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Gene therapy methods in bone and joint disorders

Evaluation of the adeno-associated virus vector in experimental models of articular cartilage disorders, periprosthetic osteolysis and bone healing

Doctor of Medical Science Thesis

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Contents

Titles of included studies

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I–VIII).

I. Ulrich-Vinther M, Maloney MD, Goater JJ, Soballe K, Goldring MB, O'Keefe RJ, Schwarz EM. Light-activated gene transduction enhances adeno-associated virus vector-mediated gene expression in human articular chondrocytes. Arthritis Rheum. 2002 Aug; 46 (8): 2095-104.

II. Ulrich-Vinther M, Duch MR, Soballe K, O'Keefe RJ, Schwarz EM, Pedersen FS. Adeno-associated virus vector mediated in vivo gene delivery to articular chondrocytes. J Orthop Res. 2004 Jul; 22 (4): 726-734.

III. Ulrich-Vinther M, Stengaard C, Schwarz EM, Goldring MB, Soballe K. Adeno-associated vector mediated gene transfer of Transforming Growth Factor-beta1 to normal and osteoarthritic human chondrocytes stimulates cartilage anabolism. Eur Cell Mater. 2005 Nov; 14 (10): 40-59.

IV. Ulrich-Vinther M, Carmody EE, Goater JJ, Soballe K, O'Keefe RJ, Schwarz EM. Recombinant adeno-associated virus-mediated osteoprotegerin gene therapy inhibits wear debris-induced osteolysis. J Bone Joint Surg Am. 2002 Aug; 84-A (8): 1405-12.

V. Ulrich-Vinther M, Andreassen TT. Osteoprotegerin treatment impairs remodelling and material properties of callus tissue without influencing structural fracture strength. Calcif Tissue Int. 2005 Apr; 76 (4): 280-6.

VI. Ulrich-Vinther M, Schwarz EM, Soballe K, Pedersen FS, Andreassen TT. Gene therapy with human osteoprotegerin decreases callus remodelling with limited effects on biomechanical properties. Bone. 2005 Dec; 37 (6): 751-8.

VII. Flick LM, Weaver JM, Ulrich-Vinther M, Abuzzahab F, Zhang X, Dougall WC, Anderson D, O'Keefe RJ, Schwarz EM. Effects of receptor activator of NFkB (RANK) signaling blockade on fracture healing. J Orthop Res. 2003 Jul; 21(4): 676-84.

VIII. Koefoed M, Ito H, Gromov K, Reynolds DG, Awad HA, Rubery PT, Ulrich-Vinther M, Soballe K, Guldberg RE, Lin AS, O'Keefe RJ, Zhang X, Schwarz EM. Biological effects of rAAV-caAlk2 coating on structural allograft healing. Mol Ther. 2005 Aug; 12(2): 212-8.

