs across the gap. This secondary or indirect fracture healing typically results in successful regenerative bone repair or healing by formation of endochondral and intramembranous bone. This type of fracture healing occurs under conditions that permit micro-motion and weight bearing (for reviews see Wraighte and Scammell 2007; Jahagirdar and Scammell 2009; Marsell and Einhorn 2011; Stocum 2012).

1.3 Morphology of Fracture Healing Region

Within the fracture healing region of the adjacent bone fragments there are four main spatial zones that contribute specific cellular and molecular components to the regeneration site: the medullary canal, the area between the cortices, the cambium layer of the periosteum, and the surrounding soft tissues. The medullary canal and the inter-cortical areas create the soft callus and go on to create bone by endochondral ossification. The subperiosteal region and immediately surrounding soft tissues create the hard callus and create bone by intramembranous ossification (reviewed by Phillips 2005). Intramembranous ossification is direct bone formation from osteoprogenitor and undifferentiated MCs that reside in the periosteum. Endochondral ossification requires recruitment, proliferation, and differentiation of undifferentiated MCs into cartilage, which later becomes calcified and eventually replaced by bone.

The phases of fracture repair have been described as four or five overlapping progressive temporal phases. The defined steps in bone regeneration consist of overlapping phases of regenerative cellular activity (Kolar et al. 2010; Dwek 2010; Schindeler et al. 2008). These stages of fracture repair are characterized by histological changes within the fracture-healing region and the later stages resemble embryonic bone development at the cellular and molecular level. Many of the same initial developmental cellular activities are present during long bone regeneration and fracture repair (Ferguson et al. 1999; Dwek 2010).

1.4 Inflammation and Hematoma Formation

The inflammatory response begins as soon as the bone break occurs. Complete fractures cause disruption of the bone tissue along with blood vessels and nerves that serve the bone. More extensive breaks can involve tearing of the periosteum and injury to adjacent soft tissues such as skeletal muscle, tendons, as well as blood vessels and nerves that serve the soft tissues. Disrupted blood vessels lead to ischemic necrosis of the affected bone ends.

Upon injury, peripheral blood and intramedullary blood and bone marrow cells fill the fracture-healing site (Fig. 2a). A fibrin clot forms and this initiates the healing cascade with the establishment of the hematoma. The fibrin clot will support the establishment of granulation tissue that serves as a template for a cartilage callus to form between and around the adjacent fracture fragments. Within the fibrin clot platelets degranulate releasing transforming growth factor beta (TGFβ) and platelet-derived growth factor (PDGF). TGFβ stimulates undifferentiated mesenchymal stem cells (MSCs) and PDGF encourages MSC and osteoblast proliferation as well as macrophage chemotaxis (reviewed by Nikolau and Tsiridis 2007; Tsiridis et al. 2007).

The hematoma forms in an environment that is ischemic, hypoxic, low pH, and high potassium and lactate concentrations. These initial conditions are not favorable for cells but some immune cells survive and there are subpopulations of cells that change over time and some support and some impair bone healing (Kolar et al. 2010, 2011; Schmidt-Bleek et al. 2011; Willie et al. 2010). A cascade of cellular and molecular events is triggered by low O_2 concentration in the injury site. Hypoxia-induced factor-α (HIFα), VEGF, and BMPs concentrations are upregulated and endothelial and osteoprogenitor cells will respond to these signals by initiating vascular in growth and initial osteogenesis (Towler 2007, 2008, 2011; Bianco 2011a, b).

The hematoma consists of coagulated circulating blood cells, immune cells, and MCs from the adjacent marrow cavities and periosteum of the bone. The initial blood cells that form the hematoma release cytokines and growth factors that fight infection, degrade the necrotic tissue at the fracture site, form a fibrin clot, stimulate neoangiogenesis and bone formation. Tumor necrosis factor-α (TNF-α) expressed by macrophages and inflammatory cells induces secondary

