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Review Article

Gene therapy used for tissue engineering applications

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Abstract

This review highlights the advances at the interface between tissue engineering and gene therapy. There are a large number of reports on gene therapy in tissue engineering, and these cover a huge range of different engineered tissues, different vectors, scaffolds and methodology. The review considers separately in-vitro and in-vivo gene transfer methods. The in-vivo gene transfer method is described first, using either viral or non-viral vectors to repair various tissues with and without the use of scaffolds. The use of a scaffold can overcome some of the challenges associated with delivery by direct injection. The ex-vivo method is described in the second half of the review. Attempts have been made to use this therapy for bone, cartilage, wound, urothelial, nerve tissue regeneration and for treating diabetes using viral or non-viral vectors. Again porous polymers can be used as scaffolds for cell transplantation. There are as yet few comparisons between these many different variables to show which is the best for any particular application. With few exceptions, all of the results were positive in showing some gene expression and some consequent effect on tissue growth and remodelling. Some of the principal advantages and disadvantages of various methods are discussed.

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Introduction

The field of tissue engineering is maturing to a stage where a range of functional tissues can be manufactured or regenerated in the laboratory or the patient. In-vivo tissue engineering, in which regeneration is stimulated within the organism by scaffolds, cells or drugs, has reached the clinic (Sandhu 2004; Ruano-Ravina & Diaz 2006) or clinical trials (Amado et al 2005; Schmoekel et al 2005). In-vitro tissue engineering, in which tissue formation is promoted within a bioreactor, has generated clinical products (Marston 2004) and breakthroughs in the formation of complex tissue structures are occurring regularly (Levenberg et al 2005; McCloskey et al 2005).

The use of cytokines and growth factors to biochemically induce regeneration is a widespread strategy in in-vivo and in-vitro tissue engineering (Whitaker et al 2001). Growth factors (or combination of factors) can be used for in-vivo applications either when there is a shortage of the required factor(s) or for inducing tissue or differentiation of stem cells. A shortage may be caused by the site of intended regeneration naturally lacking the factor (e.g. spinal fusion), underlying disease, age or the size of defect. For in-vitro applications it is essential to mimic the regenerative niche by adding factors that would stimulate regeneration in-vivo. The use of protein therapeutics to provide growth factor signals has proven successful in spinal fusion and wound healing. Growth factors for skin and wound healing have the potential to reduce scarring and accelerate healing directed by cells present in neighbouring tissues (Chen & Mooney 2003). However, direct protein delivery has a number of disadvantages including limited protein stability, even when encapsulated into a polymeric delivery vehicle, cost of delivering significant quantities of proteins and short half-lives of proteins. When intravenously injected, the biological half-lives of platelet-derived growth factor (PDGF), basic fibroblast growth factor and vascular endothelial growth factor (VEGF) are 2, 3 and 50 min, respectively (Chen & Mooney 2003). An alternative strategy is to use gene therapy to trigger intrinsic production of factors by the cells of the tissue. Gene therapy is typically used to treat diseases involving deficient or mutated proteins by delivering genes that encode intact proteins to target cells. The small amounts of DNA needed to trigger protein production are technically easier to produce, result in protein production within the target cells and in the long-term offer the possibility of extended

expression and the potential for greater control over amounts of protein expressed.

The challenge of gene therapy is to target the right gene to the right location in the right cells and express it sufficiently at the right time while minimizing any adverse reactions. In addition to the technical challenges of gene therapy, there are specific needs posed by tissue engineering. It is difficult to achieve a sustained long-term expression of the therapeutic gene, while a short controlled expression is desirable and often sufficient to accelerate tissue repair, healing of cartilage defects and bone, repair of tendon and ligaments, and nerve regeneration. The adaptation of gene therapy to tissue engineering to provide this sufficient and controlled expression is thus a key aspect in this field.

This review highlights advances at the interface between tissue engineering and gene therapy. The review is divided into two main sections relating to the strategy used, either in-vivo or ex-vivo gene transfer. Within each of these sections different vectors and tissues are examined with and without the use of scaffolds.

In-vivo gene transfer

The in-vivo strategy for gene delivery consists of the injection or implantation of genetic material directly into the host. In-vivo gene therapy targets the cells or tissue locally at the injection site. This approach minimizes the risk of contamination since there is only one procedure required for the patient. It is, however, extremely difficult to transfect cells in-vivo resulting in low levels of protein expression and, critically, it is difficult to avoid expression of the growth factor in secondary tissues. To overcome some of the challenges associated with delivery by direct injection, which include clearance from the delivery site and degradation or inactivation of the vector, scaffolds can be employed together with the bioactive signal. The use of direct injection is considered first.

Without scaffold

Viral approach Vectors for gene delivery can be classed as viral or non-viral (reviewed in Partridge & Oreffo 2004). Viruses have evolved over long periods of time to introduce their genetic material into host cells, hence virus-based vectors have been a popular choice in many gene therapy experiments. Table 1 provides a list of the most common viral vectors and summary of their main features. Direct adenoviral gene transfer has been used to enhance bone formation and to reduce thrombus formation. Adenovirus systems are designed to transiently and efficiently express therapeutic genes. There is, however, a high risk of inflammatory reaction to adenoviral vectors leading to serious adverse events and consequently limiting repeat administration.

Bone, cartilage and tendon repair The osteoinductive factors of choice for bone and cartilage repair are the bone morphogenetic proteins (BMPs) (Urist 1965). Over 30 distinct forms of the BMPs exist although the most widely studied are BMP-1 through 7. BMP-2 and -4 are highly homologous, differing only in their amino terminal region. Larger proteins include BMP-5, -6 and -7 (also called osteogenic protein-1, OP-1) and BMP-8 (OP-2) (Wozney 2002). BMP-3 may be a negative regulator of bone formation and share less homology to the other members (Daluiski et al 2001). BMP-2, -4 and -7 are considered to be the most important for bone healing. BMP-13, also known as growth and differentiation factor (GDF) 6 or cartilage-derived morphogenetic protein (CDMP) 2, has been shown to induce the formation of tendon and ligament tissues in animal experiments (Helm et al 2001).

Musgrave et al (1999) used a replication defective adenoviral vector to carry the recombinant human BMP-2 gene (Ad.BMP-2). This was injected into the thigh muscles of both immunodeficient and immunocompetent mice. Bone formation was noted, but the amount of bone formed was significantly less in immunocompetent animals. Adenoviral vectors induce an immune response limiting the effectiveness of the gene expression. The extent of immune response depends on the route of administration; intra-osseous administration seems to be more effective as shown by Baltzer et al (2000).

Immunodeficient animals were used in a study by Jane et al (2002). The calf muscles of rats were injected with Ad.BMP-4, Ad.BMP-6, Ad.BMP-2 and Ad.β-galactosidase (Ad.β-gal) and results showed that the constructs Ad.BMP-4 and Ad.BMP-6 were more potent in producing ectopic bone in-vivo compared with Ad.BMP-2. Ad.BMP-4 formed bone through mechanisms similar to endochondral bone formation, whereas Ad.BMP-6 seemed to induce bone by way of mechanisms similar to both intramembranous and endochondral ossification pathways. In another study athymic nude rats were injected with Ad.BMP-13 or Ad.β-gal in the thigh musculature, and the region was examined using light and electron microscopy at various time points between 2 and 100 days post-injection (Helm et al 2001). After 100 days the treated tissue displayed the histological and ultrastructural appearance of neotendon/neoligament. At all time points, the control injection sites were found to contain only normal muscle. Transient immunosuppression using cyclophosphamide was required to stimulate ectopic bone formation in rats using an Ad.BMP-2 vector (Okubo et al 2000). Immunosuppressed rats were also used in a study to treat dorsal osseous nasal defects (Lindsey 2001). A micro-syringe was used to deliver Ad.BMP-2 on the defect edge and on underlying nasal mucosal membranes. After 120 days radio-densitometric measurements showed that BMP-2 treated rats had significantly

Table 1 Viral vectors used in gene delivery

Viral vector	Packaging capacity	Infection	Gene delivery method
Retrovirus (including lentivirus)	8 kb	Dividing cells (lentivirus: dividing and non-dividing)	Integrated
Herpes simplex virus	30–40 kb	Dividing cells	Episomal
Adenovirus	8 kb	Dividing and non-dividing cells	Episomal
Adeno-associated virus (AAV)	5 kb	Dividing and non-dividing cells	Episomal (>90%) Integrated (<10%)

enhanced osseous repair compared with control groups. Transient suppression of the immune system allows re-administration of the viral-based vector but this may not be an acceptable strategy to enhance bone formation in otherwise healthy patients, and could have adverse effects on the bone itself. Helper dependent vectors, which have been deleted of all viral coding sequences (Kochanek et al 1996), can be used to lengthen the expression and to contribute to a solution for the immune response problems. Many papers have provided evidence that second- or third-generation adenoviral vectors reduce the host immune response and prolong the expression of transduced genes in immunocompetent animals compared with first generations (O'Neal et al 1998; Alba et al 2005). However, a study by Li et al (2006a) showed that the deletion of the *E1* and *E2b* genes and the use of different sources of the BMP-4 and -6 genes (rat or human) did not improve osteogenic potential of direct BMP adenovirus vector gene therapy. This data demonstrates that recombinant BMP adenoviral vectors are significantly limited in their ability to induce bone formation in immunocompetent animals. An adeno-associated viral vector was constructed by Li et al (2006b) to deliver human BMP-6 (AAV5.hBMP-6) and the osteogenic potential was compared with that of Ad.hBMP-6 in immunodeficient (2-month old) and immmunocompetent rats (2-month and 18-month old). The mean volumes of ectopic bone 90 days after viral injection were $0.31 \pm 0.14\,cm^3$ (AAV5.BMP-6) and $4.17 \pm 0.05\,cm^3$ (Ad.hBMP-6) in athymic nude rats, $0.64 \pm 0.12\,cm^3$ (AAV5.BMP-6) and $0.06 \pm 0.03\,cm^3$ (Ad.hBMP-6) in 2-month old rats and $0.21 \pm 0.10\,cm^3$ (AAV5.BMP-6) and no bone formation (Ad.hBMP-6) in 18-month old rats. Both types of viruses induced an immune response in immunocompetent animals.

The possible use of gene therapy for treating various disorders of the intervertebral disc was investigated by Nishida et al (2000). They showed the direct delivery of a reporter gene (β-galactosidase, β-gal) into the nucleus pulposus of rabbit lumbar intervertebral discs using the adenovirus as the vector (Nishida et al 1998). The expression persisted for at least 12 weeks in-vivo. Further experiments were done using an adenovirus construct containing the human transforming growth factor-β1 encoding gene (Nishida et al 1999, 2000). An increase in transforming growth factor-β1 production and a significant up-regulation of proteoglycan synthesis were observed within the disc. Moon et al (2000) demonstrated the successful in-vitro transduction of human intervertebral cells from degenerated and non-degenerated discs using Ad.β-gal and Ad.Luciferase (Ad.Luc). A potential less immunogenic and possible safer vector for transgene delivery is the adeno-associated virus (AAV) (Sobajima et al 2004). This vector was used to deliver reporter genes (β-gal and Luc) to human and rabbit nucleus pulposus cells in-vitro and in-vivo (Lattermann et al 2005). The transgene expression was approximately half of that seen with the adenovirus and the in-vivo gene expression was associated with a four- to six-weeks latency period. This study also showed a significant humoral immune response to the AAV vector that decreased expression in pre-immunized animals.

Vascular tissue engineering An in-vivo viral gene transfer method was used to prevent vascular thrombus formation (Kuo et al 1998). Rabbit femoral veins were transfected in-situ with Ad.β-gal, Ad.tPA (tissue plasminogen activator) or a non-viral control (phosphate-buffered saline, PBS). The segment was harvested after 15 min and inserted into the ipsilateral left common femoral artery as an interposition vein graft. A stimulus for thrombus induction was introduced into the artery downstream of the graft. Thrombus formation within both the graft and the downstream artery was significantly reduced compared with the controls. An increase in smooth muscle α-actin positive cells was observed in the vein graft.