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Tabula gratitudinis

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Abbreviations

AAT	a1-antitrypsin deficiency
AAV	adeno-associated virus
ACI	autologous chondrocyte implantation
Ad	adenovirus
AIDS	acquired immunodeficiency syndrome
BMP	bone morphogenetic protein
Cap	genes coding for capsid proteins
caALK2	constitutively active activin receptor-like kinase-2
CCL-64	mink lung epithelial cells
CD	cluster of differentiation (groups of monoclonal antibodies)
CDMP	cartilage-derived-morphogenetic protein
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CNS	central nervous system
CsCl	cesium chloride
DNA	deoxyribonucleotide acid
E1	E1 region of the Ad genome (involved in regulation of transcription of the genes of host infection)
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein (marker gene)
ELISA	enzyme-linked immunosorbent assay
env	genes coding for retroviral envelope
FGF	fibroblast growth factor
FADD	fas-associated death domain protein
gag	genes coding for structural proteins of the retroviral virion core
GH	growth hormone
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IFN-γ	interferon produced by mitogenically or antigenically stimulated lymphocytes
IGF	insulin-like growth factor
IL	interleukine
IL-1Ra	IL-1 receptor antagonist
IRES	internal ribosome entry site
ITR	invers terminal repeats
kb	kilobases
LacZ	β-galactosidase (marker gene)
LAGT	light-activated gene transduction
LTR	long terminal repeats
M-CSF	macrophage colony stimulating factor
MMP	metalloproteinase
MOI	multiplicity of infection
MPEG	monomethoxypolyethylene glycol
NGF	nerve growth factor
OPG	osteoprotegerin
OTCD	ornithine transcarbamylase deficiency
p	plasmid (*prefix*)
PDGF	platelet-derived growth factor
pHPMA	poly-hydroxypropyl methacrylamide
PMMA	polymethyl methacrylate
pol	genes coding for retroviral enzymatic proteins
PTH	parathyroid hormone
r	recombinant (*prefix*)
RANK	receptor activator of nuclear factor-κB
RANK:Fc	RANK fusion protein; blocking the activity of RANK
RANKL	receptor activator of nuclear factor-κB ligand
RCR	replication-competent retroviruses
Rep	genes coding for replication proteins
RNA	ribonucleotide acid
RT-PCR	reverse transcriptase polymerase chain reaction
RU486	mifepristone (progestational and glucocorticoid hormone antagonist)
SCID-X1	X chromosome-linked severe combined immune deficiency
SIV	simian immunodeficiency virus (retrovirus)
SV40	simian virus-40 (polyomavirus)
Tet	tetracycline (antibiotic)
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α
TNFR:Fc	TNF receptor fusion protein (blocking the activity of TNF)
tsT/AC62	immortalized human adult articular chondrocyte cell line
UHMWPE	ultra-high molecular weight polyethylene
VEGF	vascular endothelial growth factor
vp	viral particles
wt	wild-type (*prefix*)

Gene therapy

Gene therapy is a technique whereby new genes are introduced into cells in order to treat disease by restoring or adding gene expression (Figure 1). Persuasive arguments in its favor were first presented by Friedmann and Roblin in 1972 (1). The technique has gained widespread acceptance for its potential therapeutic effects, even if manipulation of somatic cells may not be entirely unproblematic. Theoretically, it may be useful for a wide spectrum of diseases, including the treatment of bone and joint disorders.

Developments in molecular biology in the early 1970s provided the basic knowledge and tools for the first clinical experiments with gene therapy in the early 1990s (2). By 1995, approximately 100 gene transfer protocols had been approved by the US National Institutes of Health, Office of Biotechnology Activities (3). Today, more than 1000 clinical gene therapy protocols have been approved worldwide: about 66% (n=708) in the United States and 28% in Europe (n=304), with the Nordic countries accounting for 9 (Finland=3; Norway=4; Sweden=2; Denmark and Iceland=0) (4) (Figure 2). Most of these trials have obviously been phase I studies (63%, n=678), but a few phase III investigations have been initiated (2%, n=20). They have primarily been targeting cancer diseases (66%, n=656), but trials on deficiency diseases (monogenetic diseases) (9%, n=95), vascular diseases (9%, n=92) and infectious diseases (7%, n=72) are also

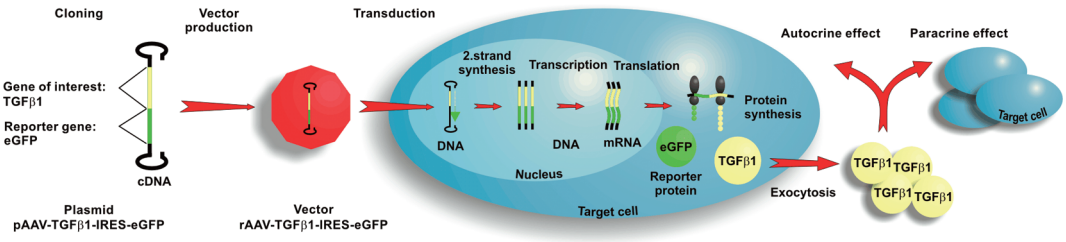

Figure 1. The principle of gene transfer exemplified by AAV vector mediated transfer of the $TGF_{\beta 1}$ gene. The gene of interest is cloned into a vector construct dedicated to transferring the gene to the target cell and responsible for initiating the cellular protein synthesis process in order to achieve an auto- / paracrine stimulation by the transgene protein.