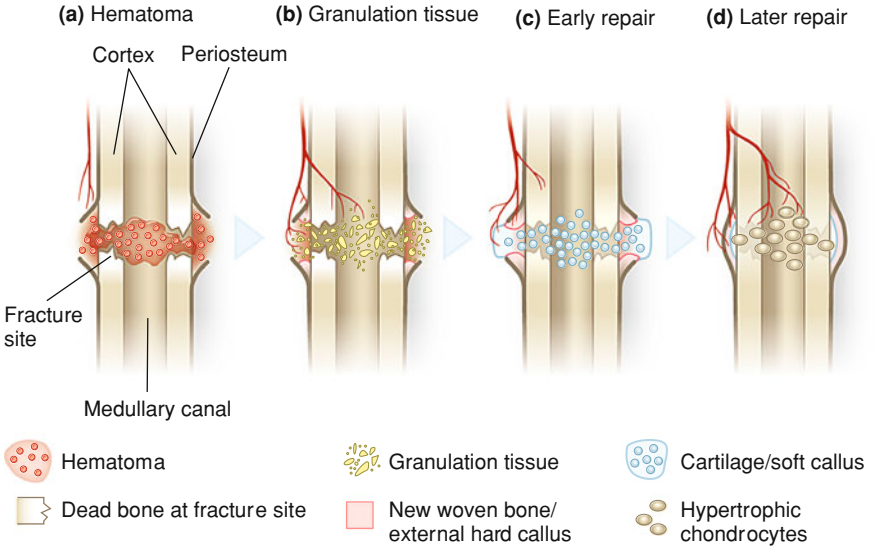

Fig. 2 Schematics of long bone fracture repair. The phases of long bone fracture repair are sequential and overlapping. Each phase may vary in length depending upon the specific bone that has been broken, the location of the break, the extent of injury, and the health status of the patient. (**a**) A hematoma forms in response to injury. Blood cells from peripheral vasculature, bone marrow (medullary canal), bone cortex, periosteum, and the adjacent surrounding soft tissues flow into the fracture site and become entrapped in a fibrin clot. (**b**) Granulation tissue forms in the fracture gap with capillary ingrowth, and inflammatory cells and MSCs migrating to the site. (**c**) A cartilaginous soft callus forms during the early repair phase. The cells that form the soft callus are osteochondral progenitors. The external hard callus is new woven bone that forms from progenitor cells originating from the cambium layer of the periosteum. (**d**) Later in the repair process, cartilage cells mature into hypertrophic chondrocytes that withdraw from the cell cycle, form mineralized cartilage matrix, and eventually undergo apoptosis. Blood vessels grow into the site and new woven bone is established across the fracture gap interior to the external hard callus. Eventually an extensive hard callus of new woven bone joins the fracture segments. The hard callus ultimately remodels to form the lamellar cortical bone and the mature trabecular bone characteristic of the bone marrow.

inflammatory signals, is chemotactic to cells, and induces osteogenesis in MSCs. TNFR1 and TNFR2 are receptors that may be specific to injury and bone regeneration (Marsell and Einhorn 2011). Coagulation activates immune cells in the hematoma as it develops and neovascularization occurs. The hematoma also has fibroblast growth factors (FGFs) released from macrophages, MSCs, chondrocytes, and osteoblasts. These are mitogenic for MSCs, chondrocytes and osteoblasts. Insulin-like growth factor (IGF) released from bone matrix, and secreted by osteoblasts and chondrocytes promotes further proliferation and differentiation of osteoprogenitor cells (Marsell and Einhorn 2011).

1.5 Formation of Granulation Tissue

The hematoma becomes transformed into granulation tissue due to increased capillary ingrowth, immune cells, and fibroblasts (Fig. 2b). Granulation tissue enhances the repair process and withstands interfragmentary deformation. Mononuclear phagocytes, macrophages, and giant cells arrive via the new vessels, remove necrotic bone, and help to build the fibrocartilaginous soft callus. Also present is macrophage colony stimulating factor (M-CSF), interleukins1 and 6 (IL-1 and IL-6), BMPs, TNFα that recruit additional inflammatory cells and attract MSCs originating from the periosteum, bone marrow, peripheral circulation, and surrounding soft tissues. Granulation tissue matures into connective tissue containing collagen types I, II, and III initially, and collagen type III gradually predominates as the granulation tissue matures (Shindeler et al. 2008). Granulation tissue is formed and the wound bed is stabilized by the periosteum (Ozaki et al. 2000). Granulation tissue forms a template and it is replaced by fibrous tissue, fibrocartilage, and then cartilage of the soft callus.