Non-viral approach From a patient's perspective using viruses to date carries more risks than benefits, hence the interest in non-viral delivery methods. Non-viral gene delivery systems include physical methods such as electroporation, microinjection, gene gun delivery or the use of uncomplexed naked DNA (Jaroszeski et al 1999; Davidson et al 2000). The use of naked plasmid DNA poses the lowest form of toxicity or other unwanted reactions; it is easy to formulate and is inexpensive. Its transfection efficiency is, however, much lower than viral-mediated gene transfer and is not always sufficient to induce a therapeutic effect for tissue repair. Over the last 15–20 years cationic polymers or lipid formulations have been developed to condense plasmid DNA to protect it from degradation and enhance uptake and transfection of plasmids (Garnett et al 1999; Agarwal et al 2005). This approach has been used to assist tendon and wound repair. All these non-viral methods have viability complications as well as evidence that unmethylated plasmid DNA amplified in bacteria is immunogenic, as reviewed by Partridge & Oreffo (2004).

Bone, cartilage and tendon repair Electroporatic gene transfer of a mouse BMP-4 plasmid vector has been used to test the ability to induce ectopic bone formation in skeletal muscle of mice (Kishimoto et al 2002). Multiple pulses were applied at a low voltage to avoid damaging the animal (Funahashi et al 1999). In all electroporated groups (plasmid containing β-gal and BMP-4, saline injection and without injection) dystrophic calcification of muscle bundles and infiltration of mesenchymal cells was observed. It is believed that the dystrophic calcification was caused by the electric pulses. Ectopic bone formation was only observed for the BMP-4 group but only in half of the animals (Figure 1). The secreted protein levels may not have been maintained long enough to induce bone formation, or it is possible the BALB/cA mice strain may be a poor responder for BMP.

Plasmid DNA (GFP and Luc) was mixed with ultrasonography contrast agent (microbubbles) and injected into coccygeal intervertebral discs of rats (Nishida et al 2006). Therapeutic ultrasound was irradiated on the surface of injected discs. The transfection efficiency using this method was significantly enhanced when compared with using plasmid DNA alone. The sustained transgene expression was possible up to 24 weeks in-vivo.

In another study, plasmid DNA (β-gal) was complexed with transferrin-poly-L-lysine and added to cationic liposomes (Goomer et al 2000). These tertiary lipopolyplexes were delivered to the tissue after permeabilizing with a mild detergent to repair flexor tendon injuries in a canine model. Very high transfection efficiencies were observed in the

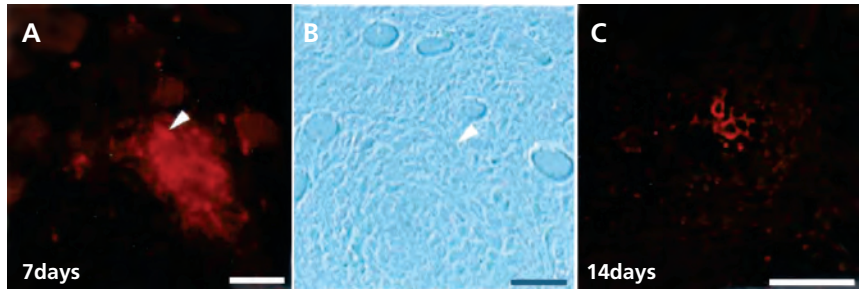

Figure 1 BMP-4 (bone morphogenetic protein 4) expression by immunohistochemistry. BMP-4 expression was detected seven days after electroporation with pMiw-BMP4 ((A) red). A Nomarski image of the same field (B) shows that spindle-shaped mesenchymal cells expressed BMP-4 (white arrowheads). Expression decreased after 14 days, even in specimens that contained newly formed bone (C). Normal control muscle had no BMP-4 signal. Scale bars = 100 μm. (Reprinted from Kishimoto et al, Copyright (2002), with permission from Elsevier.)

flexor tendon, tendon sheaths, tendon pulleys, surrounding tissues and skin.

Wound healing Wound healing growth factors or cytokines control many of the key cellular activities involved in the tissue repair process (Lawrence & Diegelmann 1994). These include epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), keratinocyte growth factor, PDGF, transforming growth factor and VEGF. VEGF improves angiogenesis during wound healing by stimulating the migration of endothelial cells through the extracellular matrix, while PDGF promotes dermal repair and EGF increases the rate of epithelialization and reduces scarring (Grazul-Bilska et al 2003).

Lipofectin-complexed plasmid DNA encoding for human VEGF was injected into the dermis of rats one week before raising a flap (Liu et al 2004). Flap survival was enhanced by 14%. A significantly greater number of vessels were seen in the VEGF-plasmid-treated animals compared with their controls (Figure 2). A particle-bombardment device (Accell) was used to stimulate the wound healing process by delivering PDGF cDNA into the skin of rats (Eming et al 1999) or a hEGF expressing plasmid to partial thickness wounds in pig skin (Andree et al 1994). In the first study (Eming et al 1999) the wound healing response was augmented for at least two weeks. After gene transfer of hEGF an externally sealed fluid-filled wound chamber was used to protect the wound. Wounds healed 2.1 days earlier than the controls. The EGF expressing plasmid was detectable at the wound site for at least 30 days.

Figure 2 Flap survival in the animal on the left was enhanced following injection of VEGF cDNA. The animal on the right was injected with vector control. (Reprinted from Liu et al, Copyright (2004), with permission from Blackwell Publishing.)

Use of a scaffold

Tissue engineering principles have been combined with gene therapy to overcome the limitations of direct in-vivo delivery. Thus plasmid DNA has been incorporated into a polymer scaffold to enhance gene transfer by delaying clearance from the desired tissue, protecting DNA from degradation and an immune response, providing sustained delivery and extending opportunities for internalization. The number of cells expressing the transgene together with the extent of gene transfer may increase, while minimizing the amount of vector used.

The combination of biomaterials and drug delivery technologies has been applied to create polymeric matrices able to deliver DNA. Delivery systems are still being developed that might be used for a specific tissue engineering application. Table 2 gives an overview of materials that have been investigated to deliver reporter genes. The release of DNA can be controlled and is able to transfect cells in-vitro and in-vivo.

Viral approach Several groups have focused on the incorporation of adenoviral vectors into a scaffold to aid bone repair. This rationale centres on the observation that adenoviral vectors have high transduction efficiency and do not integrate into the host genome.

Bone repair Gene therapy has been used in dental tissue engineering applications. An adenoviral vector carrying the PDGF gene (Jin et al 2004), which has potent effects on wound healing of tooth-supporting structures, and BMP-7 gene (Dunn et al 2005), which stimulates bone regeneration around teeth, have been incorporated into a collagen matrix. In the latter study, a titanium dental implant was placed into position followed by the delivery of the collagen matrix. A sustained transgene expression at the osteotomy site was found for up to 10–35 days. The percentage bone defect fill was increased by 50% compared with the control group. Results from the study by Jin et al (2004) are presented in Figure 3. It should be noted that PDGF-1308 possesses antagonistic effects through the formation of inactive and unstable

Table 2 Studies involving reporter genes delivered from polymeric systems

Material	Vector (gene)	Results
Hyaluronan	Plasmid (β-gal)	Injected into hind-limb muscle of rats. After three weeks positive DNA transcription was observed (Yen et al 2004).
Collagen	Polyethyleneimine (PEI), N-[1-(2,3-Dioleoyl-oxy)propyl]-N,N, N-trimethylammonium propane (DOTAP)/cholesterol and copolymer [P6YE5C with P6 = PEG_{6000} derived product and YE5C = (acetyl-YEEEEE)$_2$K-6-aminohexanoic acid-C] (Finsinger et al 2000) protected PEI complexed plasmid (Luciferase, Luc)	Sponges containing naked DNA lost 77% of their dose in an initial burst. PEI and lipid complexes were slowly released and the copolymer protected PEI–plasmid was characterized by an intermediate release. Highest expression levels were obtained with the protective copolymer in-vitro and in-vivo (Scherer et al 2002).
Collagen	Lipofectamine or PEI complexed plasmid or collagen–PLL complexed plasmid (Luc)	Subdermal: transfection levels for condensed DNA were higher than naked DNA. Perivascular: only naked DNA and non-condensed pDNA matrices were found to be active (Cohen-Sacks et al 2004).
Collagen	PEI complexed plasmid (Luc)	Increasing film thickness or the volume of DNA soak solution increased expression levels. Decreasing parameters decreased cell number but did not significantly change expression/cell (Katz et al 2005).
Agarose (gel) and lipid microtubules embedded in agarose (MT-Gel)	CK30PEG10k/TFA complexed plasmid (Luc)	Able to transfect bovine aortic smooth muscle cells, efficiency was less than freshly formed complexes, but was greater than naked DNA (Meilander et al 2003).
Alginate	Plasmid (β-gal)	Injected into rats. Capable of transfecting cells in the intestine, spleen and liver (Aggarwal et al 1999).
Gelatine	Adenoviral (β-gal)	Stents were coated with a photopolymerized hydrogel containing reporter gene. Implanted in rabbit common carotid arteries. Gene expression was observed after three weeks (Nakayama et al 2001).
Chitosan	Chitosan complexed plasmid (Luc & green fluorescent protein, GFP)	Conjugation of KNOB protein (C-terminal globular domain of the fibre protein on adenovirus capsid) after nanoparticle preparation increased transfection efficiency (Mao et al 2001).
Chitosan	Two plasmids (β-gal and Luc)	Microspheres containing two different plasmid DNAs showed sustained and longer period of the protein expression (Özbas-Turan et al 2003).
Chitosan	Plasmid (β-gal)	Microparticles, made by coacervation method, were orally administered to mice. Stomach and small intestines were stained: endogenous and exogenous β-gal activity was observed (Guliyeva et al 2006).

(*cont*)

Table 2 Studies involving reporter genes delivered from polymeric systems (*cont*)

Material	Vector (gene)	Results
Poly(lactide-co-glycolide) (PLGA)	Plasmid (National gene vector laboratories, pNGVL-1 β-gal)	DNA incorporated into PLGA microspheres (double emulsion method). Scaffolds were made using a gas foaming/particulate leaching (GF/PL) process. Sustained delivery for at least 21 days (Nof & Shea 2002).
PLGA	Plasmid (β-gal)	DNA incorporated into PLGA microspheres (cryogenic double emulsion). Scaffold was made using GF/PL process. The release of DNA can be controlled through the microsphere properties, polymer properties & processing conditions (Jang & Shea 2003).
PLGA	Plasmid (β-gal)	Increased encapsulation efficiency by using a cryopreparation process (Ando et al 1999).
PLGA	PEI or Lipofectamine 2000 complexed plasmid (Luc & GFP)	NIH 3T3s were seeded onto the PLGA disk and transfected cells were visible across the substrate (Bengali et al 2005).
PLGA	PEI complexed oligonucleotides	The release profile depended upon the size, loading and pore structure of the spheres. The sustained release resulted in improved intracellular penetration of the delivered vector as compared with uncomplexed DNA (De Rosa et al 2002, 2003).
PLGA	PLL complexed plasmid DNA	Encapsulation efficiencies ranged from 30–45% and was released over approximately 35 days. The encapsulation protected the pDNA from enzymatic degradation and the stability of pDNA was increased by complexing it with PLL (Capan et al 1999).
2D & 3D calcium phosphate/PLGA	λ-DNA	DNA was released over 42 days. Human osteosarcoma cells were plated onto 3D-PLGA/DNA/calcium phosphate matrices. Structurally intact λ-DNA was released and able to transfect cells (Kofron & Laurencin 2004).
Poly(lactic acid)-poly(ethylene glycol)-poly(lactic acid) (PLA-PEG-PLA)/PLGA	Plasmid (β-gal)	Made by electrospinning. Released plasmid DNA over a 20-day study. Tensile moduli of ~35 MPa (similar to those of skin and cartilage) (Luu et al 2003).
Dimethacrylated PLA-PEG-PLA	Plasmid (secreted human placental alkaline phosphatase, SEAP, & GFP) and Lipofectamine complexed plasmid	The photoencapsulated plasmid was delivered in active, supercoiled form. Primary chondrocytes were co-encapsulated with complexed plasmid. Transfection levels increased as the gel started to erode (Quick & Anseth 2003).
Poly(ethylene-co-vinyl acetate)	Herring sperm (HS) DNA	HS-DNA was continuously released from matrix over the duration of experiment (>1 month) (Luo et al 1999).
Poly(ethylene oxide) (PEO) based networks	Plasmid DNA (SEAP)	In-situ cross-linking of networks did not affect supercoiled form. Injection into skeletal muscle of immunocompetent mice resulted in a prolonged expression (Roy et al 2003).

