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Gene Therapy Applications for Fracture Repair

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1. Introduction

Each year more than 15 million fractures are treated in the United States and billions of dollars are spent on the treatment musculoskeletal injuries (Brinker, 2004). Ten to fifteen percent of fractures fail to heal correctly resulting in non-union or delayed union healing (Einhorn 1995; Klamut et al., 2004; Pelled, et al., 2010). The underlying medical conditions, including osteoporosis, contributing to inadequate bone healing during fracture repair are numerous and have been previously reviewed (Komatsu and Warden, 2010; Evans, 2010). With regard to normal or impaired fracture repair, stimulation and/or acceleration of bone healing would yield considerable health and economic benefits. In orthopedics, there is great interest in reducing fracture healing time, increasing the rates of unions or complete regeneration and in being able to treat non-unions more successfully.

One non-traditional approach of accomplishing this is by applying gene therapy to alter the cellular and/or molecular milieu in areas in need of regeneration. Gene therapy could be used to increase the local concentration of osteoinductive molecules at the fracture site thereby stimulating healing at sites of non-union or accelerating union in the case of normal fracture healing. Regenerative genes that replace recombinant osteogenic proteins are attractive alternatives to the costly therapeutics now available in the clinic. Direct gene therapy should be regarded as the most straight forward approach to fracture repair and this approach will be the main focus of this review.

Gene therapy is a new frontier that incorporates the current scientific understanding of human physiology, cellular and molecular biology, and in some cases virology to develop new and more effective treatments for diseases and disorders. In some cases this involves direct correcting of defective genes and other times it is merely a means to enhance production of selected proteins for a specified period of time such as during the healing of a fractured bone. Although gene therapy was initially conceived to treat often lethal genetic disorders, its applications have expanded to non-lethal conditions such as non-union fractures and will one day be used to expedite normal human healing processes such as fracture repair.

2. Clinical treatment of bone fractures

Traditional approaches to treating fractures have varied according to the subject and their condition. Standard treatment for a simple or "green-stick" fracture is immobilization usually by the application of a cast for approximately 6 weeks. The decision of whether to surgically intervene in bone repair is dictated by developing circumstances. Obviously, one would not intervene in a younger patient without a severe injury or impaired healing. Additionally, in elderly patients, the risk of surgical stress to the subject might outweigh the potential benefits of surgical intervention. Bone repair clinically often involves the "wait and see" approach to healing, during which time physicians hope that the bone heals. Surgical intervention, despite the risks, is often necessary to deal with severe musculoskeletal injuries that accompany major trauma. These injuries often require surgical reduction of the fracture, involve significant soft tissue damage or infection, any of which can alter the surgical approach beyond that normally used for simple fractures.

There are also chronic situations of impaired healing in patients with health conditions in which surgery is risky, or in which it has failed, and that might benefit from novel molecular therapeutic approaches to improve healing. A variety of conditions present potential risks and benefits to fracture therapy, necessitating careful consideration of the need and the type of intervention. The application of gene therapy certainly could avoid some of the stress of the traditional and more invasive approaches such as surgical intervention. Additionally, with proper development, gene therapy could also be used to accelerate fracture repair and return to function in cases generally not deemed in need of surgical intervention.

3. Fracture repair

Fracture healing coordinates several molecular pathways and is initiated by the inflammatory response of the injury. There are generally four recognized stages of fracture repair that involve different processes of cell recruitment, proliferation, differentiation and remodeling (Fig. 1): 1) the infiltration of inflammatory cells to the wound, 2) intramembranous bone formation, 3) cartilage/endochondral bone formation by the cells of the soft callus, and 4) endochondral bone formation and remodeling of the fracture callus (Bolander, 1992; Franceschi, 2005). When a fracture is sustained, an inflammatory response occurs, recruiting granulocytes, polymorphonuclear neutrophils and macrophages that remove debris and microorganisms and release osteoinductive molecules. A hematoma is formed in response to platelet derived growth factor (PDGF), insulin-like growth factors (IGFs), tumor necrosis factor (TNF) and fibroblast growth factor-2 (FGF-2). Various growth factors stimulate periosteal cell proliferation and the differentiation of chondrocytes that compose fracture cartilage that eventually bridges the fracture gap with cartilage. Bone formation and resorption, mediated by osteoblasts and osteoclasts, respectively function to convert the cartilage to a woven, low-density (trabecular-type) bone that is subsequently remodeled to lamellar cortical bone of pre-fracture structural and functional integrity. The underlying physiological and biomechanical conditions that regulate the transition of the cells through the stages of healing affect the rate of fracture repair (Fig. 1).

Fracture repair involves the interplay of the well regulated interdependent processes of chondrogenesis and osteogenesis. These processes have been characterized by gene profiling of the regulated expression of numerous growth factors, growth factor receptors, cytokines and transcription factors (Hadjiargyrou et al., 2002; Li et al., 2005; Rundle et al., 2006; Bais et al., 2009). The effect of growth factors and other regulatory proteins on fracture

repair have also been characterized by observational studies of fracture repair in normal animal subjects compared to those with genetically defined deficiencies, i.e., knockout mice. These approaches have identified candidate genes through protein and gene expression studies *in situ* or global studies in different models of fracture repair (Zhang, et al., 2002; Rundle, et al., 2006; Ota, et al., 2009; Komatsu, et al., 2010). Subsequent development of knockout mice deficient in the expression of the gene(s) has then established the functional significance of their expression during fracture healing, and key molecular pathways have been elucidated. In this way the study of various knockout mouse strains with different gene deficiencies has provided valuable information on molecular regulation of fracture repair. These studies have also revealed two other problems. There is considerable redundancy in the regulation of fracture repair, such that the predicted phenotype is often masked by the expression of genes with redundant functions. Also, these studies have been forced to adapt to the problem that several growth factors that cannot be compensated for are embryonic or perinatal lethal, and very difficult to use in bone repair studies on live animals. The fracture model has therefore been further adapted to use conditional knockout mice that express the gene of interest from a regulatable system, such as tamoxifen or tet-related regulation. In these cases the investment in the development of the mouse strain has to be considered, but these studies have nevertheless proven extremely valuable.

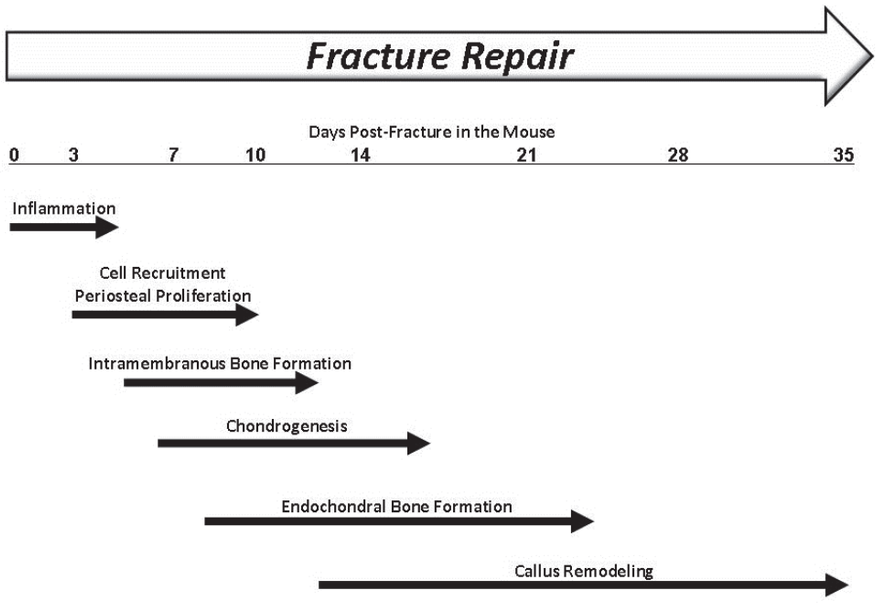

Fig. 1. Fracture repair. The time of appearance and duration of the different processes that regulate the stages of fracture callus development are shown. Bony union of the fracture callus in the mouse and the rat fracture models occurs during the fourth week of healing, and remodeling follows.