1.6 Periosteum

A bony collar forms in the subperiosteal region adjacent to distal and proximal ends of the fracture by intramembranous bone formation. This healing process is promoted by motion within the fracture site and inhibited by internal rigid fixation. The cambium layer is composed of MCs, differentiated osteoprogenitor cells, osteoblasts, and fibroblasts. A rich peripheral vascular and sympathetic neural network is also present. In addition there are endothelial pericyte cells known to have osteoblastic potential. Upon injury the cambium layer of periosteum adjacent to the fracture site thickens, undergoing a proliferative response called the periosteal reaction. The periosteal reaction produces a mass of cartilage about the fracture site that eventually contributes to the formation of an external callus of woven bone or hard callus as well as the internal soft callus. Macrophages and inflammatory cells and MCs in periosteum secrete pro-inflammatory cytokines. IL-1, IL-6, and TNF-α and these cells induce secondary inflammatory signals. Undifferentiated MSCs release BMPs that induce angiogenesis, chemotaxis, mitogenesis, and cell differentiation into osteoblasts and chondroblasts (Malizos and Papatheodorou 2005; Dwek 2010).

Osteoprogenitor cells already in the subperiosteum are ready to begin intramembranous ossification. Necessary MSCs are recruited, proliferated, and differentiated into osteogenic cells. MSCs come from surrounding soft tissues, bone marrow, and circulating MSCs to the injury site. BMP7 may be important in recruitment (Bais et al. 2009). Stromal cell-derived factor-1 (SDF-1) and the receptor CXCR-4 a G-protein-coupled receptor are important for homing MSCs to fracture site periosteum at the edges of the fracture. SDF-1 recruits CXCR-4

expressing MSCs. FGF is expressed in cells of the expanded cambial layer and it is associated with increase of fibroblast-like MCs that increases callus and bone formation (Marsell and Einhorn 2011). The external hard callus of new woven bone helps stabilize the fracture site during early repair stages through later repair stages (Fig. 2c, d).

In addition to the soft callus of the fracture site, the periosteum also participates in a process resembling endochondral ossification that forms a hard callus by recapitulating fetal skeletogenesis (Dwek 2010). The hyaline cartilage ECM becomes mineralized; osteogenic cells associated with capillaries lay down bone matrix forming a bony collar at the periphery of the callus and regions of woven bone in the core of the hard callus.

1.7 Soft Callus Formation

An internal cartilaginous soft callus forms within the fracture site (Fig. 2c). The sources of proliferating chondrocytes within the soft callus are multiple. Mesenchymal cells (MCs) within the cambium layer of periosteum, endosteum, bone marrow, and adjacent soft tissues differentiate into chondrocytes to produce the semi-rigid, avascular soft callus. Endochondral formation occurs within the granulation tissue between fracture ends and external to periosteal sites. Granulation tissue is replaced by fibrous tissue, fibrocartilage, and then hyaline cartilage. The early soft callus ECM consists of type II collagen and proteoglycan core biomarkers that are gradually replaced with type X collagen as the soft callus matures. Adhesion molecule osteonectin is present as early cartilage forms (Gerstenfeld et al. 2003, 2006).

There are several growth factors that are associated with the establishment and eventual maturation of the soft callus. TGF-β, BMPs, fibroblast growth factor-1 (FGF1), and insulin-like growth factor-II (IGF-II) influence chemotaxis, proliferation, and differentiation of progenitor cells into chondrocytes or osteoblasts. TGF-β2 and TGF-β3, and growth and differentiation factor-5 (GDF-5) are also involved with chondrogenesis and eventual endochondral ossification. The healing cascade may be initiated during the inflammation phase (Kolar et al. 2010). TGF-β2 and TFG-β3 peak during chondrogenesis. TGFs attract MSCs, pre-osteoblasts, chondrocytes, and osteoblasts and they act during chondrogenesis and endochondral bone formation. TGFs might induce synthesis of BMPs.