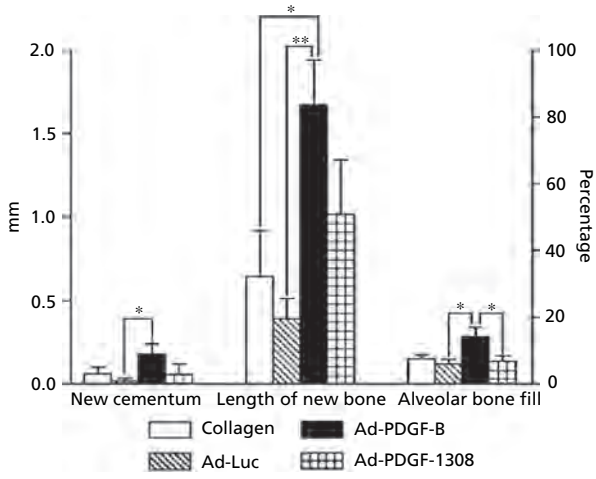

Figure 3 Histomorphometric analysis of specimens 14 days post-surgery and gene delivery. Ad-PDGF-B treatment increased cementum formation, compared with Ad-Luc treatment ($*P < 0.05$). Furthermore, Ad-PDGF-B treatment not only improved the bridging length of newly formed alveolar bone compared with Ad-Luc ($**P < 0.01$) and collagen matrix alone ($*P < 0.05$) groups, but also enhanced the percentage of alveolar bone fill compared with Ad-Luc and Ad-PDGF-1308 treatments ($*P < 0.05$). n = 6–8 specimens/group. (Reprinted from Jin et al, Copyright (2004), with permission from Elsevier.)

heterodimers with wild-type PDGF-B or -A chains (Mercola et al 1990).

Growth plate injuries may lead to a progressive angular deformity or longitudinal growth disturbance (Lee et al 2002). Ad.insulin-like growth factor-1 (IGF-1) or Ad.BMP-2 was injected into the tibial physeal defects of rabbits filled with a free autologous muscle biopsy. The release of IGF-1 protein showed a supportive effect on the restoration of the injured growth plate. However, the release of BMP-2 protein caused an increased osteogenic activity, which resulted in a premature closure of the injured growth plate, and could thus not be used to restore the injured growth plate.

Non-viral approach The delivery of plasmid DNA through localized polymeric scaffolds has been used to deliver genes safely to target cells. A gene-activated matrix (GAM) consists of plasmid DNA and a biodegradable structural matrix carrier. Plasmid DNA is economical and relatively simple to manufacture and is non-toxic to tissues. Diffusion from the delivery site should not cause systemic toxicity because of the high efficiency of DNA catabolism in the bloodstream (Bonadio 2000). Plasmid DNA is stable and flexible, and therefore likely to be compatible with the polymer-based delivery systems.

Bone and cartilage repair The first GAM feasibility study involved direct plasmid gene transfer to cells participating in bone repair in rats (Fang et al 1996). Collagen sponge GAM formulations containing β-gal or Luc plasmids were implanted into segmental gaps created in the femur of rats. Plasmid DNA, a high molecular weight polyanion, is incapable of diffusing through the collagen barrier so the GAM matrix serves as a scaffold that holds DNA in-situ until endogenous fibroblasts arrive. Implantation of a GAM containing either a BMP-4 plasmid or a parathyroid hormone 1–34 plasmid (PTH 1-34) resulted in a biological response of new bone filling the gap. Implantation of a two-plasmid GAM (BMP-4 plus PTH 1-34, which act synergistically in-vitro) caused new bone to form faster than with either factor alone. Similar results were achieved using a canine bone defect model, in which bone physiology mimics that of man (Bonadio et al 1999). GAM collagen sponges were produced containing a pMat-1 plasmid, which encodes for a secreted peptide fragment of human PTH 1-34. Implantation of the sponges at sites of bone injury was associated with retention and expression of plasmid DNA for at least six weeks. At least picogram quantities of secreted peptide were produced. Local delivery of pMat-1 induced the formation of normal new bone in a time-, plasmid dose- and bone gap size-dependent manner (Figure 4). A chitosan–gelatine scaffold has been fabricated which is able to release plasmid DNA encoding transforming growth factor-β for cartilage regeneration (Guo et al 2006). Sustained release of structurally-intact DNA was observed for up to three weeks with a burst release in the first week. Cell morphology was better in the GAM group than the control (without plasmid DNA). The round cell shape was maintained and the major components of the extracellular matrix were produced, enhancing cartilage tissue regeneration.

To increase transfection levels in-vivo, plasmid DNA can be condensed with cationic polymers or lipid formulations. Huang et al (2003, 2005a, b, c) complexed plasmid DNA with polyethyleneimine (PEI) and incorporated the resulting complexes in poly(lactide-co-glycolide) (PLGA) sponges. Scaffolds delivering plasmid DNA encoding for BMP-4 were implanted into a critical sized rat cranial defect for time periods up to 15 weeks (Huang et al 2005b). Blank scaffolds and scaffolds containing non-condensed DNA were used as controls. Bone regeneration was significant at the edges and within the defect site when scaffolds containing condensed DNA were used. Bone formation was restricted to the edges for the controls. The delivery of complexed DNA led to more complete mineralized tissue regeneration. To closely copy the natural process of bone formation Huang et al (2005c) also fabricated PLGA scaffolds containing combinations of PEI condensed plasmid DNA encoding for BMP-4, VEGF and human bone marrow stromal cells (BMSCs). Scaffolds were implanted into the subcutaneous tissue of severe combined immune deficient (SCID) mice and bone and blood vessel formation was determined at 3, 8 and 15 weeks after implantation. The combined delivery resulted in a significant increase in the quality of regenerated bone. The elastic moduli were also significantly higher than any other condition. In another study plasmid DNA encoding BMP-2 was combined with cationic liposomes and incorporated into porous hydroxyapatite scaffolds (Ono et al 2004). These scaffolds were introduced into bone defects created on rabbit cranium. Four groups were compared, BMP-2 plasmids with liposomes with and without scaffold and a control gene with liposomes with and without scaffold. Osteogenesis was faster in the group without scaffold treated with BMP-2 than the hydroxyapatite-containing group (Figure 5). New bone formation was evident surrounding the scaffold at three weeks; however, the bone tissue did

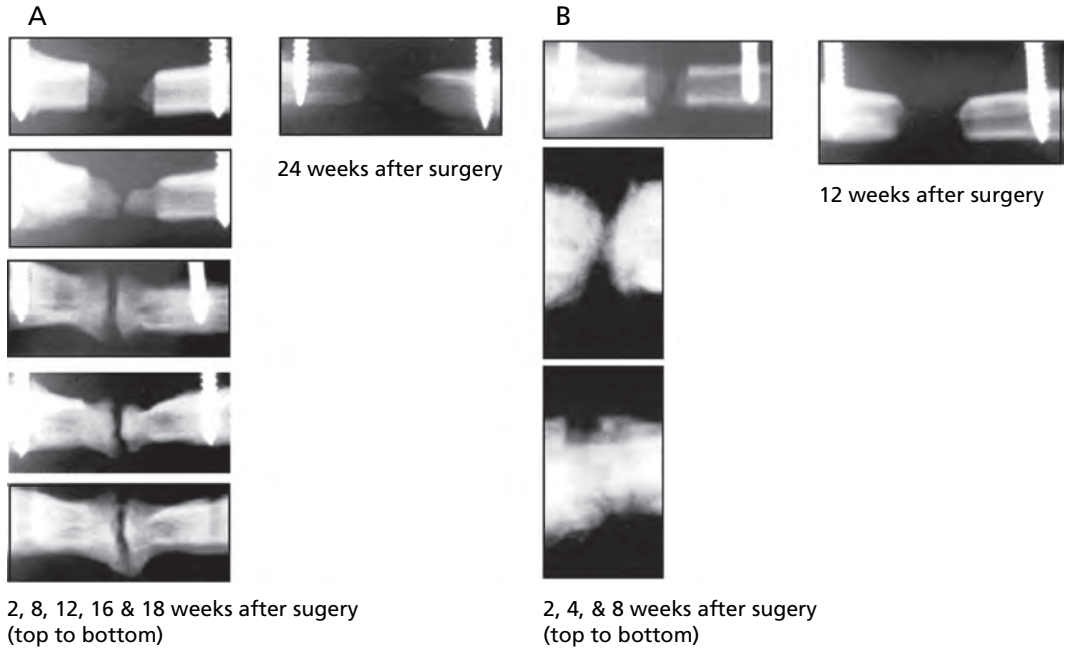

Figure 4 Bone formation dose–response: part 2. A, 1.6-cm gap. Serial radiographs obtained after surgery from dogs that received GAM implants with 100 mg pMat-1 (left) or control sponges (right). There was formation of a bony callus and bone bridging at multiple points by 18 weeks in the beagle receiving a GAM sponge. B, 1.0-cm gap. Serial radiographs obtained after surgery from dogs that received GAM implants with 100 mg pMat-1 (left) or control sponges (right). A bone bridge formed by four weeks; at eight weeks (anterior–posterior radiograph), bridging along the periosteal margin was complete. (Reprinted from Bonadio et al, Copyright (1999), with permission from Macmillan Publishers Ltd.)

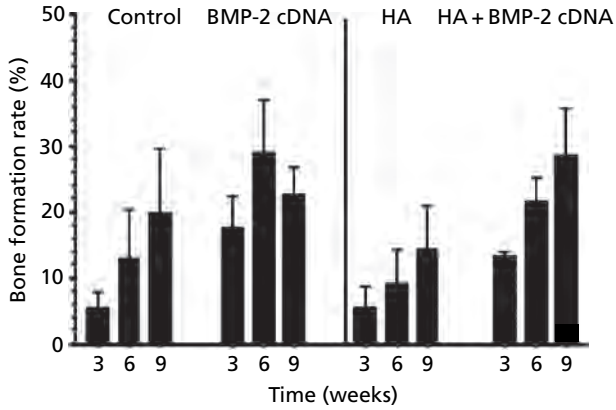

Figure 5 Results of image analysis: total amount of osteogenesis. HA, hydroxyapatite; BMP-2, bone morphogenetic protein 2. (Reprinted from Ono et al, Copyright (2004), with permission from Elsevier.)

not fill all the pores at nine weeks. It was likely cells were not able to enter the scaffold quickly enough to produce enough BMP-2 protein to induce osteogenesis inside the pores.

Vascular tissue engineering Plasmids have also been incorporated into PLGA scaffolds or fibrin sealants to promote angiogenesis. The development of blood vessels is required for the metabolic demands of the new tissue. Scaffolds have been made by a range of different techniques and researchers have tried to increase loading efficiencies. Porous PLGA scaffolds were made either by a gas foaming procedure in which wet granulation was used to increase the encapsulation efficiency of the plasmid (Jang et al 2005) or by a high pressure gas foaming process (Shea et al 1999). In the former study scaffolds were loaded with a VEGF encoding plasmid and were subcutaneously implanted into mice. An increase of the blood vessel density ($102.7 \pm 37.3\,mm^{-2}$) was observed in comparison with the control, a plasmid encoding luciferase ($63.5 \pm 27.4\,mm^{-2}$). Following implantation of the GAM created by Shea et al (19999) in rat dermis, delivery of a plasmid encoding for PDGF led to an increased vascularization near the implant site (Figure 6). Direct injection of the plasmid showed no significant enhancement of local tissue formation. In a different study an injectable implant solution was made by mixing plasmid DNA in water with PLGA in glycofurol (Eliaz & Szoka 2002). The ability of the released plasmid to transfect cells in mice was assessed using three independent plasmids. Tissues in contact with the implant containing a Luc plasmid expressed large amounts of luciferase. When using the plasmid encoding secreted human placental alkaline phosphatase (SEAP) high levels of the enzyme were expressed for 67 days. The developmental endothelial locus (pDL-1) gene encodes Del-1, a matrix protein that is believed to regulate vascular morphogenesis in embryonic development (Hidai et al 1998). Tissues containing devices releasing the pDL-1 showed a visible increase in blood vessel formation in the vicinity of the implant compared with the controls (Luc and SEAP).