4. Bone development

The similarities between bone repair and bone formation extend to intramembranous bone and endochondral bone repair, two different types of bone that heal through the molecular

mechanisms that were functional during their development. This concept is stated as "repair recapitulates development" and has been associated with bone, as it has with other tissues (Gerstenfeld et al., 2003; Bais et al., 2009). It is highly informative to view fracture repair in the context of the cells that mediate the development of the different types of bone during embryogenesis: osteoblast maturation for intramembranous bone repair and chondrocyte maturation and osteoblast maturation for endochondral bone repair. Both intramembranous and endochondral bone formation are observed during embryonic skeletal development and both of these processes of bone formation are recapitulated during fracture repair. As encountered with other tissues during healing, an examination of the molecular pathways of fracture repair has established that bone repair shares several similarities with embryonic bone formation.

4.1 Intramembranous bone formation

The flat bones of the skeleton are formed by membranous bone formation, and include the vertebrae and the bones of the skull vault, mandible, and scapula. In contrast to endochondral bone formation, in areas of intramembranous bone formation mesenchymal cells differentiate directly into osteoblasts without a cartilage intermediate. As the osteoblasts proliferate and differentiate they synthesize the osteoid that eventually mineralizes. During intramembranous bone formation, angiogenesis is essential and bone formation occurs in regions rich in blood vessels. Osteoblast development is promoted through the expression and reaction to the expression of different transcription factors and families of growth factors (Fig. 2).

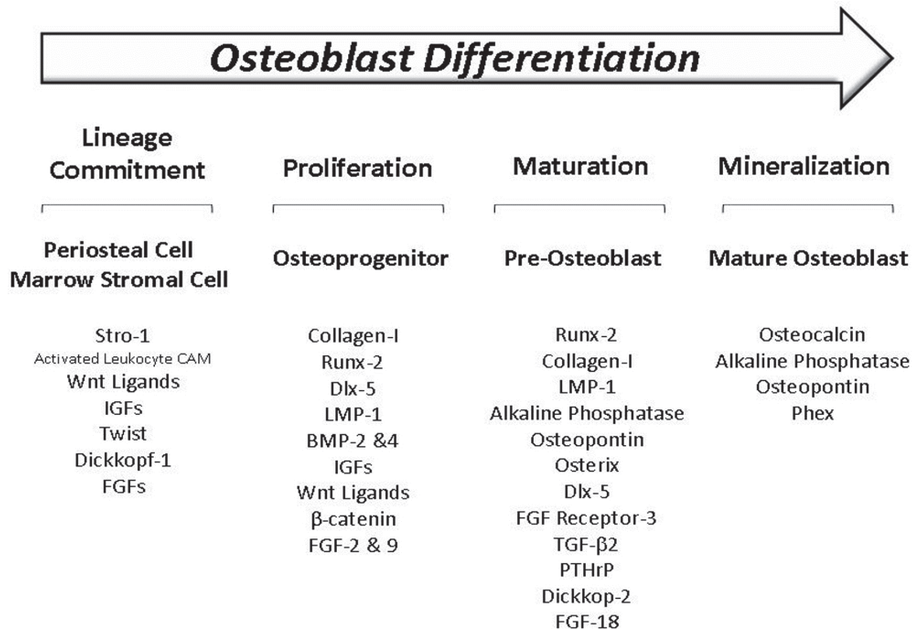

Fig. 2. Osteoblast development. Major transcription factors and growth factors that promote the different stages of osteoblast development are illustrated (adapted from Karsenty, 2009).

Bones of intramembranous origin heal entirely through intramembranous bone formation. Additionally, an endochondral bone fracture that is sufficiently stable can also heal entirely

through a fracture callus formed by intramembranous bone formation, without a cartilage intermediate. Intramembranous bone formation also occurs in "gap" repair during direct bone healing, when woven bone bridges the injury between the ends of the cortical bone without any callus formation; that bone is subsequently remodeled to lamellar bone. This type of bone repair is the principle upon which distraction osteogenesis by the Ilizarov technique is based.

4.2 Endochondral bone formation

The long bones of the limbs, the ribs and vertebrae are all formed through endochondral bone formation. During embryonic development, in areas where the aforementioned bones are created, mesenchymal stem cells (MSCs) concentrate and create regions of high cell density that represent outlines of future bones. As MSCs within long bone structures differentiate, they proceed down the endochondral bone formation path. The cells either differentiate into bone-forming osteoblasts (Fig. 2) or differentiate into cartilage-forming chondrocytes (Fig. 3). This type of bone formation occurs in areas from which blood vessels are reduced or excluded. As endochondral bone formation proceeds, the proliferating chondrocytes in the avascular regions cease to proliferate and hypertrophy. At this point, blood vessels invade the hypertrophic cartilage and osteoblasts form bone around the hypertrophic chondrocytes, resulting in the formation of a primary ossification center. The hypertrophic cartilage is progressively replaced with (trabecular-type) woven bone of the bony fracture callus that is itself eventually remodeled to cortical bone to complete healing.

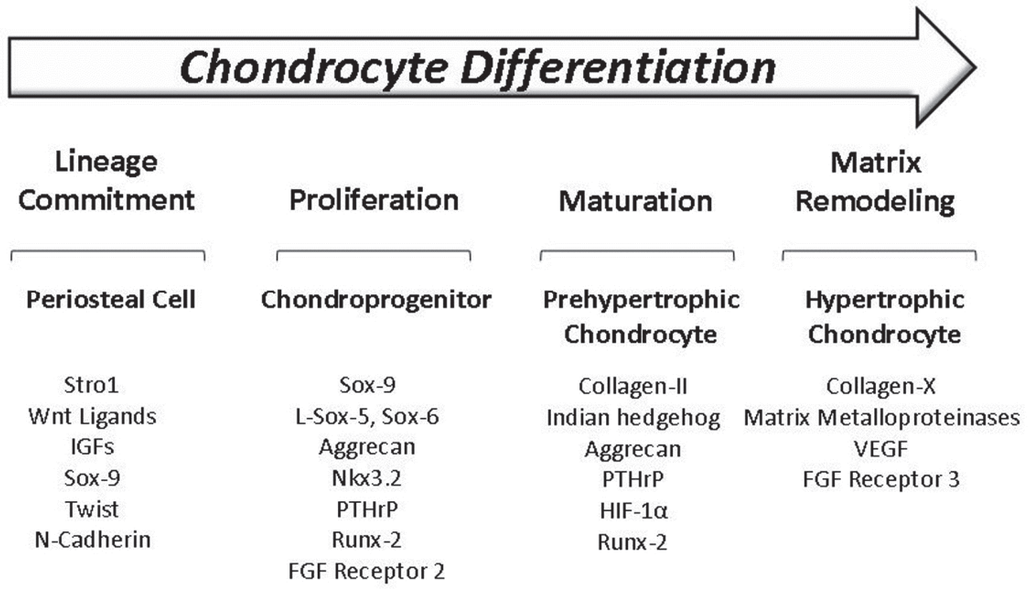

Fig. 3. Chondrocyte development. Major transcription factors and growth factors that promote the different stages of chondrocyte development are illustrated (adapted from de Crombrugghe and Akiyama, 2009; Kronenberg et al., 2009).