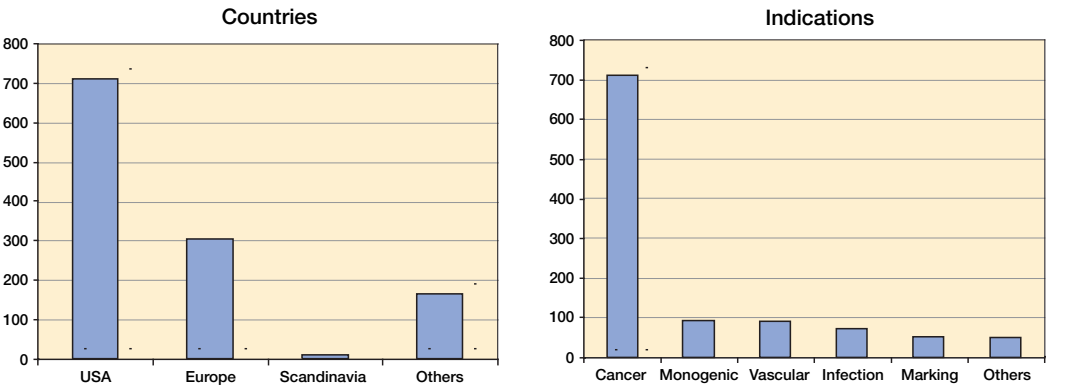

Figure 2. Diagrams showing the number of approved, ongoing or completed clinical trials worldwide. Data are available for countries where trials are being performed, indications addressed, vectors used, and clinical trials at different phases conducted (4).

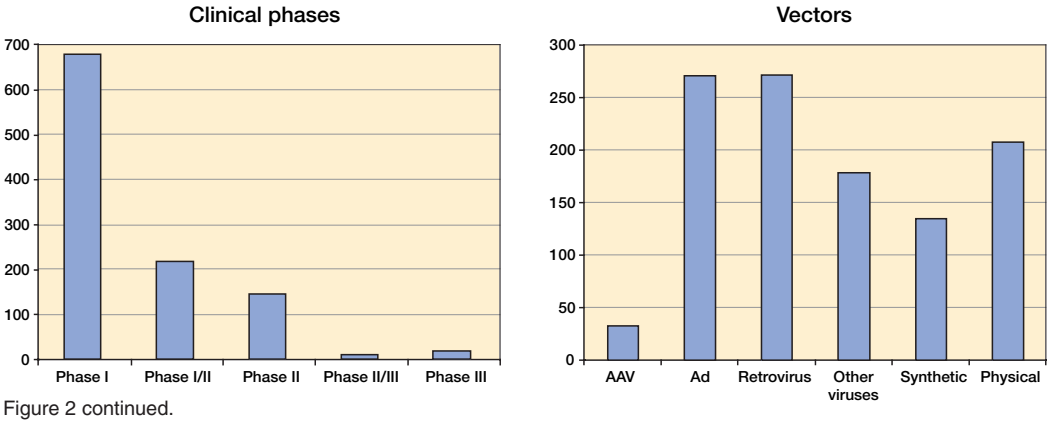

Figure 2 continued.

Table 1. Gene therapy clinical trials for non-malignant diseases

Approach	Disease	Vector system	Study phase	References
Monogenic disorders				
Direct	Cystic fibrosis	AAV	2	(91;93)
	Cystic fibrosis	Adenovirus	1	(538;539)
	Cystic fibrosis	Cationic liposomes	2	(540)
	AAT-deficiency	AAV	1	(90)
Ex vivo	SCID	Retrovirus	1	(541)
	Haemophilia B	AAV	1	(89)
Multiaetiological diseases				
Direct	Coronary artery disease	Naked DNA	1	(542)
		Adenovirus	2	(543)
		Cationic liposomes	2	(544)
	Peripheral arterial disease	Adenovirus	2	(545)
Ex vivo	Amyotrophic lateral sclerosis	Calcium phosphate coprecipitation	1/2	(546)
Infectious diseases				
Direct	HIV	Retrovirus (lentivirus)	2	(402)

ongoing. In these studies, cytokines (26%, n=274), antigenes (15%, n=162) and tumour suppressor genes (12%, n=132) are the most typical transgenes. Published clinical reports on gene therapy presently available comprise small phase I or II trials (Table 1). No peer-reviewed data from phase III trials are yet available demonstrating the clinical effectiveness of gene therapy in any disease.