Fibrin sealants are used in a wide range of surgeries, primarily as haemostatic agents, but also to assist tissue sealing and wound healing. They have also been employed for the delivery of plasmids encoding for human VEGF (Jozkowicz et al 2003) and pleiotrophin (Christman et al 2005). A GAM

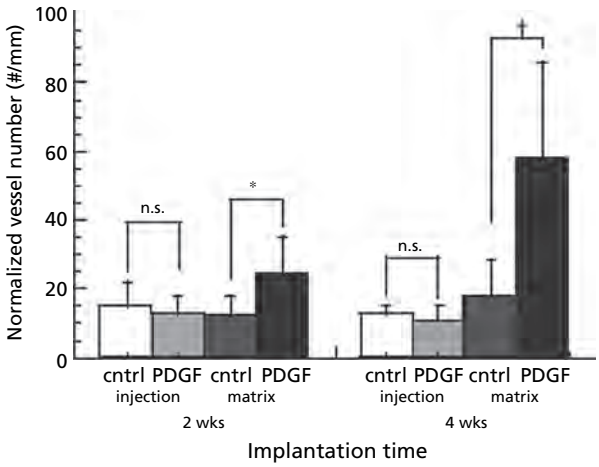

Figure 6 Blood vessel number at two and four weeks for platelet-derived growth factor (PDGF) and control (nt β-gal) plasmids for delivery by injection and release from a matrix. n.s. indicates no statistical difference; $*P<0.05$; $\dagger P<0.01$. Statistical analysis was performed using the software program Instat. (Reprinted from Shea et al, Copyright (1999), with permission from Macmillan Publishers Ltd.)

containing the pleiotrophin plasmid was injected into the ischaemic left ventricle of rats. The fibrin glue is responsible for maintaining the geometry of the left ventricle and cardiac function following myocardial infarction. The delivery of pleiotrophin plasmid increased neo-vascularization compared with injection of either pleiotrophin or fibrin glue alone. Additionally these newly formed vessels were functionally connected with the existing coronary vasculature.

Nerve regeneration Collagen has been used to deliver therapeutic agents to the central nervous system after trauma. A GAM consisting of plasmid DNA complexed with poly-D-lysine in a collagen paste was placed between the proximal and distal ends of severed rat optic nerve (Berry et al 2001). Plasmids encoding for basic FGF2, brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) promoted retinal ganglion cells survival for over three months after injury (Figure 7). Plasmids encoding for nerve growth factor (NGF) were used to elicit neurite outgrowth by the cell line PC12, and by neurons dissociated from dorsal root ganglia (DRG) (Whittlesey & Shea 2006). These plasmids were complexed with Lipofectamine2000 and incorporated into PLGA microspheres. Transfection of PC12 cells produced bioactive NGF resulting in a neurite outgrowth. In the same study a model system was developed for the localized production of neurotrophic factors by transfecting an accessory cell type. Dissociated chick DRG neurons were co-cultured with PLGA transfected HEK293T cells. The survival of the DRG cells was found to be at least 10-fold higher and resulted in strong neurite outgrowth compared with the controls (Figure 8).

Ex-vivo gene transfer

Ex-vivo gene transfer methods involve the in-vitro genetic manipulation of cells and reintroduction of the cells into the host with or without a scaffold. The ex-vivo strategy is in many respects safer for the patient, since immune responses to viral particles or inflammatory and toxic effects from transfection agents are minimized. The disadvantage is that it involves two separate invasive procedures for a patient, which increases the risk for contamination. It is however possible to choose the type of transduced or transfected cells.

Without scaffold

Attempts have been made to use the ex-vivo therapy for bone, cartilage, wound, urothelial, nerve tissue regeneration and for treating diabetes using viral or non-viral vectors.

Viral approach Adenoviral and retroviral vectors were employed to transduce cells. Lentiviruses are a type of retrovirus that can infect both dividing and non-dividing cells such as nerve cells, hence these viruses have been used for nerve regeneration.

Bone and cartilage repair Different cell populations were used to investigate the use of ex-vivo gene therapy to produce bone (Musgrave et al 2000). BMSC lines, primary muscle derived cells (MDC), primary BMSCs, primary articular

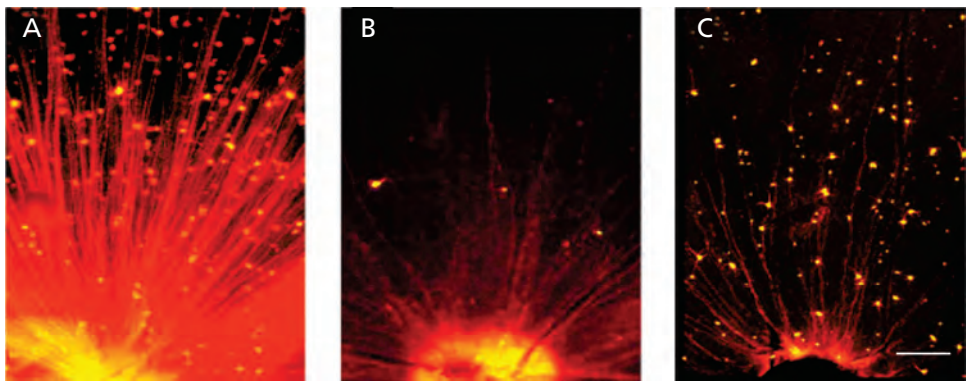

Figure 7 Functional effects of delivering plasmids that encode neurotrophic factors. Lysinated rhodamine–dextran-filled retinal ganglion cells somata and axons in whole-mounted retinae. A. Retina from an intact, untreated control. B. Retina 40 days after implantation of a GAM containing a plasmid encoding GFP. C. Retina 40 days after implantation of a GAM containing a combination of plasmids encoding FGF2/BDNF/NT-3 (NTFs). Magnification bar, 250 μm. (Reprinted from Berry et al, Copyright (2001), with permission of Elsevier.)

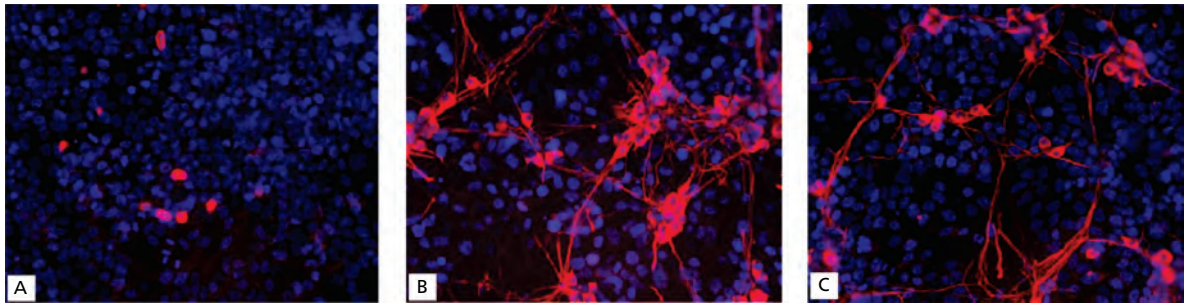

Figure 8 Dissociated chick DRG neurons co-cultured with PLG-transfected HEK293T. HEK293T cells incubated with PLGA disks releasing pRK5 (A) or pRK5-NGF (B) lipoplexes for 24 h before the addition of E8 DRG neurons. Control co-culture (C) was untransfected with 25 ng mL^{-1} NGF added with the DRGs. Images of TUJ1 (class III beta-tubuli, red) and nuclear stain Hoechst 33258 (blue) were captured independently and overlaid. All micrographs were taken at magnification × 200. B and C are representative of five random fields of view. A is not representative of random fields, due to poor DRG survival in the absence of NGF. (Reprinted from Whittlesey & Shea, Copyright (2006), with permission from Elsevier.)

chondrocytes and primary fibroblasts were all transduced with Ad.BMP-2. All cell types were capable of secreting BMP-2. The transduced cells were injected in hindlimbs of SCID mice and bone formation was evaluated. BMSC and MDC were more efficient in producing bone than the primary BMSC and chondrocytes. No ectopic radiographic densities were detectable with Ad.BMP-2 transduced fibroblasts. A study was performed to improve healing of the meniscus by using Ad.β-gal transduced myoblasts and injecting them in the menisci of rabbits (Kasemkijwattana et al 2000). Staining of desmin, a muscle specific protein, revealed the presence of myoblasts, myotubes and myofibres in the same area where β-gal was expressed. β-Gal expression was observed for up to six weeks.

Wound healing Human oral mucosal keratinocytes were infected with a retroviral vector carrying the β-gal or human clotting factor IX gene, which is able to promote wound healing and to prevent excess bleeding (Mizuno et al 1999). Next, the cells were cultured on a mitomycin C-treated 3T3 feeder layer to make a stratified epithelial sheet. The membrane was then grafted onto SCID mice. β-Gal expression was observed five weeks after grafting and Factor IX was detected in serum for more than three weeks in-vivo. The results showed that the oral mucosal epithelium was an ideal target tissue.

Urothelial tissue engineering The treatment of bladder dysfunction is limited by the inability to treat impaired detrusor contractility. Yokoyama et al (2001) explored a new treatment for urologic dysfunction using skeletal MDCs. MDCs were transduced with Ad.β-gal and injected into the lateral bladder wall. A high amount of β-gal expressing cells were observed at day 5, 35 and 70. The injected MDCs spread between the smooth muscle layer and bladder mucosa. Myotubes and myofibres expressing β-gal and positively stained for fast myosin heavy chain were seen at day 35 and 70 (Figure 9).

Nerve regeneration The ex-vivo gene therapy has been used for the treatment of central nervous system and peripheral nervous system injury. Lentiviral vectors encoding ciliary neurotrophic factor were used to transduce purified adult Schwann cells (Hu et al 2005). These cells were injected into peripheral nerve sheets and subsequently transplanted onto transected optic nerve in rats. The viability and axonal regeneration of injured adult rat retinal ganglion cells were significantly enhanced after four weeks and increased levels of the growth factor in the grafts were observed.

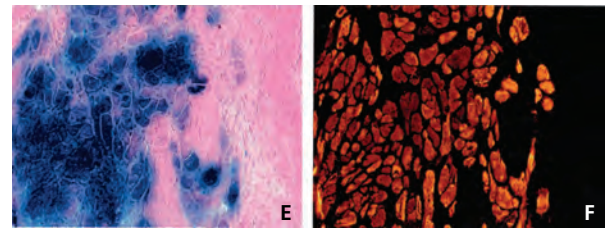

Figure 9 Bladder sections showing staining of lacZ (E) and fast myosin heavy chain (F) at 70 days after injection. Blue area shows lacZ-positive injected MDCs (E). Fast myosin heavy chain immunohistochemistry revealed that at 70 days after injection, almost all injected MDCs have differentiated into myotubes and myofibres (F). Original magnification × 200. (Reprinted from Yokoyama et al, Copyright (2001), with permission from Elsevier.)

Diabetes A study was performed by Sasaki et al (2003) to help restore insulin secretion to treat diabetes mellitus. 3T3-L1 pre-adipocytes were transduced by a retroviral vector plasmid expressing the cDNA for insulin (INS/fur), whose DNA sequences were modified to allow cleavage by furin. When the transduced cells were induced to differentiate in-vitro, insulin secretion was significantly increased compared with the undifferentiated cells. Differentiated cells were then transplanted into the intraperitoneal space of streptozotocin-induced diabetic nude mice. Glucose levels were significantly lowered compared with the controls (non-transformed cells) where no significant reduction was detected. The therapeutic effect lasted for one month after transplantation.