Two important tissue repair processes do differ from embryonic development: 1) inflammation and 2) angiogenesis. Inflammation characteristic of fracture repair is completely absent during bone development. Repair studies conducted *in utero* have

introduced inflammation in an embryo and impaired embryonic tissue repair developed to the point where embryonic tissues uncharacteristically formed scars. These characteristics are important considerations for bone therapy, a tissue that does undergo an acute inflammatory phase mediated from the innate immune system that is necessary for fracture repair. However, evidence from T cell and B cell deficient mice has also suggested that even in the absence of an immune response, the adaptive immune system mediates an inflammation that can impede fracture repair, suggesting immune regulation of the pro- and anti-inflammatory cytokines (Toben et al., 2011). Additionally, bone therapy must also consider the sources of chronic or infectious inflammation that can impede healing and produce delayed healing or scarring. Therefore, inflammation offers the chance to regulate and promote an important early stage of healing that is distinct to tissue repair, but one in which poor regulation could ultimately severely impair healing. Studies on knockout mice have demonstrated the requirement for innate inflammatory cells and signaling molecules for efficient bone repair.

Angiogenesis, the development of new blood vessels from existing blood vessels, is characteristic of fracture repair, while vasculogenesis, the *de novo* development of blood vessels, is the mechanism by which the embryonic vasculature develops. Perfusion of the fracture callus vasculature in a rat femur osteotomy model revealed that the invasion of the vasculature in the healing fracture, while similar in structure and progression to the growth plate during development, did seem to display a variable spatial organization different from the linear orientation of the developmental growth plate (Mark et al., 2004). This might be due to the chondrocyte organization affected by the enlarged circumference of the fracture callus relative to the growth plate. Street et al. (2002) demonstrated that locally applied vascular endothelial growth factor (VEGF) promoted angiogenesis and bone healing in two different models of bone repair: mouse femur fractures that heal by endochondral bone formation, and in rabbit tibial unicortical defects that heal by intramembranous bone formation. These results establish that angiogenic factors are important in regulating the different types of bone repair and suggest that repair and development utilize the same growth factor families; it seems likely that they would also act through the same mechanisms. However, the regulation of angiogenesis involves several different families of growth factors, signaling molecules and extracellular matrix enzymes, and intervention to improve angiogenesis probably involves complex regulation that requires a detailed understanding, for dysregulated angiogenesis has been associated with cancer.

5. Development of gene therapy for fracture repair

5.1 Bone healing

For the description of the effects of therapeutic efficacy, of gene therapy, it is first important to agree on the definition of bone healing. The ultimate objective of bone repair is the bridging of the cortical defect with bone and the return to pre-fracture mechanical strength and function. An orthopedic definition of healing concludes that healing is successful when there is bridging of the cortical injury in three of four aspects when viewed by X-ray (the two cortices viewed in the anterior-posterior aspect, and those cortices viewed in the lateral-medial aspect). However, a definition often used to evaluate the healing of *in vivo* models of fracture repair for research purposes simply defines healing as the appearance of a bony callus over the cortical injury, a condition that will remodel to pre-fracture bone absent complicating factors, such as infection. Both definitions describe the same process, although

at different stages. It is critical to develop atherapy that actually promotes bony union, rather than a therapy that simply produces a larger fracture callus. It is therefore necessary to evaluate the characteristics of the healing fracture to identify the target tissues or the particular defect, engineer the vehicle and select the transgene that provides the best opportunity for timely expression in the appropriate fracture tissues.

5.2 Source of cells

There has been considerable effort dedicated to identifying the source of the cells that contribute to the fracture callus tissues, and some debate as to whether the cells required for fracture callus development are derived from the circulation, as would be expected for stem cells, or whether they are derived from cells resident in the periosteum and bone marrow. The studies of Meyers et al. (2010) support mesenchymal stem cell (MSC) homing to the fracture site mediated by CXCR4 expression and that these cells contribute to healing. On the other hand, the experiments of Colnot et al. (2006, 2009) seem to indicate that the bone formation capacity of the fracture callus originates locally and support the periosteum as the primary source of cells for bone and cartilage formation in the fracture callus. Through bone marrow transplant studies, this work determined that the bone formation cells were derived from the recipient and not from the donor through the circulation; the inflammatory and osteoclast populations were derived from the donor through the circulation, which is in agreement with the hematopoietic origin of these lineages. Other studies demonstrated that the periosteum responded to chondrogenic stimulation by BMP-2 (Yu et al., 2010). Finally, these studies have established that while the endosteum has osteogenic potential, the periosteum possesses chondrogenic and osteogenic potential, evidence that bone formation potential is local in origin. Other populations of cells that are not derived from the periosteum are also required for fracture repair, most notably the inflammatory cells and osteoclasts that are derived from the hematopoietic cell repertoire and arrive at the fracture site through the bone marrow or the reforming periosteal circulation. This conclusion argues against developing a gene therapy with circulating multipotent stem cells as the primary source of cells for the repair of bone tissue. Whatever the defect, it is critical to identify and target the cell populations and the particular function that might be deficient.

If the periosteum is the tissue targeted for gene therapy in the clinic, the delivery approach and the vector/transgene combination must account for the morphology and the cellular composition of the periosteum during fracture healing. Immediately post-fracture, the most obvious time to use gene therapy to intervene to improve fracture repair, the periosteum is only a few layers of fibroblast and pre-osteoblast cells in thickness, making accurate therapeutic gene delivery possible but vector retention very difficult. Additionally, the periosteum is usually disrupted by fracture, and a significant interruption in the periosteum can impede the therapeutic application, as well as more significantly impair normal fracture repair. While the fracture periosteum thickens very rapidly in the initial stages of fracture repair, the therapy must be applied to dividing cells to disperse the therapy in the callus that forms. This problem is addressed by using a targeting system that can best transfect the resident cells present in the tissue at that time, such as a Murine Leukemia Virus (MLV)-based vector or an electroporated non-viral vector that would transduce or transfect the cells that proliferate in the first few days post-fracture (Iwaki et al., 1997). The therapy can then be propagated throughout the fracture tissues as the host cells proliferate (Rundle et al., 2003). The choice of vector for a particular stage of healing is therefore very important.

Cells derived from the circulation are commonly thought to be MSC in origin or function, are attracted to the fracture site and subsequently proliferate to become soft tissue. Another concern with stem cells is that the probability of transformation to a cancerous state appears to be more characteristic than with other types of cells. Stem cells have great potential for fracture therapy, but their contributions to the fracture tissues and their responses to manipulations as therapeutic vehicles must be carefully considered.

5.3 Growth factor genes

A considerable effort has been expended in determining the genes that are critical at different stages of repair for the healing process. Gene therapy attempts to intervene with factors that improve the normal healing process. The skeletal gene therapy strategies to date have targeted the osteoblasts, osteogenic progenitors, chrondrogenic progenitors and other cells within the fracture repair region.

To begin to identify appropriate target genes that might be used to accelerate healing, several laboratories subjected mRNA isolated from different aspects of fracture repair to gene profiling procedures to gain insight to the molecular pathways regulating each situation, including the stages of fracture repair (Hadjiargyrou et al., 2002; Li et al., 2005; Rundle et al., 2006) and the expression of genes of current therapeutic interest, such as stem cell genes (Bais et al., 2009). A variety of genes, involved in activation and repression, were discovered to participate in each stage of the repair process and suggest therapeutic candidates for fracture repair, but the first target genes selected for fracture therapy were those that influenced the rate of osteoblast differentiation and thus bone formation (Fig. 2).

5.4 Fracture model

Fracture models have taken different paths for research purposes: 1) genetic models to describe the functional importance of a gene or gene pathway for healing, and 2) procedural manipulations that examine mechanisms of healing or model clinical situations for the evaluation of therapeutic intervention.