Gene therapy in orthopaedics

Some diseases of the locomotive system cannot be cured successfully due to the limited healing capacity of most of the tissues constituting the musculoskeletal system. Thus, ligaments, tendons, menisci, and articular cartilage all have low blood supply and reduced cell turnover. Even bone, which is normally capable of regeneration, can be problematic, especially in deteriorative disorders such as osteoporosis.

Numerous growth factors and other proteins (e.g. bone preservative cytokines, antibiotics and anti-inflammatory drugs) capable of promoting regeneration of these tissues have been identified, but delivery problems often hinder their use. These problems may be overcome if relevant genes can be delivered to the target tissues. The advantages of gene delivery include the ability to establish a local, endogenous synthesis of authentically processed therapeutic proteins at the site of deterioration or injury, whereby therapeutic substances are persistently produced directly by local cells "on location". Novel techniques are addressing the problem of ensuring targeted therapeutic gene delivery in sufficient quantity to improve local healing process.

Chronic diseases constitute an obvious group of promising orthopaedic targets for gene therapy (**5-9**). Progress has been most promising in arthritis (**6**) and disc degeneration (**10**), but the principles may also be applied to aseptic loosening occurring through pathologic resorption of bone around prosthetic joints (**11**). Osteoporosis, another chronic orthopaedic condition of enormous socioeconomic importance, is another attractive target for gene therapy. The only clinical trials yet initiated in the orthopaedic area involve gene transfer to joints (**12;13**). At present state, gene therapy has been applied to various orthopaedic models, including: 1) articular cartilage repair, 2) inhibition of wear debris induced osteolysis and 3) bone repair. These models and their results will be reviewed here with special attention to the recombinant AAV vector for gene delivery.

Genes of orthopaedic interest

Signal proteins

Various cytokines and growth factors have been found to enhance the healing process in tissues of the musculoskeletal system (Table 2) (**14-17**). Cytokines are non-antibody proteins primarily secreted by inflammatory leukocytes and acting as intercellular mediators. They differ from classical hormones in that they are produced by a number of tissue or cell types rather than by specialized glands. They generally act locally in a paracrine or autocrine rather than endocrine manner.

Growth factors are signal proteins synthesized by the resident cells at the ill site (e.g. fibroblasts, endothelial cells or mesenchymal stem cells) and by the infiltrating reparatory or inflammatory cells (e.g. platelets, macrophages or monocytes). Growth factors are involved in stimulating cell proliferation, differentiation and migration as well as matrix synthesis.

The genes encoding for many of the known growth factors and cytokines have been determined and it has therefore become possible to use these signal proteins in therapeutic gene therapy.

Table 2. Effects of signal proteins in various tissues of musculoskeletal system

	Skeletal muscle	Articular cartilage	Meniscus	Ligament /tendon	Bone
IGF-1	+	+	+/–	+	+
bFGF	+	+	+	+	+
aFGF	+/–		–	+/–	
NGF	+		–	–	
PDGF	+/–		+/–	+/–	
EGF	–	+	+	+	–
TGFα	–		+	–	
TGFβ	–	+/–	+	+/–	+
BMP		+	+		+
CDMP		+			
VEGF					+/–
OPG		(+)			+/(–)

Application of signal proteins

The effectiveness of medical therapy depends on the interaction between the pharmacological molecule and the cellular target receptor. The cellular response reflects the quality and duration of the stimulation. Although direct application of human recombinant proteins can have beneficial effects on the healing process (**16;18-20**) and in anti-inflammatory therapy (**21-23**), very high dosages and repeated injections of these proteins are often required due to their short biological half-lives. The use of growth factors to promote healing and other cytokine to inhibit auto-immunologous inflammatory reactions in joints or skeletal deterioration (e.g. osteoporosis) is also severely hindered by the difficulty of ensuring their delivery to a specific injury site. Finally, the effect of some of the most essential and potent of these signal molecules, such as TGF-β, is highly dependent on the momentous concentration of the protein.

Many strategies, including the use of polymers, pumps and coated implants, have been investigated as possible methods for achieving constant growth factor levels at a given site (**24-28**). Although capable of improving local persistence of signal proteins, their success remains limited. Transfer of genes encoding relevant signal proteins has the potential to overcome the delivery problems associated with the use of the proteins themselves (**24;28-31**).