Non-viral approach There is an interest in developing non-viral approaches since the immune response to viral vectors can adversely affect the phenotype of cells (Yang et al 1996) and hence decrease tissue regeneration. Gene transfer efficiencies are low but strategies exist to optimize non-viral systems to enable them to reach their full potential.

Bone and tendon repair A nucleofection method was used to deliver the osteogenic genes hBMP-2 and hBMP-9 to human mesenchymal stem cells (MSCs) to form bone tissue (Aslan et al 2006). Cell viability after nucleofection was 53.6% and gene efficiency was 51–88%. The nucleofected cells were then injected into the distal part of the thigh muscle in six- to eight-weeks old female NOD/SCID mice. Bone formation was detected in the BMP-2 and -9 transplants at three and four weeks postinjection, while no bone formation was observed in the control group (EGFP). The efficacy and cytotoxicity of cationic agent mediated non-viral gene transfer was evaluated in osteoblastic cells for bone tissue engineering applications (Kim et al 2004). Three different agents were used: a lipid, gelatine and PEI. All of them were able to transfect the osteoblastic cells. However, the cationic lipid and PEI were more effective in rat primary osteoblasts. Cell survival rate was decreased as the amount of lipid or PEI was increased. Gelatine was better in transfecting the human fetal osteoblasts and the dose did not affect cell survival rate (Figure 10). In another study highly purified MDCs were transfected with Lipofectamine-complexed plasmid carrying the β-gal reporter gene and the neomycin resistance gene (Pelinkovic et al 2003). After selection in medium containing G418, a colony containing β-gal genes was isolated and cultured in the medium. The cells were injected into the supraspinatus tendons of nude rats. β-Gal gene expression was detected for up to 21 days. The injected cells changed from a round morphology to a more spindle-shaped morphology and were surrounded by collagen matrix. The cells showed differentiation into vimentin-expressing fibroblastic cells.

Bladder tissue engineering The non-viral ex-vivo gene transfer method has been used to treat bladder dysfunction. Highly purified MDCs were transfected using a Lipofectamine-complexed plasmid encoding for β-gal, minidystrophin- and neomycin-resistance genes (Huard et al 2002). At 72-h after transfection, the cells were selected using G418 for 10 days until colonies appeared. The cells were injected into the bladder walls of SCID mice and were capable of surviving. Some of the cells differentiated into the smooth muscle lineage, while some of them differentiated into myotubes and myofibres that became innervated and subsequently improved the contractility of the bladder.

Use of a scaffold

Injected transfected cells can have low in-vivo survival levels and they can migrate through the circulation to other sites. This makes the delivery of a sufficient level of therapeutic protein difficult. Porous polymer matrices can be used as potential scaffolds for cell transplantation. The selection of the scaffold is as important as the choice of cell type, vector or gene and will be influenced by the anatomical location.

Viral approach The greatest proportion of researchers have used the viral approach to regenerate tissues because transfection levels are high compared with non-viral methods.

Bone, cartilage and tendon repair The use of scaffolds is particularly important in repair of structural tissues such as bone, and offers an excellent opportunity for the incorporation of gene vectors. A number of different BMP genes and cells have been used in this area. The cells that have received the most attention are bone marrow, fibroblasts, mesenchymal stem cells and chondrocytes.

Bone marrow cells Demineralized bone matrix, collagen, PLGA, hydroxyapatite and grafts have been used as scaffolds for transduced bone marrow cells.

Bone marrow cells were harvested from rats, expanded in tissue culture and transduced with an Ad.BMP-2 vector. The

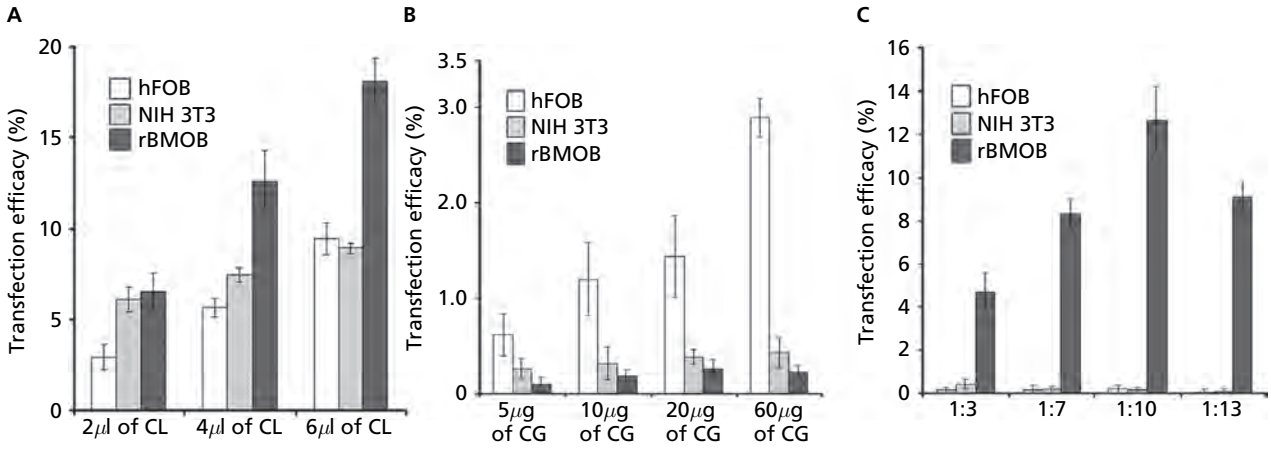

Figure 10 A. Cationic-lipid-mediated gene transfection. Clonal human fetal osteoblasts (hFOB), NIH 3T3 fibroblasts (NIH 3T3), and rat bone-marrow-derived primary osteoblasts (rBMOB) are transfected with pDsRed2 plasmid DNA encoding red fluorescent protein using 2, 4, or 6 μL cationic lipid (CL). Transfection efficacy defined as the number of transfected cells divided by the total number of the cells. B. Cationic-gelatin-mediated gene transfection. Four different doses of cationic gelatin (CG; 5, 10, 20, or 60 μg in 30 μL 0.1X PBS) were used to transfect hFOB, NIH 3T3, or rBMOB. Transfection efficacy obtained from different dosage of cationic gelatin. C. Cationic-polymer-mediated gene transfection. hFOB, NIH 3T3, and rBMOB were transfected with pDsRed2 plasmid DNA using cationic polymer (CP), polyethyleneimine (PEI) with P/N (phosphate in DNA/nitrogen in polyethyleneimine) ratio of 1:3, 1:7, 1:10, or 1:13 in 150 μM NaCl. Transfection efficacy obtained from different P/N ratio of cationic polymer. Data are means ± 1 s.d. from triplicate experiments. (Reprinted from Kim et al, Copyright (2004), with permission from John Wiley & Sons, Inc.)

cells were either implanted in an 8-mm femoral defect in rats together with a guanidine hydrochloride-extracted demineralized bone matrix (Lieberman et al 1999) or were seeded onto the same matrix or a collagen sponge and applied to the spine after posterolateral spinal arthrodesis in rats (Wang et al 2003). Histomorphometric data from the first study showed that the defects healed with transduced cells had significantly denser trabecular bone than defects treated with recombinant BMP-2 (rhBMP-2) protein. However, no difference in defect strength was observed. All spines were fused at four weeks postoperatively in the second study. Histological data showed that spines that had received BMP-2 producing bone marrow cells were filled with coarse trabecular bone, whereas those that received rhBMP-2 were filled with thin trabecular bone (Figure 11). A similar study was performed to deliver the LIM mineralization protein-1 (Ad.LMP-1) gene (Viggeswarapu et al 2001). This protein is directly involved in osteoblast differentiation. After arthrodesis of the lumbar spine, demineralized bone matrix or collagen were loaded with transduced bone marrow-derived buffy-coat cells and implanted. A solid continuous spinal fusion mass was observed at five weeks. In a second study peripheral blood derived buffy-coat cells and a collagen–ceramic composite with 15% hydroxyapatite and 85% tricalcium phosphate were used (Viggeswarapu et al 2001). Solid spinal fusion was observed at four weeks. Hidaka et al (2003) reported successful posterolateral spinal fusion in athymic rats in an ex-vivo study using bone marrow cells. The cells, expanded for four weeks, were transduced using an adenovirus encoding the osteoinductive growth factor BMP-7 and seeded onto an allograft osteoconductive scaffold. At eight weeks radiographic fusion was 70% and mechanical fusion 80% vs 0% in controls. A high rate of bone formation was observed and the fusion masses had the appearance of normal trabecular bone. In another study primary human bone marrow osteoprogenitor cells were transduced with Ad.BMP-2 or Ad.β-gal and injected into diffusion chambers containing PLGA porous scaffolds that were produced by supercritical carbon dioxide processing (Partridge et al 2002). The scaffolds were implanted intraperitoneally in nude mice. Bone formation was observed after four weeks. These results indicated osteoblastic differentiation of the cells.

All the previous studies used adenoviral vectors. However, lentiviral vectors offer the potential for long-term gene expression because the genetic material is integrated into the host. Prolonged gene expression may be advantageous when large bone defects need to be repaired. A lentiviral vector was developed to enhance osteogenesis (Sugiyama et al 2005). The vector encoding BMP-2 downstream of either the cytomegalovirus immediate early (CMV) promoter or the murine leukaemia virus long terminal repeat (RhMLV) promotor was designed. BMP-2 producing BMSCs were seeded on a collagen sponge and the construct was implanted into the hind limb of SCID mice. This study demonstrated that the RhMLV promoter induced higher gene expression than the CMV promoter in BMSCs allowing prolonged gene expression (Figure 12).

Fibroblasts Fibroblasts, transduced to express BMP-7, were seeded onto scaffolds such as poly(lactic acid) (PLA)/hydroxyapatite, hydroxyapatite, polypropylene fumarate/β-tricalcium phosphate, polycaprolactone and gelatine to induce bone and cartilage formation. A PLA/hydroxyapatite composite scaffold was made by Schek et al (2004). The shape and internal pore architecture of the hydroxyapatite phase

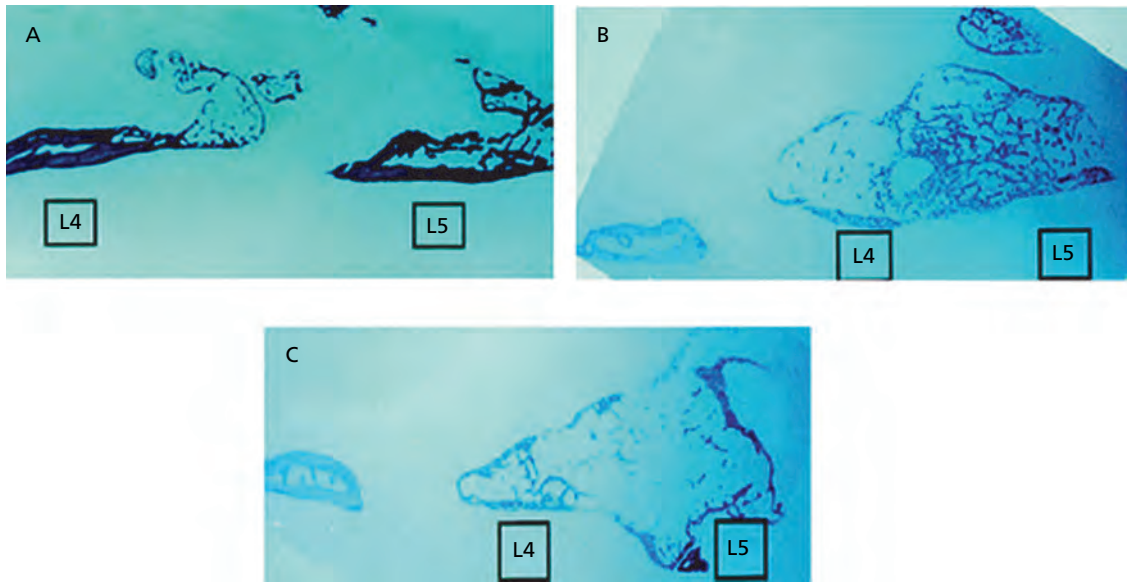

Figure 11 Non-decalcified histological sections of the intertransverse process fusion mass in rats (1% methylene blue and 0.3% basic fuchsin, magnification × 10). A. Section obtained eight weeks after arthrodesis with autogenous iliac crest bone grafting demonstrated no evidence of bone formation between the transverse processes of L4 and L5. B. Section obtained four weeks after arthrodesis with transduced bone marrow cells producing BMP-2 showed new trabecular bone formation bridging the transverse processes of L4 and L5. C. Section obtained four weeks after arthrodesis with recombinant BMP-2 shows new trabecular bone formation bridging the transverse processes of L4 and L5. (Reprinted from Wang et al, Copyright (2003), with permission of The Journal of Bone and Joint Surgery, Inc.)