Intramembranous bone repair models have modeled bone repair on those bones that undergo intramembranous bone formation, often using a rodent calvarial defect model. In this case, a defect of a critical size that will not heal without intervention is produced in the calvaria and viral or cell-mediated therapy applied on a scaffold (Gysin et al., 2002; Pelled et al., 2010). These models have been successful in promoting the healing of the critical size defect and show great potential for developing therapy to repair skull and facial injuries, or conditions resulting from birth defects or cancer.

Endochondral bone repair models have become the most common model for bone repair, traditionally using rodents. The rat has been the preferred model for pharmacological studies, and the femur fracture model was originally developed in the rat (Bonnarens and Einhorn, 1984). The phases of endochondral bone healing in rodents have been characterized in detail in numerous reviews, but the initial description of endochondral bone healing in the rat (Bolander, 1992) is still the standard of fracture callus development to which impaired healing is compared and by which therapy is evaluated (Fig. 2).

The development of genetically defined (knockout) mouse strains has necessitated adaptation of the rat model to the mouse. There is an obvious problem with the size of the animal model; because the mouse tibia was larger than other limb bones and more accessible for surgery, tibial fracture models were developed first (Hiltunen et al., 1993).

This model is used quite often, but the variable architecture of the tibia makes fracture production and healing more variable, and fracture calluses usually present with some asymmetry. The mouse femur has a much more consistent architecture, and although smaller and very close to the body and therefore more difficult to surgically manipulate, the mouse femur facture model has been used. Fracture repair studies also tend to be driven by the availability of the desired mouse strain and whether it is viable to serve as a subject of bone repair. This problem has necessitated the development of conditional knockout mouse strains as described above. To add to the difficulties of fracture analysis, the genetic background of the mouse strain under study can impact healing, and result in variations in callus size and tissue content that should be considered when analyzing bone development and fracture repair in knockout mice (Jepsen et al., 2008). Even with consistent fractures and bone architecture, differences in fracture repair can be subtle, especially among strains of knockout mice. Traditional approaches using X-ray examination to visualize the fracture callus have been confined to fracture production, or at best preliminary screening of healing. It is necessary to refine analysis techniques to improve the sensitivity. In this respect, micro-CT analysis of fracture repair has become invaluable (Morgan et al., 2009; Bouxsein et al., 2010). The disadvantage here is that the soft tissue callus is excluded from the analysis, so that conclusions on fracture repair are limited to bone formation. However, recent adaptations of the micro-CT have resolved differences in the vasculature of osteopontin knockout mice, a powerful application for evaluating angiogenesis in bone repair (Duvall et al., 2007). Histomorphometry is also used quite frequently to evaluate soft and hard fracture callus tissues with high resolution (Gerstenfeld et al., 2005).

The development of the even more challenging models of fracture repair that one would expect to encounter in a clinical situation has emphasized the study of surgical segmental defects. Recently, investigations have characterized endochondral bone formation during fracture healing in response to delayed stabilization, or when stabilization of the fracture is reduced or delayed (Miclau et al., 2007). Although the periosteum is disrupted during the standard closed fracture model, it is severely interrupted in these models. The periosteum is a critical source of cells for fracture repair; segmental cortical bone graft transplantations from ROSA 26A mice demonstrated that approximately 70% of the recipient graft was derived from the donor, as observed by β-galactosidase staining. Moreover, the donor cell transplants mediated BMP-2 therapy that improved segmental defect vascularization and healing (Zhang et al., 2005). A recent study demonstrated that a periosteal covering was necessary for healing a segmental defect in a "trabecular" metal cylinder implant to the femur diaphysis of the goat. In the absence of a periosteum, the implant lacked the bone ingrowth associated with healing (Bullens et al., 2010). Whether on not the periosteum is the main source of cells for fracture repair, impaired fracture repair with periosteal resection indicates that it is a major mediator of fracture callus development. Other approaches have induced ischemia through femoral artery resection which demonstrated the importance of the circulation to fracture repair (Lu et al., 2007). These models produce significantly delayed or non-union fracture healing. Evaluation of PTH therapy in an open rat femur fracture model revealed that it was not as successful as in the closed fracture model (Tagil et al., 2010), indicating that fracture therapy cannot be extrapolated to all clinical conditions.

5.5 Delivery of therapy to the fracture model

The delivery of gene therapy can be critical for the success of bone repair. Without injury, the periosteum consists of only a few cell layers of osteoblasts and fibroblasts. Its

development into a fracture callus is dramatic. However, at the time of fracture and for a few days thereafter, the periosteum presents a small target for accurate application of the vector and transformation of the resident cells. The two options that exist for the delivery of therapy to a rodent fracture are percutaneous applications to the bone surface, and intramedullary injections. Most of our fracture therapy has utilized percutaneous injections. Injections of a radio-opaque contrast dye soon after fracture (Fig. 4A) established that even small volumes applied percutaneously leak from the periosteal surface into the surrounding tissues (Fig. 4B). The percutaneous application to the lateral aspect of the mouse femur fracture limited transgene expression to that aspect of the fracture callus. Intramedullary injections can distribute the injection around the fracture circumference, but leakage into the supra-periosteal tissues still occurs. The vector can facilitate transformation of the target tissues; in the case of rapidly proliferating periosteal cells in early fracture healing (Iwaki et al, 1997), transgene expression was propagated quite well from the MLV-based vector following percutaneous injection (Roe et al., 1993). The efficiency of delivery might also be compensated for by the transgene; a BMP transgene produced heterotopic bone in the surrounding tissue (Rundle et al., 2003), while a Cyclooxygenase (COX)-2 transgene did not (Rundle et al., 2008). Hence, the choice of vector and transgene can compensate for some of the difficulties in accurate delivery of the therapy and growth factor genes are discussed below with the gene therapy vectors they utilized for gene transduction and expression.

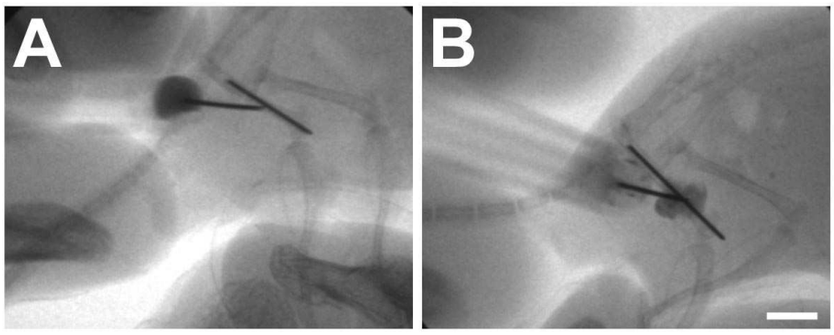

Fig. 4. Fracture therapy delivery by lateral percutaneous injection to the mouse femur fracture site. To illustrate the delivery technique, a radio-opaque contrast dye is injected and tracked by fluoroscopy. The injection needle is placed on the fracture at midshaft, and 25 ul of the dye injected. The intramedullary stabilizing pin of this model is visible perpendicular to the needle. (A) Immediately before injection. (B) Immediately after injection. Scale bar = 5 mm.

6. Vectors for fracture therapy

6.1 Viral vectors

Because there are several significant advantages to viral vectors, they are by far the most extensively studied vector for gene therapy. Viral vectors possess the ability to transduce their DNA into a variety of cells. They are relatively easy and cheap to produce and some will integrate into the host genomic DNA. The two main types of viral vectors used for *in vivo* fracture repair gene therapy are adenoviruses and retroviruses (Klamut et al., 2004; Evans, 2010; Pelled et al., 2010).