Gene delivery systems

The principle

Foreign genes are not easily transferred to a recipient cell. *In vivo,* four extra-cellular barriers to gene transfer exist (**32**): 1) opsonins, 2) phagocytes, 3) extra-cellular matrices and 4) degradation enzymes. The major cellular obstacle to naked DNA uptake is the negative molecular charge of the cell membrane, which causes DNA repellation. Once intracellular, the gene must be protected from degradation by cytoplasmatic endonucleases and endosomal entrapment. Hence, gene delivery vehicles (*lat.* vectors) capable of efficiently carrying the DNA into the target cell nucleus are necessary for any practical transfer.

The ideal gene delivery vector is non-toxic, non-immunogenic, easy to produce in large quantities, and efficient in protecting and delivering DNA into cells, preferably with specificity for the target cell and capable of regulating and controling the levels of transgene protein expression in the transduced cells (Table 3). This ideal vector remains to be discovered.

Various techniques have been deployed for introducing new genes into mammalian cells for the purpose of gene expression (**33-36**). Based on vector genesis and their cellular approach, these systems are divided into three major categories: 1) viral vectors, 2) synthetic vectors and 3) physical methods. Viruses are designed by nature to transfer genetic material into eukaryotic cells and they have therefore served as a starting point. However, safety concerns have directed attention towards the development of non-viral, synthetic vectors and physical DNA-delivery methods. The advantages of the non-viral vectors include their low immunogenicity, low acute toxicity, simplicity and large-scale production. Their drawbacks include lower gene transfer efficiency than viral vectors and transient gene expression in the recipient cells.

Table 3. Characteristics of ideal vector system

1	High transgene packaging capacity
2	High concentration that allows many cells to be transduced
3	Inexpensive, convenient and reproducible manufacturing
4	Appropriate route of delivery
5	Vector remains intact during delivery
6	Ability to target the desired cell type
7	High efficiency of foreign gene expression
8	Non-toxic to the host cell
9	Non-immunogenic
10	Post-transductional control of transgene expression
11	No viral replication / re-activation in host cells

Viral vectors

Viral gene delivery involves a number of vectors based on recombinant viruses including AAV, adenovirus, retrovirus and herpes simplex virus. The main advantage of viral vectors is the high gene delivery efficiency compared with non-viral transfer systems. However, important drawbacks associated with their practical use are evident, notably immunological toxicity and the potential replication of competent viruses (Table 4).

Adeno-associated virus vectors

AAV is a non-pathogenic, non-enveloped, small (20 nm), single-stranded DNA (5 kb of nucleotides) parvovirus (**37**) that has been known since 1965 (**38**), but did not become widely recognized until it emerged as a major player in the viral gene delivery arena. The viral genome contains two open reading frames that express four replication and three capsid proteins and is flanked by the 145 bp inverse terminal repeats (ITRs), which are the only cis-acting elements essential for replication, packaging or integration (**37**). Recent studies indicate that the ITRs also have weak transcriptional promoter activity (**39**). Thus, the entire AAV genome, except from these repeats, can be replaced by a transgene cassette of interest to form a biologically active recombinant AAV vector (**37**).

The natural tissue tropism of *wt*AAV is for lung epithelial cells; however, the AAV vectors have

Table 4. Comparison of vector systems

Feature	AAV	Adenovirus	Retrovirus	Herpes simplex	Non-viral
Maximum insert size	5 kb	37 kb	7 kb	30 kb	Unlimited
Maximum titre (particles/mL)	10^{12}	10^{11}	10^{8}	10^{8}	Unlimited
Spectrum of host cells	Broad	Broad	Dividing cells (Non-dividing cells; lenti virus)	Mainly neurons	Broad
Chromosomal integration	Episomal	Episomal	Random	Episomal	Episomal // unknown
Duration of expression	Long	Short	Long	Short	Short
In vivo risks	Immunogenic	Immunogenic Cytotoxic	Mutagenic (Carcinogenic)	Cytotoxic	Variable

been used to also efficiently transduce both dividing and non-dividing cells in liver, retina, central nervous system, intestinal epithelium, mesenchymal stem cells, skeletal muscle, osteoblasts and articular cartilage (**I-V;VI;VIII;40-48**) . This nearly ubiquitous tropism can be partly explained by the fact that AAV apparently uses widely expressed molecules as co-receptors, including heparan sulfate proteoglycan (primary attachment receptor) (**49**), fibroblast growth factor receptor 1 (**50**) and αVβ5-integrin (sub-receptors) (**51**).