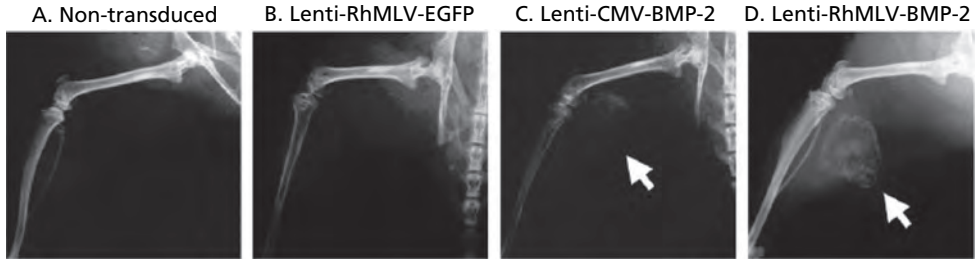

Figure 12 Radiographs of SCID mice at three weeks after implantation of rat BMSCs (2.5×10^6 cells). New bone formation was induced by implantation of both (C) Lenti-CMV-BMP-2-transduced cells and (D) Lenti-RhMLV-BMP-2-transduced cells. However, increased bone formation was noted with implantation of Lenti-RhMLV-BMP-2-transduced cells in comparison with Lenti-CMV-BMP-2-transduced cells. No changes in density were seen in hind limbs on radiographs of mice that received either (A) nontransduced cells or (B) EGFP-transduced cells. (Reprinted from Sugiyama et al, Copyright (2005), with permission from Elsevier.)

was defined using image based design and created using solid-free form. A thin poly(glycolic acid) (PGA) polymer film was placed between the PLA and hydroxyapatite before assembly to serve as a barrier to cell infiltration during seeding and cell migration during growth. Ad.BMP-7-transduced primary human gingival fibroblasts were suspended in fibrinogen and pipetted into the ceramic side. The scaffolds were then immediately placed onto a drop of thrombin to induce gelation. Chondrocytes were seeded into the PLA phase. In the next step the composites were implanted into immunocompromised mice. These scaffolds promoted the growth of bone, cartilage and a mineralized interface tissue. Solid-free form was also used to create scaffolds from hydroxyapatite and a biodegradable polypropylene fumarate/β-tricalcium phosphate composite (Lin et al 2005; Schek et al 2006). New bone was formed on the biodegradable scaffold when seeded with Ad.BMP-7 transduced human gingival fibroblasts, and closely followed its contours (Lin et al 2005). The total stiffness was retained for up to 12 weeks after implantation, as scaffold degradation and tissue invasion took place. In the study by Schek et al (2006) fibrin hydrogels or PLA sponges were used as a second carrier because the surface area of the solid scaffolds is low and the material itself is not absorbent. Scaffolds were again seeded with Ad.BMP-7-transduced human gingival fibroblasts. Significant amounts of bone were observed on nearly all the implants. The choice of secondary carrier had an effect on bone formation, the use of fibrin led to large amounts of bone compared with PLA sponges. Both base materials supported bone growth, however hydroxyapatite scaffolds were more osteoconductive (Figure 13).

In a different study porous polycaprolactone scaffolds were fabricated using selective laser sintering, seeded with Ad.BMP-7-transduced primary human gingival fibroblasts and subcutaneously implanted into immunocompromised mice (Williams et al 2005). The bone mineral density ($513.36 \pm 14.23\,\mathrm{mg\,cm^{-3}}$) of the newly formed bone lay within the range of normal bone mineral density measures of human trabecular and cortical bone (120 and 1100 mg $\mathrm{cm^{-3}}$, respectively). To demonstrate the clinical application of this technology a minipig condyle scaffold was designed based on an actual pig condyle. This scaffold replicated the anatomy well and could be built within three hours.

Bone reconstruction in the head and neck region is frequently performed after radiation therapy. Rat dermal fibroblasts were transduced with Ad.BMP-7 and seeded on gelatine sponges (Nussenbaum et al 2005). Two weeks before surgery rats received either no radiation or a 12-Gray radiation dose to the calvarium. Critical size calvarial defects were created and the scaffold was placed into the defect. An autologous bone graft was used as control. None of the bone grafts showed obvious evidence of healing at the wound margins. For the non-radiated cranial defect a near 100% bone healing was observed. However, the defects that were pre-operatively treated with radiation were characterized by bone healing of 10–25%. These findings have implications for translating this tissue engineering approach to patients with cancer-related segmental bone defects.

Mesenchymal stem cells Mesenchymal stem cells were transduced with a viral vector containing the BMP-2, -4 and -7 gene or the sonic hedgehog gene (Shh). These cells were either mixed with collagen or seeded on PLGA/hydroxyapatite, PLA, PGA, hydroxyapatite and collagen scaffolds. Viruses used for stem cell transduction include retroviruses and adenoviruses. Retroviruses are not able to transduce non-dividing cells, while adenoviral vectors provide high transfection efficiency even in non-proliferating cells.

W-20 cells, a murine stromal cell line which expresses the common markers for the osteoblastic phenotype, were transfected with the retroviral BMP-2 gene and plated on PLGA/hydroxyapatite composites (Laurencin et al 2001). In-vitro BMP-2 producing cells rapidly attached to the degradable PLGA/hydroxyapatite scaffold and continued to express BMP-2. Implantation of this system in a SCID mouse model was able to induce heterotopic bone formation. In another study muscle-derived stem cells (MDSCs) were transduced with a self-inactivating retroviral vector that enabled doxycycline-inducible expression of human BMP-4 (Peng et al 2004). Type I collagen based scaffolds (Gelfoam) containing transduced MDSCs were able to induce bone formation in mice that received doxycycline in comparison with no bone formation in the absence of doxycycline (Figure 14). When a Gelfoam disk containing these MDSC transduced cells was implanted in critical sized calvarial defects complete bone healing was observed in the +doxycycline group (Peng et al 2005). Surprisingly, residual bone regeneration was detected in the −doxycycline group. To overcome this problem MDSC-Nog (noggin expressing cells) were co-implanted

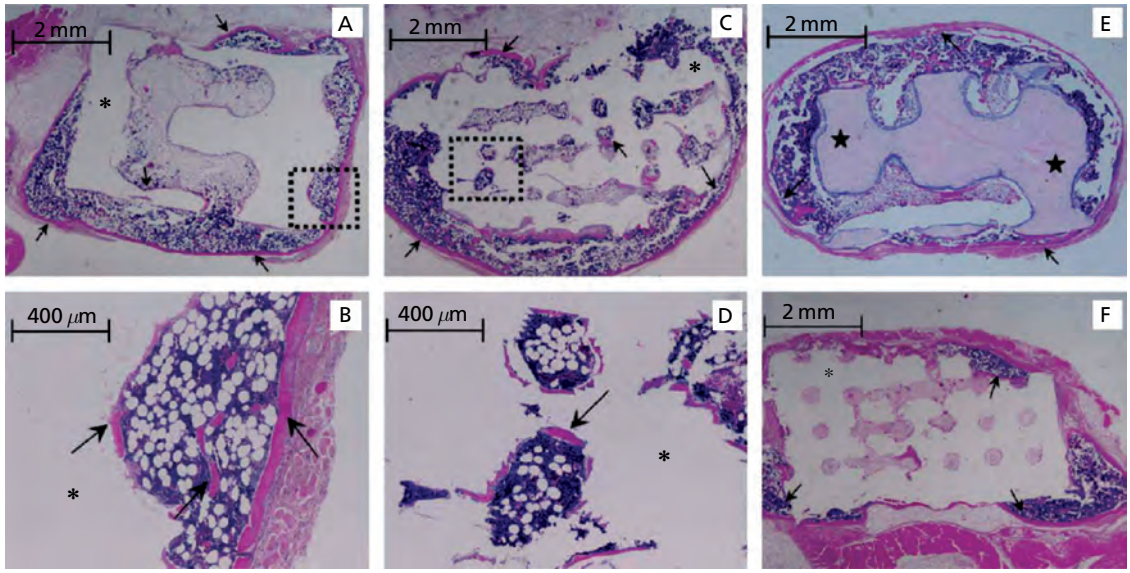

Figure 13 Haematoxylin and eosin staining confirmed the presence of bone on the implanted scaffolds. Residual PPF-TCP was visible in sections (star) and empty areas were previously occupied by hydroxyapatite (HA) that was removed during demineralization (asterisks). Large amounts of bone (arrows) that fully surrounded the scaffolds were observed when transduced cells were used in conjunction with fibrin, regardless of whether scaffolds were hydroxyapatite (A and C) or PPF-TCP (E). Bone formation patterns were also similar on scaffolds whether they contained 800-μm pores (A) or 300-μm pores (C). Higher magnification of areas within the dashed boxes (B and D correspond with boxes shown in A and C, respectively) showed the bone was morphologically normal, possessed marrow space, and formed in direct contact with the scaffold base material. In contrast to the ''shell'' of bone that fully surrounded the scaffold seeded using fibrin gel, scaffolds seeded with transduced cells using the poly(lactic acid) (PLA) sponge showed the presence of only small, isolated bone growth (F). (Reprinted from Schek et al, Copyright (2006), with permission from Elsevier.)

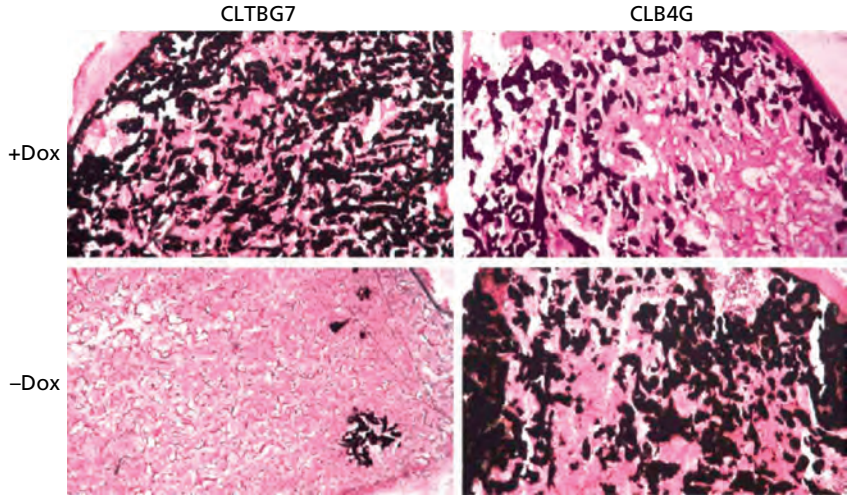

Figure 14 Regulated bone formation elicited by transduced MDSCs. Von Kossa staining shows dense mineralized bone trabeculae that formed after doxycycline (Dox) induction at the sites implanted with CLTBG7-transduced MDSCs. In contrast, in the absence of doxycycline, only small, isolated bone nodules formed at the implantation sites. As expected, bone formation at the sites implanted with CLB4G transduced MDSCs (note from author: noninducible retroviral vector) was independent of doxycycline induction. Original magnification ×100. (Reprinted from Peng et al, Copyright (2004), with permission from Elsevier.)

with the transduced MDSCs. In a natural healing process, expression of growth factors is followed by expression of their specific antagonist (Yoshimura et al 2001). Noggin is the antagonist of BMP-4 and the simultaneous expression can improve bone regeneration that closely mimics the original tissue. It also prevents undesired bone regeneration and bone overgrowth. Gelfoam was also used as a scaffold for MDSCs and primary MDCs retrovirally transduced to express BMP-4 (Shen et al 2004). The scaffold was implanted into the thigh muscles of syngeneic mice. All cell populations secreted high levels of BMP-4, but the MDSCs produced significantly more bone than the MDCs. The BMP-4 expression in MDSCs lasted for a longer period at the bone forming sites than the MDCs.