Adenoviruses are double-stranded DNA viruses that do not generally insert into the genome of the host cell but remain extrachromosomal. Adenoviruses are highly efficient in that they have a wide host cell range, transduction is followed by rapid transgene expression and they can transduce dividing and non-dividing cells; but these vectors provide only transient gene expression because they do not integrate into genomic DNA. Direct injection of a transgene into the fracture site in small and large animal models, performed percutaneously, was accomplished using adenoviral vectors encoding bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF) and other growth factors (Evans, 2010). Adenovirus directed BMP-2, BMP-4, and BMP-6 have all been reported to stimulate bone formation when directly injected at the fracture site (Baltzer et al., 2000a; Baltzer et al., 2000b; Chen et al., 2002; Bertone et al., 2004). However, in some models adenovirus directed BMP delayed healing (Egermann et al., 2006) and even when enhanced healing is reported, it is often accompanied by ectopic bone formation in the musculature, which is counterproductive. Clinical trials with adenoviral vectors have indicated that these vectors stimulate an immune response and this feature impedes the repair process and opportunities for multiple applications. Thus, adenoviral vectors have not yet been used successfully and are unlikely to be used in the clinic to accelerate fracture repair.

Adeno-associated vectors (AAV) are single stranded-DNA viruses that do not integrate readily into the host-cell genome but are capable of transducing a number of cell types whether they are dividing or not. Problems with producing the virus in large quantities, the small transgene carrying capacity and other technological considerations have limited the use of this vector for gene therapy applications. However, AAVs have been incorporated into Gene Activated Matrices (GAMs) to reduce their immune response and to effectively deliver a gene therapeutic to a bone defect. AAV in a GAM (collagen implant) transduced host cells surrounding the implant and led to remodeling of the allograft and replacement with bone (Koefoed et al., 2005; Evans, 2010).

Retroviruses are RNA viruses and therefore in order to replicate they must integrate into the host DNA. Retroviruses are highly regarded as orthopedic vectors because they possess the ability to transduce osteoblasts, bone marrow stromal cells, chondrocytes, and muscle-derived stem cells. Retroviruses transduce a variety of cells with high efficiency; they are highly stable; they have a large transgene capacity (i.e., more than 8kb of genomic information can be incorporated into the cassette), are easy to construct, do not elicit an immune response and expression of the transgene is robust and persistent because of DNA integration. However, there is a risk of insertional mutagenesis which is discussed below. Although retroviruses can only infect dividing cells (Roe et al., 1993), this disadvantage can make them efficient vectors for expressing transgenes during wound repair (Fig. 5).

Certain growth factors or signaling molecules that act early in the normal fracture repair process (BMP-4, COX-2, LMP-1) stimulate bone formation in small animal fracture models using retroviral gene therapy (Rundle et al., 2003; Rundle et al., 2008; Strohbach et al., 2008). Studies with single growth factor gene therapy with retroviral vectors in closed fractures in the rat femur fracture model demonstrated that retroviral-based gene therapy produced specific effects on fracture healing, and was even capable of improving it. A MLV-based vector expressing a hybrid BMP-2/4 gene that increased BMP-4 secretion, and therefore osteogenesis, was the first retroviral-based gene therapy studied by our group. Post-fracture administration of the MLV-BMP-4 vector to the rat femur fracture periosteum produced a dramatic enhancement of hard callus bone formation when compared to controls, with results somewhat similar to the BMP-2 protein administrations in a similar study (Einhorn

et al., 2003). No evidence of viral vector expression in extraskeletal tissues was detected, suggesting that transfection was limited to the injury. MLV-based retroviral FGF-2 vectors were also tested for enhancement of fracture repair. FGF-2 has been proposed to function through cell proliferation, and recombinant FGF therapy had demonstrated increased callus size (Nakajima et al., 2001). FGF-2 gene therapy stimulated proliferation of periosteal and adjacent cells of the soft callus but did not lead to the development of hard callus or accelerate bony bridging (unpublished data). Therefore, FGF alone is not an ideal therapeutic candidate, but FGF could potentially be used in a combination therapy to enhance the early formation of cartilage callus if combined with a transgene that would enhance the subsequent ossification of the cartilage callus. Studies introducing single growth factor genes into a fracture site have generally shown an augmentation of hard and soft callus formation without improved bony union of bone tissues at the fracture gap, as determined by X-ray examination.

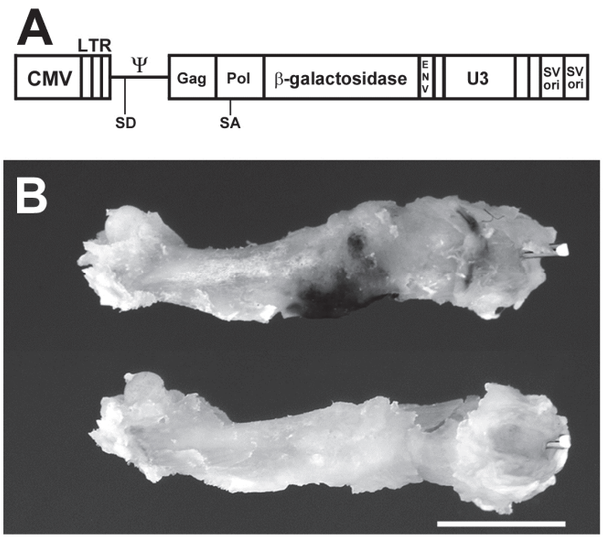

Fig. 5. Retroviral vector transgene expression in fracture tissues. The MLV-based vector construct expressed a β-galactosidase transgene. Femur fractures were harvested at 7 days post-fracture and stained for β-galactosidase expression. Injections were performed at one day post-fracture as described in Fig. 4. Staining revealed β-galactosidase transgene expression on the lateral aspect of the fracture callus (dark spot, upper bone). The lower fracture received a negative control gene expressed from the same vector and injected in the same manner. Scale bar = 5 mm.

Retroviral-based COX-2 gene therapy for fracture repair did differ from other transgene therapies. COX-2 is the rate limiting enzyme in the synthesis of prostaglandins during tissue repair. The COX-2 product Prostaglandin-E_2 induces the expression of multiple osteogenic growth factors, stimulates osteogenesis and supports cortical and trabecular bone formation, angiogenesis, and reduces apoptosis. COX-2 expression increases the osteoblastic potential of MSCs and supports osteoblast differentiation. COX-2 deficient mice also demonstrated that COX-2 was essential for the fracture repair process (Simon et al., 2002; Zhang et al., 2002). To prepare a retroviral gene therapeutic, the COX-2 gene was manipulated to remove sequences that destabilize mRNA, AU-rich elements (AREs) in the 3'translated region, to

increase mRNA half-life. In addition, the Kozak sequence was modified to improve translation. The COX-2 expressing retrovirus was injected 24 hours after fracture to target proliferating cells at the fracture site to maximize the number of cells that were transduced within the fracture tissue. COX-2 expression did not produce inflammation at the fracture site, nor were systemic effects of prostaglandin production observed. The COX-2 gene was expressed in osteoblasts and other cells at the fracture site. Unlike BMP-2 and FGF-2 that were expressed from retroviral vectors, application of the COX-2 retroviral vector accelerated bony union of the callus tissues and hence improved healing of the fractured bone, possibly through angiogenic mechanisms (Rundle et al., 2008). Similar results were observed in PTH treatments in the rat femur fracture, although unlike COX-2 gene therapy, this approach required repeated applications of PTH (Alkhiary et al., 2005).