The human *wt*AAV serotypes have shown remarkably different expression patterns because of differences in cell entry and intracellular activities. At present, more than 40 genomic variants have been identified (**52**). Their use as potential serotypes or pseudotypes for gene transfer is currently being investigated. The commonly used AAV-2 serotype vectors are characterised in terms of their wide target-range, long-term and relatively poor expression levels (**45**). The recent discovery of the novel rhesus monkey serotypes AAV-7 and AAV-8 may therefore be of interest because they may be more efficient human gene therapy applications (**53**). In particular, AAV-8 vectors have demonstrated an up to 100-fold higher factor IX expression in liver cells than any other AAV serotype. Moreover, the expression levels were not compromised by preimmunization with other AAV serotypes. Therefore, receptor-targeted capsid mutants and novel serotypes seem to broaden the recipient spectrum for AAV vectors and may improve the efficiency of AAV vector mediated gene transfer.

The relative inefficiency of AAV-2-based gene delivery has inspired studies of potential barriers to efficient gene transfer in tissues like lung (**54**). The first of these barriers to be clearly identified was the relative paucity of the common AAV attachment receptor, heparan sulphate proteoglycan, on the apical surface of respiratory epithelial cells (**55**). But even in tissues like skeletal muscle, where AAV2-based vector transduction appears to be relatively robust (**56**), substantial increases in vector transduction efficiency can be observed with alternative serotype capsids (**57**). For instance, a several-hundred fold improvement of transduction efficiency was observed in factor IX and AAT vectors cross-packaged into AAV-1 capsids (**57**). Similar improvements were observed in the central nervous system with other serotypes (**45**).

Neutralizing antibodies often prevent the readministration of AAV-2 vectors (**58**). However, successful readministration was achieved in mouse lung when the transgene was packaged into a different serotype (**59**). There is some cross-reactivity between neutralizing antibodies of certain serotypes, but this may depend on the infection route (**60**). Future clinical application of AAV vectors would therefore possibly require prior evaluation of the type of neutralizing antibodies present to determine which AAV serotype and which delivery route should be used.

As members of the dependovirus genus, the AAVs generally require a helper virus, like adenovirus, herpesviruses or vaccinia virus, in order to replicate themselves in the productive phase of their life cycle. The helper virus super-infection provides the AAV with essential helper functions needed to complete the reproductive life cycle. In the absence of such helper virus co-infection,