BMPs regulate growth, differentiation, and apoptosis of osteoblasts and chondroblasts. They induce the developmental cascade for chondro-osteogenesis. BMP2 is especially crucial for initiation of the healing cascade and possibly callus formation. BMP2 may play an important role in inducing osteoblast differentiation; most BMPs can stimulate osteogenesis in mature osteoblasts. BMPs may stimulate synthesis and secretion of IGF and VEGF, or they may directly activate endothelial cells to undergo angiogenesis. BMP7 has been especially effective in stimulating osteogenesis and is already used clinically. GDF-5 and cartilage-

derived morphogenetic protein-1 (CDMP-1) induce endochondral bone growth. Endothelial cells, osteoblasts, and chondrocytes produce IGFs. IGF-11 (skeletal growth factor) acts later during endochondral bone formation. TNF-α initiates chondrocyte apoptosis (Gerstenfeld et al. 2003; 2006).

1.8 Maturation of the Soft Callus

Fracture callus chondrocytes proliferate and mature into hypertrophic chondrocytes (Fig. 2d). Mineralization of soft callus ECM proceeds from the fragment ends toward the center of the fracture site. Chondrocyte mitochondria accumulate calcium phosphate granules that are transported through the cytoplasm and released in the ECM to become seeds for growth of apatite microcrystals. A cascade involving M-CF, RANKL OPG, and TNF-α accomplishes this. Mineralization and resorption of the cartilaginous callus is a recapitulation of embryological bone development. Eventually the hypertrophic chondrocytes undergo apoptosis. Hypertrophic chondrocytes secrete VEGF, a factor that is central to neovascularization during endochondral bone formation (Carlevaro et al. 2000). In addition, VEGF is a key player in promoting both angiogenesis and osteogenesis during fracture repair (Street et al. 2002; Keramis et al. 2008). VEGF acts synergistically with BMP4 to improve bone regeneration (Peng et al. 2002; Li et al. 2009; Feng et al. 2011).

1.9 Hard Callus Formation and Primary Bone Formation

This is an active period of osteogenesis and the formation of mineralized bone matrix proceeds from the periphery toward the center of the fracture site. Primary bone formation at the fracture site resembles endochondral ossification. Matrix metalloproteinases 9 and 13 (MMP-9, MMP-13) are responsible for ECM degradation during the soft callus remodeling. These are expressed in osteoclasts and osteoblasts and mature hypertrophic chondrocytes. In addition, MMPs are expressed in vascular endothelial cells and perivascular cells associated with angiogenesis during endochondral bone repair. Resorption of mineralized cartilage takes place and a cascade involving M-CF, RANKL OPG, and TNF-α accomplishes this. M-CSF, RANKL, and OPG help to recruit bone cells and osteoclasts to form woven bone. TNF-α initiates chondrocyte apoptosis. Eventually the calcified cartilage is replaced with woven bone and the fracture site becomes more solid and mechanically stable. There is no more cell proliferation (Shindeler et al. 2008).

The external hard callus derived from the periosteum and internal soft callus together are called the bridging callus. This structure increases the strength and stiffness within the fracture gap and allows formation of lamellar or secondary bone. At the end of the repair phase the injured bone has regained enough strength and rigidity to allow low impact exercise.

1.10 Bone Remodeling and Secondary Bone Formation

Woven bone from either intramembranous or endochondral ossification is replaced by lamellar bone, which will form either cortical or trabecular bone depending on the location. This process can continue for years after successful union of the fracture segments. The original external bony hard callus and the internal hard callus are gradually remodeled to integrate with the adjacent bone segments. If the callus is formed across a relatively small size gap, lamellar bone first forms perpendicular to the adjacent bone segment then it is remodeled to the correct orientation. If the callus is formed across a large gap woven bone is formed first then transformed into lamellar bone with the correct orientation to the adjacent bone segments (Shindeler et al. 2008).

There are several key features of bone formation that are associated with bone regeneration as well. First, bone formation is always intimately associated with neoangiogenesis or vasculogenesis. Another example of the intimate association of bone formation with blood vessels is in ectopically induced bone in tissues or organs that would not normally be associated with bone (Ripamonti et al. 2006, 2010). Second, within both the developing bone and the regenerating fracture, osteogenic cells associated with the external periosteal region form a bony collar that becomes the first cortical bone in the developing bone and repairs the cortical bone defect during fracture repair. Third, a cartilage model in the developing bone or a cartilaginous soft callus in fracture repair characterizes endochondral bone formation. These cartilaginous tissues are eventually replaced by woven bone that matures mainly into trabecular bone found in the bone marrow (Ferguson et al. 1999; Kanzcler and Oreffo 2008).