Lim Mineralizing Protein (LMP)-1 was also examined for its therapeutic efficacy. LMP-1 is an osteogenic factor that is a part of the BMP pathway and is a critical regulator of osteoblast differentiation. It is especially novel because it is an intracellular factor, unlike most transgenes utilized in gene therapy which are secreted soluble factors. Therefore, the effects of LMP-1 gene therapy are limited to the cells that are transduced by the retrovirus, an important consideration if the target cells of the host tissue are limited. LMP-1 affects osteoblast differentiation by binding to Smurf1, an E3 ligase, preventing it from ubiquitinating BMP-activated Smads and Runx2 and targeting them for proteosomal degradation (Boden et al., 1998; Sangadala et al., 2006). The LMP-1 retroviral gene therapeutic contained the human LMP-1 coding region and an optimized Kozak sequence which was ligated into the 6.8-kb CSLA retroviral backbone. LMP-1 expressing retrovirus (Fig. 6A) was injected 24 hours post-fracture. Immunohistochemistry of the fracture callus at 21 days revealed LMP-1 expression in chondroblasts and osteoblasts. Radiographs demonstrated bridging of the fracture callus at 21 days in LMP-1 treated animals and not in control animals (Fig. 6B). Additionally, the bone mineral content at the fracture site was increased in LMP-1 treated animals and these findings demonstrated that injection of the LMP-1 retroviral vector enhanced fracture healing (Strohbach et al., 2008).

Direct injection of MLV-based vectors have been used extensively in gene therapy studies including those described above, and are generally successful (Evans, 2010). However, there is a risk of insertional mutagenesis associated with each type of retrovirus (Mitchell, et al., 2004; Lewinski et al., 2006). Such mutagenesis has been conclusively demonstrated in MLV-based gene therapy for the Interleukin-2 receptor gamma chain deficiency in Severe Combined Immunodeficiency (SCID)-X1 (Howe et al., 2008) where transduction of cells led to the development of a clonal T cell acute lymphocytic leukemia. The leukemia was caused by retroviral integration near to proto-oncogenes, although other genetic abnormalities unrelated to the vector also contributed. The majority of gene therapy applications with lentiviral vectors have been *ex vivo*, because of this vector's association with the human immunodeficiency virus. Repair of bone defects treated with syngeneic rat bone marrow cells transduced with lentiviral vectors expressing BMPs have been compared to repair with cells transduced with adenoviral vectors (Hsu, et al., 2007; Miyazaki, et al., 2008; Virk, et al., 2008). Prolonged therapeutic BMP expression provided with the lentiviral vector in a critical sized femoral defect model provided superior repair associated with regional gene therapy than that observed with adenoviral vectors (Hsu et al., 2007; Virk, et al., 2008). In a spinal fusion model, MSCs expressing BMP after lentiviral transfer induced more abundant bone within the spinal fusion mass then cells transduced with adenovirus. Cells transduced with lentivirus or adenovirus expressing BMP both outperformed recombinant BMP protein

therapy (Miyazaki, et al., 2008). Both the MLV-based and the lentiviral-based retroviral vectors have displayed characteristic preferences for integration sites, with the MLV-based vector integration associated with transcription start regions and lentiviral vector integration associated with sites of active gene transcription (Mitchell et al., 2004). Studies with chimeric viruses demonstrated that these characteristics were determined, at least for MLV, by the integrase and gag proteins (Lewinsky et al., 2006). This tendency toward activation of adjacent genes could also be minimized by the removal of segments of the U3 enhancer sequence of the viral long terminal repeat, although this step necessitated further modification of the vector to maximize its production (Jang et al., 2011).

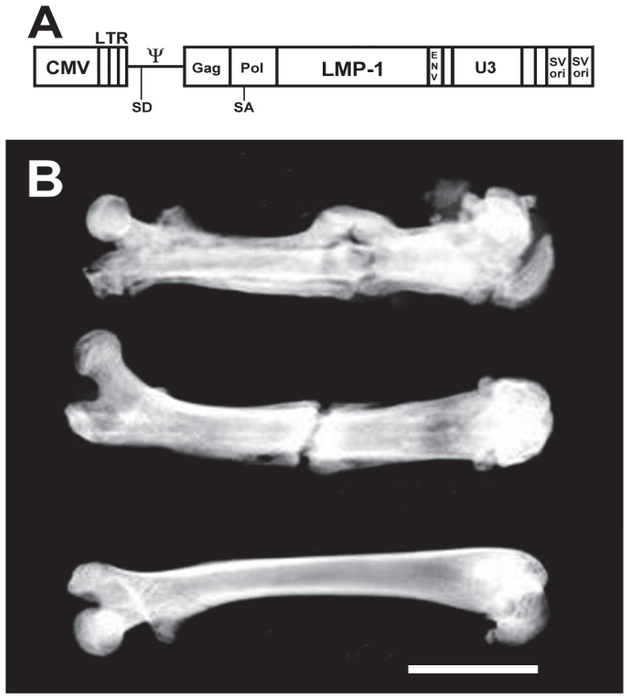

Fig. 6. LMP-1 gene therapy in the rat femur fracture. The MLV-based vector construct expressed a LMP-1 transgene. (B) Percutaneous injections were performed at one day post-fracture to the lateral and medial aspects of the fracture site. X-rays examination at 21 days post-fracture revealed that the LMP-1 transgene promoted bone formation that bridged the fracture gap (top). The control transgene injected in the same manner did not promote bone formation and healing appeared normal (middle). The unfractured contralateral bone (bottom) is presented for comparison. Scale bar = 10 mm.

Obviously, in spite of having the pathologic elements of the genome engineered so that they are absent from the vector, again lentiviral vectors suffer from the association with human immunodeficiency virus (HIV) in public opinion. Given this association, patients might explore other therapeutic options, even for severe or chronic clinical conditions. Because of their efficiency for gene transduction and expression, their ease of use and the number of studies in which they have been utilized, the viral vectors will probably continue to be used mainly for experimental purposes. Problems with viral vector safety could be obviated by the development of efficient non-viral gene therapy vectors for gene therapy in bone (Pountos et al., 2010). Progress towards this goal is discussed below.

6.2 Cell vectors

Using cells as a method of gene therapy can be looked at in two broad categories: use of stem cells or somatic cells. When stem cells were first discovered, there was great hope from the scientific community for the untold potential that these cells possessed. However, with the scientific hope came public concern. These concerns have been somewhat alleviated with the discovery of adult stem cells. Orthopedic gene therapy with adult stem cells has focused on the use of mesenchymal stem cells (MSCs). MSCs can be found in the bone marrow and obtained by a relatively simple bone marrow biopsy. After biopsy the cells can be expanded in culture and then used to treat the same individual that they were taken from. The advantage of this approach is that there is little risk of an immune response as the cells are unaltered and genetically identical to the individual they are being implanted into. However, there are a few fairly obvious disadvantages of this approach. First, a bone marrow biopsy is painful and there is a risk of infection. Second, and more importantly, if the individual from which the cells were taken has a defect in the healing process at the molecular level, use of their bone marrow MSCs (BMSCs) to treat a non-union fracture would not likely provide a benefit. A major limitation for BMSCs in allogeneic applications is, of course, the immune response, which could eliminate the possibility of further applications, including those from donors who share a significant portion of the original donor haplotype, much as has been observed in clinical transfusions.

Delivering osteogenic transgenes or cells with transgenes in a minimally invasive way remains the goal for development of clinical therapeutics. MSCs expressing BMP-2, TGF-β1, LMP-1, IGF-I, growth and differentiation factor (GDF)-5 have been shown to enhance bone, cartilage and tendon repair. Delivering osteogenic transgenes or cells with transgenes in a minimally invasive way remains the goal for development of clinical therapeutics. The use of allogeneic cells or *ex vivo* gene delivery may be attractive with regard to safety but would dramatically increase the cost of the gene therapy. MSC and skin fibroblasts may be able to differentiate into osteoblasts and chondrocytes, but difficulties with the isolation of large quantities of genetically modified cells and with the choice of cell scaffold material to seed genetically modified cells is likely to impede the development of these approaches.