A retroviral vector was used to introduce BMP-7 cDNA into periosteal-derived rabbit MSC (Mason et al 1998, 2000) or to introduce the BMP-7 gene or the Shh gene into periosteal-derived mesenchymal stem cells (Grande et al 2003). In the first study cells were expanded, seeded onto PGA grafts and implanted into a rabbit knee osteochondral defect. Grafts containing BMP-7 gene modified cells showed complete or near complete bone and articular cartilage regeneration at eight and twelve weeks. The transduced periosteal stem cells in the study by Grande et al (2003) were cultured and seeded onto a PGA scaffold. Scaffolds were implanted into full thickness osteochondral defects in knees of rabbits. The quality of repair tissue was significantly enhanced resulting in a smoother surface and a larger amount of hyaline cartilage. Shh transduced cells exhibited the highest quality of hyaline cartilage but bone formation in the subchondral compartment was reduced because of the maintenance of the cartilage phenotype over a longer period compared with the BMP-7 transduced cells (Figure 15).

A study by Chang et al (2003a, b) made use of an adenovirus to introduce BMP-2 to marrow stromal cells of miniature swine seven days before implantation. Cells were mixed with collagen type I and the constructs were implanted to fill a cranial defect. Near complete repair of the defects was observed and the new bone area was significantly greater than the control group after three months. Biomechanical tests illustrated that the maximal compressive strength of the new bone was comparable with that of normal cranial bone. Bilateral maxillary defects were created and a biodegradable PLA plate was used as an internal splint (Chang et al 2003b). The collagen/transduced cells construct was slowly gelled and implanted to fill the defect. Visual bone formation was noted at the defect site after three months. Histological examination showed mature woven bone with good mineralization. The repaired defects showed no sign of the adenoviral vector after three months. Tsuda et al (2003) transduced bone marrow derived MSCs with an adenoviral vector carrying the human BMP-2 gene and containing a RGD containing peptide. Higher amounts of BMP-2 were produced in those cells compared with cells infected with the BMP-2 carrying gene containing the wild-type fibre (Figure 16). Bone formation was enhanced in-vivo when transduced MSCs were seeded onto a hydroxyapatite scaffold and subcutaneously implanted in syngeneic rats.

Human liposuction aspirates contain a heterogeneous population of cells, including multipotent cells called processed lipoaspirate cells. These cells are easy to obtain, carry a relatively lower donor site morbidity and are available in large numbers. The processed lipoaspirate cells were exposed to Ad.BMP-2 for four to seven days, seeded onto collagen sponges and placed in SCID mice to test for bone induction (Dragoo et al 2003). Haematoxylin and eosin sections confirmed bone formation at six weeks. In a different study Ad.BMP-7-transduced adipose-derived adult stem cells were mixed with a collagen Type I matrix and placed into the subcutaneous pocket of rats (Yang et al 2005). After four weeks trabecular-like new bone was formed while in contrast no new bone was found after implantation of untransfected stem cells.

Other cells Muscle-derived cells (MDC) and chondrocytes have also been transduced using the ex-vivo method and seeded onto a scaffold. Primary cultures of MDCs from male mice were transduced with Ad.BMP-2. These cells were implanted into a critical-sized skull defect in female SCID mice by using a collagen sponge (Lee et al 2001). The defects had >85% closure within two weeks and 95–100% within four weeks. Control groups showed at most 30–40% closure. The majority of the cells were located on the surfaces of the newly formed bone. However, a small fraction occupied the lacunae of the new bone where osteocytes normally reside. Some of these cells were co-localized with osteocalcin immunohistochemistry, indicating that they were able to differentiate into the osteogenic lineage. Rabbit MDC and chondrocytes were transduced with a retrovirus encoding for the β-gal gene (Adachi et al 2002). The cells were seeded onto a type I collagen gel and cultured for three weeks. The implants were grafted in full thickness articular cartilage defects of rabbits. Transgene expression was detected in the defects only up to four weeks after implantation; however, the repaired tissue in the MDC and chondrocyte groups were better histologically than the control groups. The tissues were almost entirely composed of type II collagen in the MDC and chondrocyte groups. Another study involved the retrovirus transduction to overexpress Runx2 in primary skeletal myoblasts (Gersbach et al 2004). Cells were subsequently seeded onto a 3D fibrous collagen scaffold. Exogenous Runx2 expression induced osteogenic differentiation and repressed myogenesis in these constructs compared with the controls. A mineralized layer of hydroxyapatite was observed at the periphery of the construct and it was sufficient to increase the

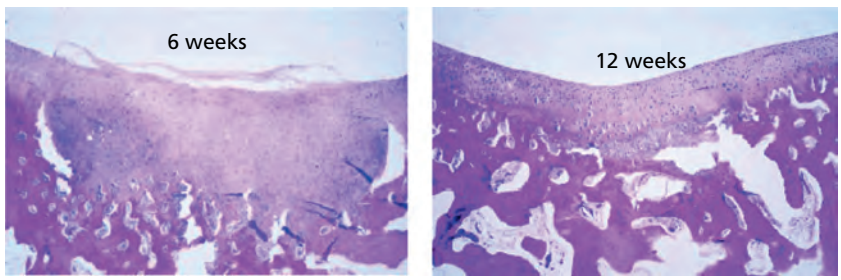

Figure 15 Histological (haematoxylin & eosin) appearance of a defect treated with the Shh gene. The eventual remodelling into bone had taken place by twelve weeks. Note especially the quality of the hyaline cartilage formed at the joint surface (original magnification ×40). (Reprinted from Grande et al, Copyright (2003), with permission of The Journal of Bone and Joint Surgery, Inc.)

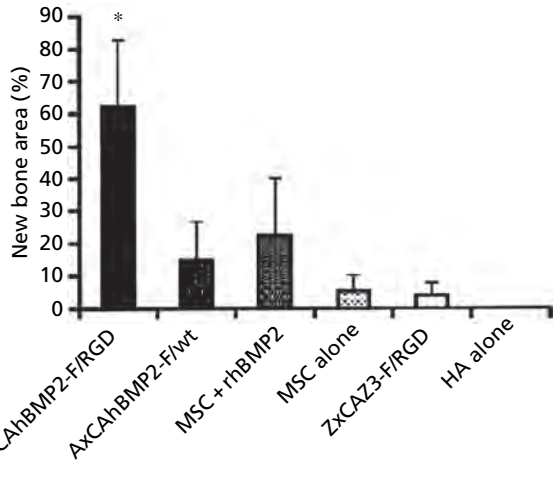

Figure 16 Quantification of new bone formation. New bone area was calculated as a percentage of bone area per total area of each pore. An asterisk denotes the statistically significant difference between the AxCAhBMP2-F/RGD-transduced MSCs group and all other groups. (Analysis of variance, $P < 0.05$). (Reprinted from Tsuda et al, Copyright (2003), with permission from Elsevier.)

compressive modulus 30-fold compared with the controls. In another study bovine nasal chondrocytes were transduced with a retrovirus bearing the human tissue inhibitors of metalloproteinase-1 (TIMP-1) gene (Kafienah et al 2003). PGA scaffolds were seeded with those cells and the constructs were grown for 40 days. In the next step the 3D constructs were incubated with or without interleukin-1 (IL-1) for four weeks. The transduced chondrocytes expressed TIMP-1 for the whole period. TIMP is known to inhibit a number of matrix metalloproteinases (MMPs) involved in the degradation of cartilage matrix. MMP activity was reduced to control basal levels in the transduced IL-1 stimulated tissue engineered cartilage (Figure 17).

Wound healing The use of a retrovirus epidermal-mediated gene transfer has been restricted to ex-vivo approaches due to their inability to transduce non-proliferating cells. Human keratinocytes, transduced using a retroviral vector encoding PDGF-A or IGF-1, were cultured on mitomycin C-treated 3T3 cells used as a feeder layer to produce epithelial sheets (Eming et al 1996). The detached epithelium was placed on a small piece of silastic membrane and implanted under a full thickness skin flap on the back of athymic mice. Seven days after grafting, modified cells expressing PDGF-A or IGF-1 underwent terminal differentiation and generated a stratified epithelium comparable to unmodified cells. Compared with control grafts of unmodified cells or grafts expressing IGF-1, the newly formed connective tissue layer subjacent to the PDGF-A modified grafts was significantly thicker and showed an increase in type I collagen and fibronectin deposition. In another study, primary rat dermal cells were transduced using a retroviral vector encoding for PDGF-B gene (Breitbart et al 1999). Cells were seeded onto a PGA fibre scaffold and were cultured for three weeks before implantation into rat excisional skin wounds. At 14 days all wounds had re-epithelialized. A decrease in the cellularity of the regenerated dermis at 14 days compared with 7 days was observed with the exception of the PDGF-B group.

Non-viral approach Non-viral vectors have been investigated for the ex-vivo gene delivery system; either naked

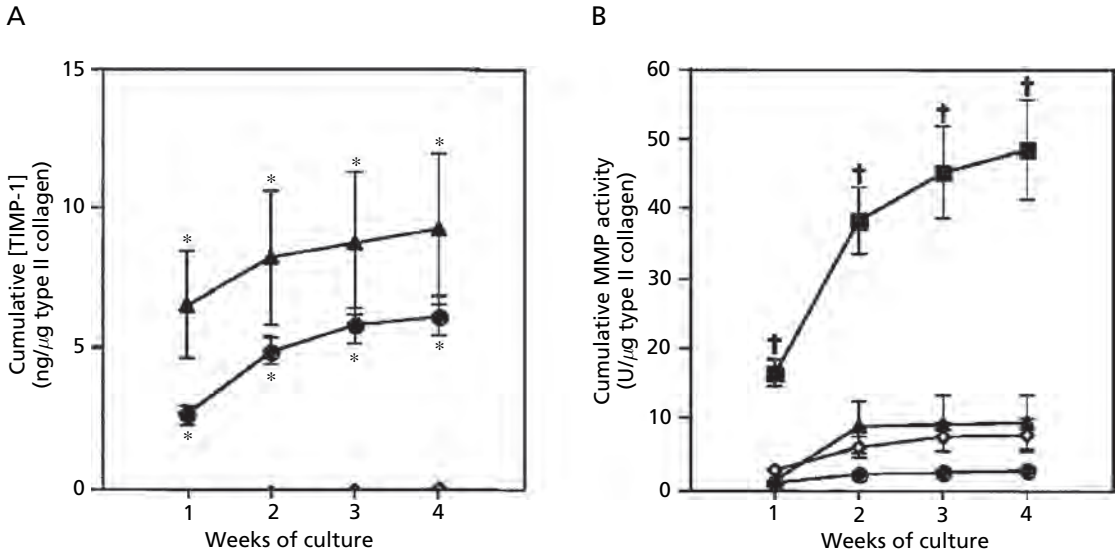

Figure 17 Up-regulation of tissue inhibitor of metalloproteinases 1 (TIMP-1) and inhibition of MMP in transduced, engineered cartilage cultures. Cartilage was grown from TIMP-1-transduced chondrocytes or untransduced control chondrocytes and then cultured with or without interleukin-1 (IL-1) for four weeks. Culture medium was collected at weekly intervals during the IL-1 stimulation period and assayed for (A) TIMP-1 by enzyme-linked immunosorbent assay, and (B) MMP activity by a quenched fluorescent substrate assay. Values are the mean ± s.e.m. For TIMP-1-transduced tissue-engineered cartilage, there were eight experiments with IL-1 (▲) and eight control experiments without IL-1 (●); for untransduced tissue-engineered cartilage, there were four experiments with IL-1 (■) and four control experiments without IL-1 (◇). *$P < 0.01$ compared with untransduced controls; †$P < 0.01$ compared with all other cultures. (Reprinted from Kafienah et al, Copyright (2003), with permission from Wiley & Sons, Inc.)

plasmid DNA or condensed DNA was used. This approach has been used for urothelial and vascular tissue engineering applications and to help bone and cartilage repair. The scaffolds used in these studies were hydroxyapatite, PGA, PLA and collagen.