2 Critical Size Defect does not Regenerate/Repair

Critical size defect (CSD) injuries in bone by definition are those that do not undergo regenerative healing. A CSD can vary depending on the species, the particular bone, and the location of the defect within the bone. Segmental long bone CSDs can be caused by several injuries such as high-energy trauma, infections and cancerous bone tumors that must be surgically removed, as well as revision surgery (surgery to correct a failed implant or results from a previous surgery). Preexisting patient risk factors such as immune compromise and osteoporosis can affect the surgical outcome resulting in delayed bone healing, cartilaginous nonunions, or infection (Willie et al. 2010).

An important study to examine the biological processes resulting in a lack of healing demonstrates that except for impaired chondrogenesis, no differences in callus tissue distribution could be observed at 2 weeks postoperation between a successful bone healing and a nonunion rat model. Differences become apparent only at 4 weeks postoperation (Kolar et al. 2010). In a second study of delayed

bone healing employing sheep tibial osteotomy model, all the stages of the regenerative response take place except that the hematoma is prolonged with a different spatial distribution of new bone, and there is delayed or prolonged endochondral bone formation present compared to the successful bone regeneration (Kolar et al. 2010, 2011).

3 Traditional and Alternative Therapeutic Approaches to Critical Size Defects

Of the traditional therapeutic methods currently available to treat CSDs, amputation is the treatment of last resort or is the emergency treatment to save the patient's life in limb crush injuries resulting from earthquakes or battlefield explosions. In cases where treatment options are available, the choice is complex and depends on the cause, size, and location of the defect and the experience of the surgeon. Ideally, treatment options should provide three essential characteristics: osteoconductivity, osteoinductivity, and osteogenic potential. Osteoconductive materials promote in-growth of local capillaries and osteoprogenitor cells from the patient to the implant. Osteoinductive materials stimulate the osteoprogenitor cells to form bone directly, while osteogenic material contains cells that can differentiate into osteoblasts and form new bone (reviewed by Willie et al. 2010; Mehta et al. 2012).

Autogenic cortical vascularized bone graft continues to be the gold standard for bone healing and restoration. However, problems can develop at the graft site due to stress fracture, bone resorption, and ultimately nonintegration of the graft. Adverse effects can occur at the site where the bone is harvested, including hematoma formation, bone fracture, infection, and nerve injury resulting in persistent pain. Autografts are restricted in availability and often result in donor site morbidity (Willie et al. 2010; De Long et al. 2007). Allogenic bone grafts from human cadaver sources will eliminate donor site complications, but these bone grafts have higher complications due to reduced revascularization and remodeling, increased bacterial and viral infection, and immune rejection (Willie et al. 2010; De Long et al. 2007).

Alternative therapeutic approaches employ bone graft substitutes that incorporate osteoconductive extracellular matrices, osteoinductive proteins, and often, osteogenic cells. BMP2 and BMP7 have been FDA approved but treatments require supraphysiological dosages to get an effect and outcomes are inconsistent. Platelet-rich plasma treatment is safe and feasible but there is no clinical evidence of benefit (reviewed by Willie et al. 2010).

Grafts of synthetic bone substitute calcium phosphate ceramics such as hydroxyapatite (HA) or tricalcium phosphate (TCP) with the addition of features that enhance the osteogenic and mechanical performance have been used in selected suitable cases. Synthetic bone substitutes must incorporate materials with

structural properties that encourage bioactivity, osteoconductivity, osteoinductivity, or osteogenesis, as well as the necessary mechanical properties or compressive strength (reviewed by Hannink and Arts 2011). A major concern with engineered tissue implants in general and bone implants in particular are that they often do not become vascularized sufficiently to keep the implant alive and become integrated into the host. Furthermore there is often a lack of sufficient vascularity when new bone grows on the bone graft substitute scaffold. Scaffolds that do not fully integrate into the bone fragment can cause delayed healing, future fractures, nonunions, and cartilaginous nonbone unions.