Use of BMSCs for *ex vivo* based gene therapy for fracture and bone repair has been explored and provided some encouraging results in animal models. Transduction of BMSCs with BMPs prior to implantation at a fracture site stimulates bone formation. These cells become incorporated into the endochondral fracture callus (Gamradt et al., 2006). Furthermore, combination therapy of more than one BMP such as BMP-2 and BMP-7 or BMP-4 was shown to produce cooperative interactions that enhance bone regeneration. Transduction of MSCs with BMP-2/7 or BMP-4/7 demonstrated enhanced osteogenesis (Zhao et al, 2005). Likewise, combination therapy of BMP-2 and BMP-7 transduction of cells implanted into a cranial defect enhanced healing (Koh et al., 2008). Combination therapy of VEGF and BMP-6 transduction of osteoprogenitor cells has also shown preliminary promising results for future fracture repair studies (Cui et al., 2010). In addition to BMPs, one study found that cells transduced with IGF-I injected post-fracture resulted in accelerated progression toward complete fracture healing as measured by percent of mineralized callus and mineralized matrix compared to controls (Shen et al., 2002). However, no functional structural testing was done to determine if IGF-I transduced cell application actually restored mechanical integrity more quickly.

A newly explored source of MSCs is from human adipose tissue. Adipose-derived stromal cells (ADSCs) are very similar to BMSCs but ADSCs have the advantage of being more readily accessible and harvesting does not involve a highly painful procedure like a bone

marrow biopsy (Kawai et al., 2009). Preliminary studies of the use of ADSCs to stimulate bone formation and fracture repair are promising. Non-transduced ADSCs transplanted into the fracture site in a rat model enhanced fracture repair when compared to transplanted fibroblasts or placebo (Shoji et al., 2010). ADSCs transduced with adenoviral BMP-2 implanted on a collagen-ceramic carrier inserted into a critical sized femoral defect demonstrated induced bone formation, which was not observed with untransduced ADSCs (Peterson et al., 2005). ADSCs expressing BMP-2 implanted in a critical size defect stimulated bone formation and healing far beyond control treatment (Li et al., 2007).

Additionally, muscle-derived stem cells (MDSCs) have been used in *ex vivo* gene therapy for repair of bone defects. Therapies for bone repair using MDSCs all involve manipulation of the BMP pathway, generally overexpression of BMP-2 or BMP-4 because they are such potent osteogenic factors. Implantation of MDSCs transduced with BMP-2 using either an adenoviral or retroviral vector stimulate bone formation in critical-size defect mouse models (Lee et al., 2001; Lee et al., 2002). Likewise, transduction of MDSCs with BMP-4 as an *ex vivo* cellular therapy to stimulate bone formation was effective in rodent models (Wright et al., 2002; Shen et al., 2004). Furthermore, combination therapy of VEGF and BMP-2 or BMP-4 overexpressing MDSCs accelerates bone healing in a skull defect mouse model (Peng et al., 2005).

Skin fibroblasts, a somatic cell, have also been explored as a potential cellular vector for *ex vivo* mediated gene therapy to enhance bone formation. When transduced with a retroviral vector for Runx-2, skin fibroblasts seeded on collagen scaffolds and implanted into mice demonstrated the capability to mineralize *in vivo* (Phillips and Garcia, 2008). Although easier to obtain than bone marrow cells, the problem of immunological reaction remains if these cells are isolated from allogeneic donors.

Hematopoietic cells offer an additional vehicle for bone therapy. Hematopoietic stem cells have been enriched for Sca-1+ expression and modified using a MLV-based viral vector to express FGF-2. These cells efficiently engrafted into the bone marrow and expressed FGF-2 that produced a dramatic increase in endosteal bone formation (Hall et al., 2007). This study supports a role for hematopoietic cells in gene therapy, at least for the bone marrow. In addition, this approach might be invaluable for prevention of fractures in osteoporotic patients, because their effect of increasing bone mineral density in conditions such as osteoporosis could dramatically reduce the incidence of fractures.

6.3 Non-viral plasmid vectors

Adenoviral, AAV, retroviral, and lentiviral based gene therapies have proven effective in fracture repair, but safety issues inherent with use of these therapeutics in clinical trials have not been resolved. A current focus of gene therapy is the development of non-viral plasmid vectors. There are several reasons for this. First, plasmid vectors do not insert into the genome of the cell into which they are introduced, and there is no risk of insertional mutagenesis. Second, non-viral plasmid vectors are not immunogenic, and there is minimal risk of a host immune response. Third, plasmids are being developed that possess specific expression time-frames and have cellular specificity (i.e., they will only be expressed in restricted target cell populations even if transfected into tissues). Non-viral vectors, such as plasmid vectors and linear DNA, have the potential to provide a much safer form of gene therapy, but they have not been optimized for the clinic to provide therapeutic levels of gene expression for a sufficient time period, at least in tissues other than muscle.

Historically, Gene Activated Matrices (GAMs) were developed with non-viral (plasmid) vectors to heal bone (Bonadio, 1999). The first GAM was a collagen sponge with plasmid

DNA containing the parathyroid hormone (PTH) 1-34 transgene. The strategy was to transfect *in situ* host cells that invaded the collagen sponge that was saturated with plasmid DNA that had been placed in the bone defect. Although shown to be effective in a rodent implant model (Bonadio, 1999; Geiger, et al., 2005), this therapeutic approach has not been further developed for extensive use in the clinic.

Currently, the major disadvantage of non-viral plasmid vectors for gene therapy is low transfection efficiency. The most widely utilized method of non-viral plasmid transfection is lipid-based or other soluble mediators that cause alterations in the cellular membrane allowing for introduction of the plasmid into a cell. More recently, electroporation has been explored in combination with plasmid vectors possessing DNA nuclear entry sequences. Efficient delivery of non-viral plasmids in muscle, cornea, lung, smooth muscle and vasculature (Dean et al., 2003, 2005; Zhou and Dean, 2007) has been achieved. However, expression of transgene from the plasmid vector continues for only 3-7 days, a time frame achieved with adenoviral vectors. With respect to bone repair, electroporation of a BMP-9 expression plasmid into a collagen sponge implant healed a murine radial segmental defect (Kimelman-Bleich et al., 2010). This result is quite promising for the future of non-viral gene therapy in the clinic, although few such studies have been performed to date.

Clinical trials have not been conducted with non-viral agents that stimulate fracture repair, however, phase I and II clinical trials with injections of FGF-1 in the pCOR DNA plasmid-based gene delivery system (called NV1FGF, Soubrier et al., 1999) into muscle provides an effective therapy for peripheral vascular disease and induces therapeutic angiogenesis (Baumgartner, et al., 2008). NV1FGF restored capillary and arteriolar density in animal models of hind limb ischemia. In phase II clinical trials, NV1FGF effectively increased FGF-1 mRNA and circulating protein levels, and reduced the number of amputations and deaths of subjects with critical limb ischemia (Maulik, 2009). These studies provide proof of concept that minimally toxic plasmid vectors are capable of providing therapeutic levels of a type of growth factor (FGF-1) that might also stimulate fracture repair. Because this vector is already undergoing clinical trials and has demonstrated minimal toxicity, it is well positioned to enter clinical trials for bone healing. These studies have served as an impetus to develop non-viral plasmid-based vectors for fracture repair.

There are several barriers to plasmid-based gene therapies in most tissues other than muscle. These include low levels of gene transfer and expression *in vivo* when targeting genes into non-replicating or slowly replicating cells. However, movement of DNA into the nucleus remains the crucial step for gene transfer and expression in non-dividing and slowly dividing cells. Early during fracture repair, periosteal cells and MSCs derived from the marrow proliferate to provide chondrogenic and osteoblastic lineage cells to repair the fracture, and a window of opportunity for application of a viral gene therapeutic was identified. We found that this same rapidly dividing cell population is also an excellent target for non-viral gene therapy if used in conjunction with *in vivo* electroporation. With regard to enhancing vector delivery and therapeutic gene expression, our group and others have focused on modifying plasmid DNA by incorporating a copy of a DNA Targeting Sequence (DTS) from the SV40 enhancer into a plasmid vector to increase nuclear entry of the DNA delivered into the cytoplasm following electroporation (Fig. 7A,B). This method has been shown to increase gene delivery and expression in muscle, blood vessels, lung and cornea tissue *in vivo* (Li, et al., 2001; Dean et al, 2003; Young, et al. 2003; Dean et al., 2005; Zhou and Dean 2007).