Bone and cartilage repair BMSCs, transfected with a plasmid encoding BMP-4, were combined with a porous hydroxyapatite scaffold and used in an ectopic rabbit model (Jiang et al 2005). This gene transfer technique enhanced expression of BMP-4 and promoted differentiation from BMSCs to osteoblasts. In a different study, cationized gelatine complexed plasmid DNA encoding BMP-2 was incorporated into a PGA fibre (Hosseinkhani et al 2005, 2006). Marrow stromal cells were seeded on these scaffolds and cultured by static and perfusion methods. The construct was then implanted into the back subcutis of rats. Homogeneous bone formation was observed throughout the scaffolds with transfected cells, using the perfusion method. Alkaline phosphatase activity and osteocalcin content at the implanted site were significantly higher compared with those seeded with other agents (PBS, MSC, naked plasmid, cationized gelatine–plasmid complexes, transfected MSC by static method) (Figure 18). In a study by Goomer et al (2001) primary perichondrial cells and chondrocytes were permealized using a mild detergent. Plasmids were first complexed with transferrin–poly-L-lysine and then added to liposomes. These tertiary lipopolyplexes were then incubated with the permeabilized

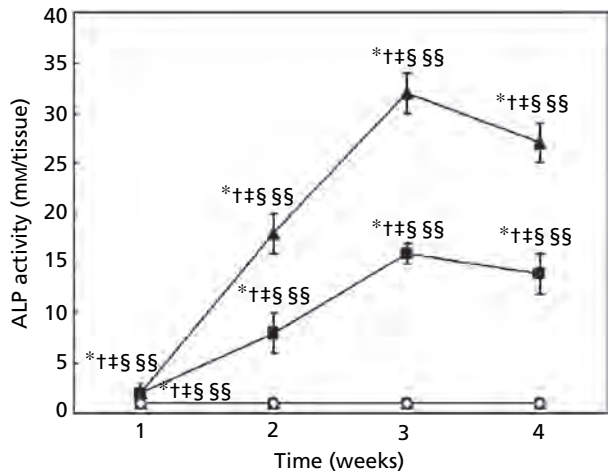

Figure 18 Time course of alkaline phosphatase (ALP) activity of tissues around the implanted site of collagen sponges with PGA fibre incorporation at collagen/PGA fibre weight ratio of 0.2 impregnated with PBS (○), MSC (□), naked plasmid DNA (△), cationized gelatin-plasmid DNA-BMP-2 complex (■), transfected MSC by static method (◇), and transfected MSC by perfusion method (▲). The dose of plasmid DNA was 10 μg mL^{-1}. The flow rate of medium perfusion was 0.2 mL min^{-1}. *$P < 0.05$ compared with the ALP activity of PBS at the corresponding week. †$P < 0.05$ compared with the ALP activity of MSC at the corresponding week. ‡$P < 0.05$ compared with the ALP activity of naked plasmid DNA at the corresponding week. §$P < 0.05$ compared with the ALP activity of cationized gelatin plasmid DNA-BMP-2 complex at the corresponding week. §§$P < 0.05$ compared with the ALP activity of transfected MSC by static method at the corresponding week. (Reprinted from Hosseinkhani et al, Copyright (2006), with permission from Elsevier.)

cells. Primary chondrocytes transfected with parathyroid hormone related protein (PTHrP) genes 1–87 and 1–173 were seeded onto a PLA scaffold and placed in a defect in the rabbit knee. The expression of the truncated PTHrP construct was higher than its wild type isomer 1–173; these differences were consistent with the observation that elimination of the carboxy-terminal sequences of PTHrP 1–173 increased production and secretion of the truncated 1–87 isomer.

A commercially available type I collagen sponge, Resorba, seeded with FuGENE complexed N2TetOnLacZ-transfected chondrocytes was implanted in full thickness defects in the knees of rabbits (Ueblacker et al 2004). Gene expression was induced by doxycycline. After three weeks the regenerated tissue appeared smooth and resembled the surrounding articular cartilage. Histological data showed that the implants were integrated into the cartilage and bone. Collagen fibres from the cell-containing sponge as well as newly formed collagen were demonstrated (Figure 19).

Urothelial tissue engineering Human urothelial cells were expanded in-vitro and seeded on PGA scaffolds (Yoo & Atala 1997). After seven days in culture the scaffolds were transfected by electroporation and were implanted in the dorsal subcutaneous space of athymic mice. Luciferase activity was observed at days 1, 3, 5 and 7 with a peak at day 5 in the engineered neo-organ structure.

Vascular tissue engineering Rat aortic smooth muscle cells were liposomally transfected with lysyl oxidase and seeded in a collagen gel and subjected to dynamic tensile testing after one week of in-vitro culture (Elbjeirami et al 2003). The tensile strength and elastic modulus was increased by approximately 2-fold compared with a control, plasmid DNA vector with no lysyl oxidase cDNA. Desmosine levels, a product of lysyl oxidase-mediated cross-linking of elastin, were higher. This approach may benefit many tissue engineering applications where there are concerns about mechanical failure.

Conclusions

This review has demonstrated that there exists a significant number of reports on gene therapy in tissue engineering, but these cover a huge range of different engineered tissues, different vectors, scaffolds and methodology. Consequently there are as yet few comparisons between these variables to show which are the best for any particular application. With a few exceptions, all of the results were positive in showing some gene expression and some consequent effect on tissue growth and remodelling.

There have been reports of direct gene transfer in the absence of scaffolds, whether in-vivo or ex-vivo, viral or non-viral. This is potentially an attractive method because it involves only one procedure for the patient. However, the difficulty of transfecting cells in-vivo with non-viral vectors and the possibility of adverse reactions with viral vectors may outweigh the attraction. The greatest proportion of papers have used a viral approach, as may be expected due to the enhanced transfection efficiency of this class of vectors. The viral approach is particularly attractive for an ex-vivo approach since high transfection levels are obtained and the

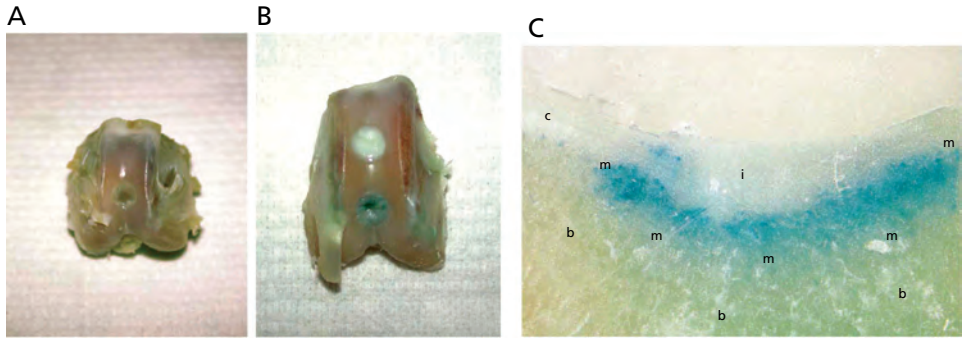

Figure 19 Femoral condyles three weeks after implantation of chondrocyte-seeded collagen sponges. A. Non-induced control specimens did not demonstrate X-gal staining macroscopically. B. As an internal control and to detect possible unspecific X-gal staining two defects were generated in one animal: non-transfected cells were implanted into the proximal and N2TetOnLacZ-transfected cells into the distal defect. X-gal staining was detected in the defect with the transfected cells after treatment with doxycycline only. C. Transversely cut section through a femoral condyle of the induced group demonstrating the depth and the cumulative effect of X-gal stained cells in the defect; b = bone, c = cartilage, i = implant, m = margins of the defect. (Reprinted from Ueblacker et al, Copyright (2004), with permission from OsteoArthritis Research Society International.)

immune responses to viral particles and components and toxic effects from transfection agents are minimized. However, one report on direct in-vivo administration highlighted that the viral vector was not fully effective due to immune interference (Musgrave et al 1999). In general, the safety issues associated with viral systems have either not been seen or not been investigated thoroughly in the area of gene therapy in tissue culture. The expected effects include intercellular trafficking and the implications of mutations and genetic alterations due to integration, which may result in generation of tumours. Eighteen year old Jesse Gelsinger, a patient who suffered from ornithine transcarbamylase (OTC) disorder, died in a gene therapy trial after the injection of a corrective gene encased in a deactivated adenovirus into his hepatic artery (Marshall 1999). The virus triggered a fatal immune response. This tragic death has prompted research to make the adenoviral gene therapy safer. Viral vectors could be made safer by the use of a suicide gene, such as HSV-1 thymidine kinase (HSVtk) (Nathwani et al 2004). In the advent of unplanned malignant transformation transduced cells could then be selectively eliminated. However, the long-term reliability of this method with respect to continued gene function and potential immunogenicity has, to our knowledge, not been thoroughly investigated.

The ex-vivo approach to gene therapy looks interesting at a research level because it reduces the challenges faced by in-vivo applications. However, in practice commercializing the individualized therapy required by ex-vivo application is likely to make this route less attractive for clinical use. In this review a number of the studies have shown significant transfection, but which in biological terms has a modest effect when compared with controls. However, in other instances excellent results have been demonstrated e.g. the cartilage regeneration described by Ueblacker et al (2004). Significantly this result was achieved with a non-viral vector, which should give more support for work with this intrinsically safer approach. This work also demonstrated the use of a regulatable gene controlled by antibiotic administration, a technology which may be particularly useful in addressing issues of temporal control.

For many types of tissue engineering a scaffold is an advantage both in terms of tissue support and providing a delivery vehicle for a gene vector. If a scaffold is used, the selection of scaffold is as important as the choice of cell type, vector or gene and is influenced by the anatomical location. For example the use of hydroxyapatite to heal bone defects on rabbit cranium was not beneficial since the cells were not able to enter the scaffold sufficiently quickly enough to produce therapeutic levels of protein to induce osteogenesis (Ono et al 2004). In contrast the delivery of PEI-complexed DNA from a PLGA scaffold led to an enhanced mineralized tissue regeneration (Huang et al 2005b). Poly(α-hydroxyacid)s can cause a mild, non-specific tissue response with fibroblast activation and the invasion of macrophages, foreign-body giant cells and neutrophils during their final stage of degradation (Weiler et al 2000). Despite these drawbacks, there are several advantages to these polymers. The rate of degradation of these polymers can be controlled; this is necessary to match resorption with appropriate developmental stages in tissue growth. FDA approval and an established history of use guarantee that these synthetic polymers will be used as scaffolds for tissue engineering applications.

The ultimate goal for gene therapy is to produce a system that involves a one step delivery of the gene of interest. The GAM approach is interesting because it can harness the advantage of the progress currently being made in tissue engineering. The scaffold provides a physical support for cell adhesion and a template for tissue formation and the delivery of DNA from the scaffold can induce specific cellular processes. The delivery of more than one gene might be more effective if they can act synergistically, as indicated by the study from Fang et al (1996). Overall a number of studies have shown in principle that gene therapy could benefit tissue engineering.

We must bear in mind however that the current studies are mainly at a research level. This review has identified a number of areas where technical improvements are needed in vector, scaffolds, materials, level and control of gene expression and choices need to be made about the correct strategy for different applications. Refining the technology and producing

systems that are effective, convenient and safe for human use (Partridge & Oreffo 2004) will still require many years of further work. We anticipate that clinical trials may initially favour the more widely used viral vectors, but in the longer-term non-viral vectors are more likely to satisfy the criteria of safety and convenience.

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