There are currently two approaches to solving the problem of providing sufficient vascularity to bioengineered implants. The first approach is to prevascularize the scaffold prior to implantation. This can be accomplished in vitro by culturing vascular endothelial cells on the scaffold. Alternatively the scaffold can be prevascularized by implanting the prospective scaffold implant in a region of the body that will promote vascularization from the patient and subsequently placing the vascularized implant in the bone defect. The second approach is to apply biomolecules known to attract blood vessel sprouting to the scaffold prior to implantation (Nomi et al. 2002; Rouwkema et al. 2008; Kanczler and Oreffo 2008; Novosel et al. 2011).

4 A Novel Therapeutic Approach

Knowledge of the cellular and molecular mechanisms that lead to successful fracture repair will be vital for establishing effective bioengineered therapies. Postnatal bone formation recapitulates, in part, the same cellular mechanisms that establish embryonic bone. Angiogenesis is intimately associated with both endochondral and intramembranous bone formation. During bone regeneration osteogenic cells are associated with vascular tissue invading from the adjacent periosteal space or the adjacent injured marrow cavity. Through the design of bioengineered scaffold material that supports these cellular mechanisms, novel therapeutic approaches can be fabricated that enhance regenerative repair of bone. Willie et al. (2010) have suggested mechanical signals necessary to initiate successful regeneration: ECM that provides growth factor release and a surface to support inflammation, cell migration and differentiation, establishment of vasculature and soft cartilage callus formation.

There are several materials that could be potentiallyused successfully in a soft tissue therapeutic approach to bone regeneration. One such material is synthetic hydrogel (Fig. 3). Recently hydrogels have been used widely as a three-dimensional (3D) soft scaffold in tissue engineering, since they can be designed and fabricated to be excellent physicochemical mimetics of natural ECMs (Tibbitt and Anseth 2009; Lutolf and Hubbell 2005; DeForest and Anseth 2012). The hydrogels are water-swollen polymer networks and the molecular architecture can result in tissue-like viscoelastic material, diffusive transport, and interstitial flow

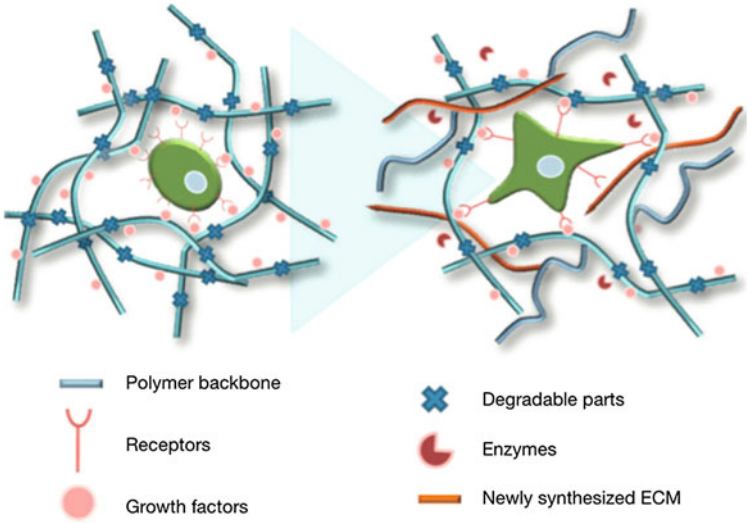

Fig. 3 Depiction of synthetic hydrogels fabricated from polymers conjugated with growth factors that facilitate binding of these factors to cell surface receptors. Hydrogel scaffolds can direct cell behavior through signaling cascades. The gradual degradation of the polymer backbone by either hydrolysis or enzymatic reactions will allow cells to create their own microenvironment and drive regenerative events

characteristics. Despite their distinctive features the hydrogels must meet several criteria to be used for bone tissue engineering. They have to be biologically compatible to minimize adverse inflammatory responses. Biodegradable hydrogels are typically desired such that they degrade, over time, to yield space for new ECM formation. Moreover, it is important to control spatial–temporal presentation of growth factors for the hydrogels to be angiogenic and osteoinductive scaffolds.