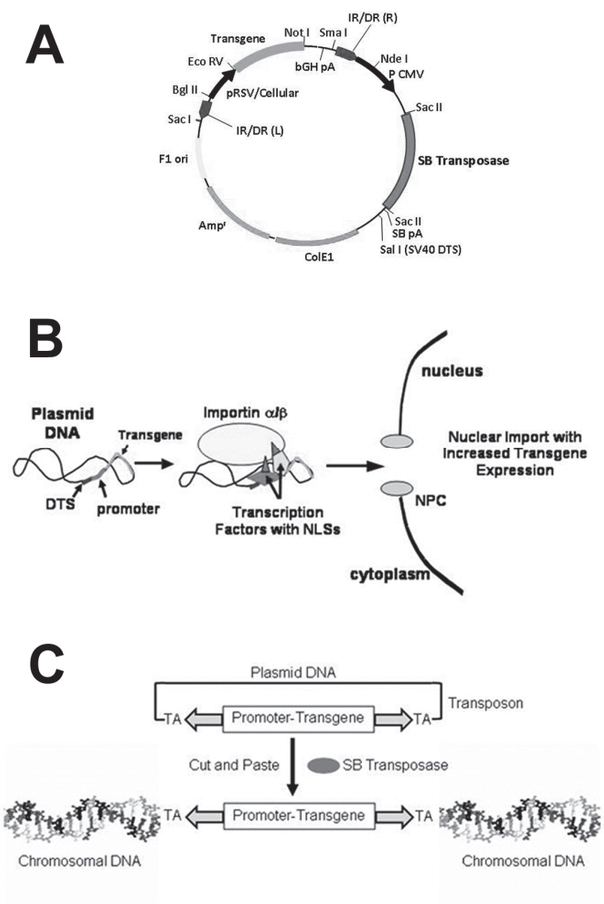

Fig. 7. Development of non-viral plasmid vectors for fracture repair. (A) A non-viral plasmid proposed for fracture repair contains two robust nonspecific viral promoters (RSV and CMV) that drive expression of the transgene and the *Sleeping Beauty* transposase, respectively. The *Sleeping Beauty* transposon (Harris, et al., 2002) stimulates transgene integration and increases the duration of transgene expression. The RSV promoter can be replaced with a cell specific promoter for cell-specific expression (Type I collagen promoter or osteocalcin promoter to target osteoblasts or a Type II collagen promoter to target chondrocytes). A SV40 DTS is inserted in the Sal I site to direct nuclear entry to enhance delivery to the nucleus and expression of the transgene. (B) For nuclear entry, natural transcription factors bind to gene promoter and enhancer regions that are present on the plasmid DNA. The transcription factors have nuclear localization signals (NLSs) that protect the plasmid DNA and facilitate its movement into the nucleus. Translocation to the nucleus requires the NLSs bind to the nuclear import machinery (importin α/β) and move the DNA/protein complex through the nuclear pore complex (NPC) into the nucleus, where it is stably integrated and transcribed. (C) Once plasmid DNA is delivered to the nucleus, transgenes are expressed and the SB transposase is produced. SB transposition of a therapeutic gene and promoter from the plasmid to the chromosome occurs with integration only into genomic regions with TA-base pairs. Insertion of the transgene and promoter extends expression until attenuated by cellular silencing mechanisms that ultimately limit transgene expression.

Lack of tissue specificity also remains a significant safety problem with currently developed non-viral therapies. The SV40 DNA nuclear targeting sequences (DTS) have supported nuclear import of plasmid DNA in a number of cells, but several laboratories have discovered that some promoter sequences undergo cell specific DNA nuclear import (Dean, 2005). The smooth muscle gamma actin (SMGA) promoter (Miller and Dean, 2008; Young, et al., 2003), the endothelial cell flk-1 (receptor) promoter, human surfactant protein C (SP-C) promoter (Degiulio, et al., 2010) and the collagen-Iα2 promoter (Strong, et al., 2006) all undergo nuclear import in restricted cell popultions. Thus, these cell-specific plasmid nuclear import sequences have the potential to provide targeted and enhanced gene expression in specific cells when incorporated into clinically tested nonviral vectors.

To increase the duration of *in vivo* transgene expression from both adenoviral and plasmid vectors that do not integrate into the host genome, the Tcl/mariner family of transposable elements (Izsvak et al., 2010) has been utilized. The Sleeping Beauty element undergoes cut-and paste transposition through a DNA intermediate (Fig. 7C). This process requires Sleeping Beauty transposase (SB) expression and short direct-repeat sequences in the transposon terminal inverted repeats that bracket the transgene and TA rich target regions in chromatin. SB transposase expression allows plasmid-based and adenovirus based transposons to integrate stably into chromatin and provide long-term transgene expression *in vivo* (Yant, et al., 2002). This system has been used to transduce human CD34+ stem/progenitor cells and achieve long-term transgene expression (Sumiyoshi, et al., 2009). Thus, this system can be used to integrate genes into stem/progenitor cells and to maintain pluripotency and could find use in fracture repair. However, insertional mutagenesis of the transgene might also become enough of a safety issue to delay clinical application.

7. Acknowledgements

Our research summarized in this review was funded by a US Congressional Appropriation and an Assistance Award from the United States Army (Award No. DAMD-17-03-2-0021) to the Musculoskeletal Disease Center at the VA Loma Linda Health Care System and by the National Medical Testbed which was funded by cooperative agreement number DAMD-17-97-2-7016. The opinions or assertions contained herein are the views of the authors and should not be assumed to reflect official policy or endorsement by the US Army, US Department of Defense or US Department of Veterans Affairs. All of our work was performed in facilities provided by the Department of Veterans Affairs.

8. Conclusion

Gene therapy for fracture repair has continually evolved over the last decade. Potential gene therapeutics now range from plasmid vectors placed in collagen supports, adenoviral vectors used in supports or in direct injections, direct retrovirus injections, *ex vivo* MSC therapies and electroporation mediated delivery of enhanced plasmid vectors. During this evolution, effective therapeutic genes have been identified based on an increased understanding of regulatory pathways involved during different stages of the fracture repair process. Based on perceived safety issues, non-viral and cell based therapeutics have recently received more attention and will likely be the vector of choice in the future. Recent studies engineering plasmid vectors to overcome problems with efficient delivery to non-dividing cells in tissues, inefficient DNA transport to the nucleus, and to increase cell specificity suggest that gene therapy for fracture repair has a bright future.

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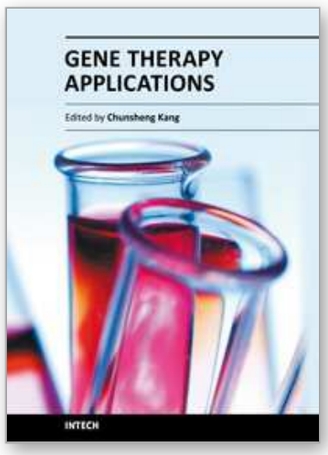

Gene Therapy Applications

Edited by Prof. Chunsheng Kang

ISBN 978-953-307-541-9
Hard cover, 492 pages
Publisher InTech
Published online 23, August, 2011
Published in print edition August, 2011

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