A novel approach for enhancing fracture repair and potentially improve regeneration repair of CSDs is to make a scaffold biomimetic of cartilage soft callus stage fracture repair. Various types of hydrogels can be formed from synthetic or naturally derived polymer materials in situ (Chung and Park 2009). Photo-crosslinked hydrogels can be prepared by using vinyl-conjugated monomers in the presence of a light source and initiator. Thermosensitive amphiphilic block copolymers form hydrogels in concentrated aqueous solutions by micelle formation and packing in response to change in temperature. Stereo-complexation or hydrogen bonding interaction between polymer backbones enables gelation. Introducing ionizable groups to copolymers can form pH sensitive hydrogels. Peptide oligomers designed to go through self-assembly are able to form hydrogels. VEGF and BMPs can be entrapped within hydrogel scaffolds. They can also be physically or chemically immobilized on the hydrogels for control of the growth factor release in a predefined manner.

5 A Novel Amphibian Bone Regeneration Research Model

The cellular and molecular mechanisms of long bone development, remodeling, and regeneration in amphibians are highly conserved evolutionarily, and these mechanisms are typical of other vertebrate bone studied (Pritchard and Ruzicka 1950; Hall 2003; Hall and Miyake 2000; Hutchison et al. 2007; Miura et al. 2008; Slack et al. 2008; Song et al. 2010). There has been a renewed interest in *Xenopus laevis* tadpoles and post-metamorphosed adult frogs as model systems for regenerative medicine due to the wide range of micromanipulative surgical procedures and transgenic methods available for studying differential gene expression and gradual loss of function as these animals transition from regeneration capable of larvae to regeneration-incapable adults (Slack et al. 2008; Feng et al. 2011). As described above for mammals, many of the same genes and cellular mechanisms are expressed during amphibian limb embryonic development, epimorphic regeneration of the vertebrate limb, and during long bone fracture repair. Transcription factors SOX9, RUNX2 and OSTERIX and GDF5, and later transcription factor Cbfa-1 and PTHrP and collagen type II are actively expressed in all vertebrate long bone development and regeneration examined so far (Miura et al. 2008; Hutchison et al. 2007).

5.1 Amphibian CSD Models

Amphibians, just as mammals, fail to regenerate CSDs (Goss 1969; Hutchison et al. 2007; Satoh et al. 2010; Feng et al. 2011). In 2007, Hutchinson reported that urodele long bone would not regenerate when the defect was greater than a certain size. Recent studies of CSDs in amphibians have demonstrated that neither larval urodeles (salamanders) nor adult anurans (frogs) can regenerate bone across a CSD without therapeutic aid. CSDs in the cartilaginous radius of juvenile axolotls underwent repair when BMP2 soaked beads were applied to the defect site (Satoh et al. 2010). CSDs in adult *Xenopus laevis* principal tarsus bone healed when 1,6 hexanediol diacrylate (HDDA) scaffolds soaked with BMP4 and VEGF were implanted in the defect (Feng et al. 2011).

5.2 Advantage Amphibians

A potentially fruitful approach to bone regeneration would be to chemically induce the regeneration of damaged tissues or whole complex structures in situ (Stocum 2012). This would involve activation of resident stem cells. Urodele amphibians can naturally reprogram dedifferentiated cells to an earlier mesenchymatous state. Identification of natural molecules that encourage regeneration in situ would

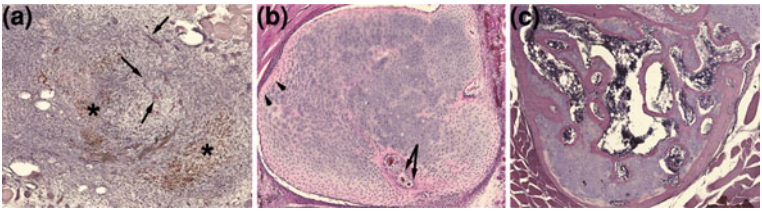

Fig. 4 Histological stages of *Xenopus* CSD bone regeneration. An HDDA scaffold with VEGF/BMP4 was applied to the CSD. (**a**) Hematoma (*asterisks*) with granulation tissue (*arrows*) is present at 2 weeks. (**b**) Cartilage rod starting to ossify (*arrows*) at 8 weeks. (**c**) Extensive woven bone ossification with a lamellar bone outer shell is present at 24 weeks

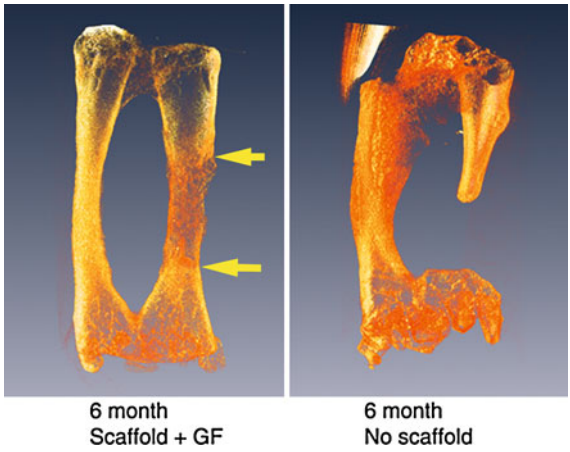

Fig. 5 MicroCT image of *Xenopus* hind limb CSD regeneration. An HDDA scaffold with VEGF/BMP4 was applied to the CSD. After 6 months of regeneration microCT image revealed that extensive woven bone was present in the growth factor treated CSD. *Arrows* indicate the original CSD boundary

permit a more effective search of resources such as combinatorial chemical libraries for synthetic small molecules that trigger the cascades activating dedifferentiation and transdifferentiation (Song et al. 2010). Since both frogs and salamanders can repair fractures like mammals and do not regenerate CSDs, both groups of amphibians could be used as inexpensive and effective screens to examine therapeutic effectiveness in enhancing bone regeneration.

5.3 *Frog Hind Limb as a Novel CSD Small Animal Regeneration Screen*

A study of CSD in the adult *Xenopus laevis* hind limb has demonstrated that successful regenerative repair can be triggered with a single application of VEGF/BMP4 after 6 months (Feng et al. 2011). Additional studies demonstrate that the progressive stages of *Xenopus* CSD healing resemble those of mammalian fracture

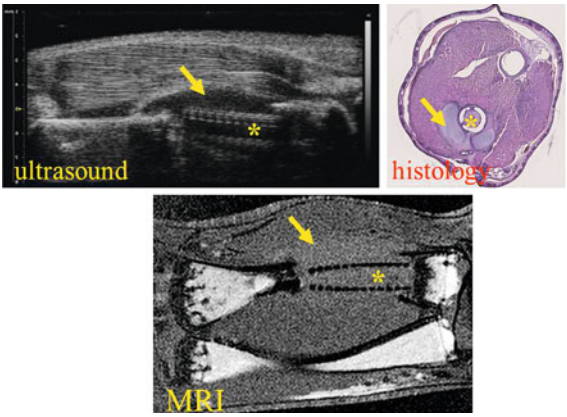

Fig. 6 Noninvasive monitoring of *Xenopus* hind limb CSD regeneration with ultrasound and MRI. An HDDA scaffold with growth factors was applied to the CSD. After 8 weeks of regeneration a cartilage callus (*arrow*) and the HDDA scaffold (*asterisk*) are visible in both ultrasound and MRI images. Histological analysis of the same case at the end of the regeneration period confirmed the presence and location of the scaffold (*asterisk*) and a cartilage callus (*arrow*)

repair (Figs. 4, 5). The progress and potential efficacy of bioengineered therapies designed and fabricated to improve regenerative repair in *Xenopus* CSDs can be monitored and evaluated by noninvasive imaging such as ultrasound and MRI (Fig. 6).

6 Conclusions and Future Directions for Healing CSDs

Fractures undergo endogenous successful regenerative healing that recapitulates many of the same cellular mechanisms and molecular cascades found in embryonic endochondral bone development. Advances in the knowledge of bone development and bone regeneration will permit more effective treatment of bone injures. In addition, vascularization is intimately involved with the initiation of every type of bone formation. Supplying regenerating bone with adequate sustainable vascularization continues to be a concern with all current therapies. Bioengineered therapies that induce and support the regenerative morphogenetic cascade and vascularization offer a potentially valuable approach to healing CSDs. One promising material is synthetic hydrogel, which can be designed and fabricated into scaffolds to trigger and extend the successful morphogenesis of fracture repair to heal CSDs. Theoretically, 3D hydrogels could be designed to support the establishment and function of any regeneration stage: hematoma, granulation tissue, soft callus, or hard callus. Adult *Xenopus laevis* hind limb is a novel small animal model system for bone regeneration research that offers several advantages. *Xenopus* hind limbs have been used successfully to screen promising scaffolds designed to heal critical size bone defects.

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