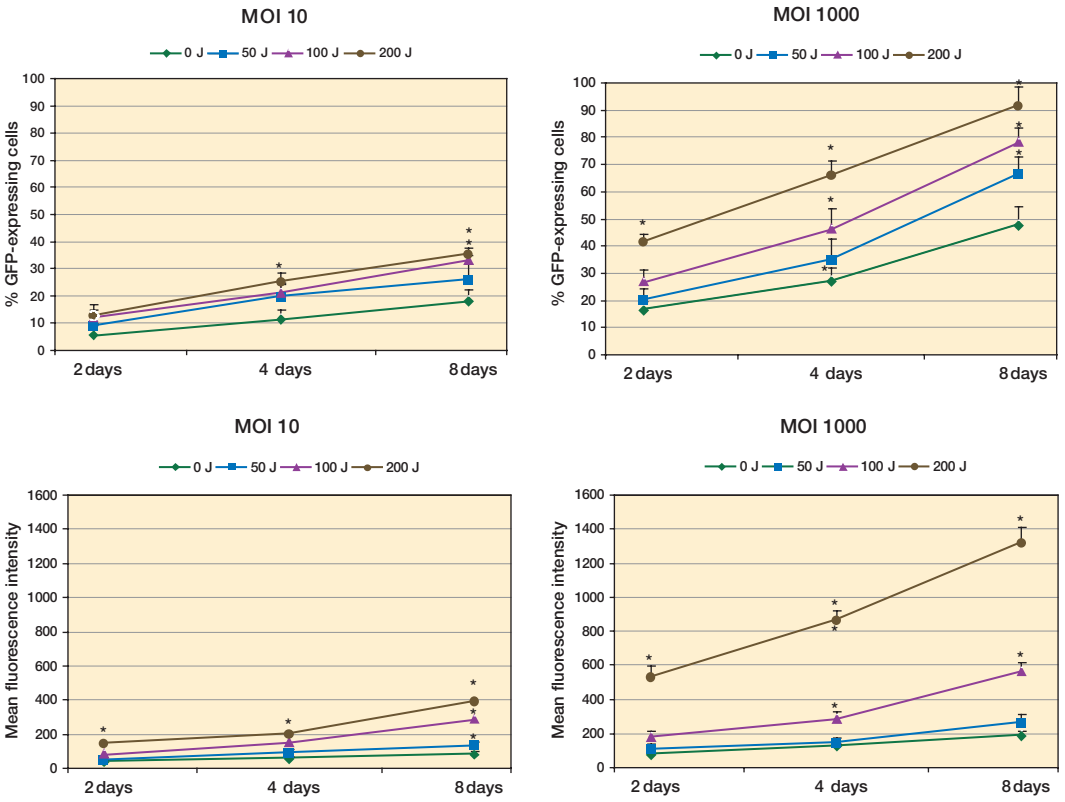

Figure 3. Ultraviolet light enhancement of transduction efficiency and gene expression in primary human articular chondrocytes transduced with AAV-eGFP (mean ± SEM). Primary human articular chondrocytes were isolated and grown in monolayer culture and exposed to ultraviolet light irradiation at doses of 0, 50, 100, or 200 J/m^2 (I). They were then infected with AAV-eGFP at MOI of 10 or 1,000, and analyzed on the indicated days for eGFP by flowcytometry. * p-value < 0.05 (exposed versus unexposed controls) (N = 6).

*wt*AAVs establish long-term latency within mammalian cells by integrating as concatamer and preferentially, but not exclusively, at the AAV-S1 locus on human chromosome 19 (**61**). This integration process requires AAV *rep* proteins and is not seen in recombinant AAV vectors (**62;63**). Upon subsequent helper virus infection of latent cells, the *wt*AAV genome is rescued and the virus reenters the replicative phase of the life cycle. The genome maturation kinetics is not well understood for the recombinant AAV vector genome. Recombinant AAVs, infecting a cell in the absence of helper virus and *rep* protein, persist as single-stranded genome for a certain period in transduced cells. Using host cell nuclear DNA polymerases, the single-stranded AAV genome is converted to double-stranded forms that may persist as linear or circular episomes and may also appear in the high-molecular-weight DNA. However, integration of AAV may occur at a very low frequency, and with a loss of chromosome 19 specificity (**64-66**).

The conversion of single-stranded AAV into double-stranded molecules appears to be the rate-limiting step in expression from AAV vectors (**67**). We therefore hypothesized that the major impediment to AAV vector mediated transduction of human articular chondrocytes was the second-strand synthesis (I), as it has been shown that such transduction is relatively inefficient, requiring an MOI >10^3 (**68**). Other studies have demonstrated a significant increase in second-strand synthesis by activation of endonucleases via ionising radiation (γ) or ultraviolet light irradiation (**69-71**). Our studies have demonstrated that ultraviolet light irradiation markedly enhances the transduction efficiency of articular chondrocytes at doses of up to 200 J/m^2, whereas it does not significantly affect cell viability (I,II) (Figure 3). These results

have been supported by subsequent studies demonstrating that the production of secreted TGFβ1 protein from AAV-TGFβ1-IRES-eGFP infected into human bone marrow derived-mesenchymal stem cells is highly inducible by ultraviolet light irradiation (**48**).

As the AAV vector genome primarily remains a nuclear episome, the duration of the transgene expression is largely dependent on the target cell lifespan. Tissues with low cellular turnover, such as skeletal muscle and articular cartilage, have hence turned out to have a promising potential for long-term AAV vector mediated systemic delivery of transgene products (**II;IV;VI;40;41;72**). In addition to sufficient AAV transducibility and low cellular turnover, they are easy to access for direct in vivo gene delivery. For systemic targeting of the transgene signal protein, the high vascularity and blood-flow makes skeletal muscles attractive for transduction, whereas articular chondrocytes, which are relatively isolated from the vascular system, are obvious targets for persistent AAV transgene expression in local intra-articular treatment (**II;73**).

Production of viral AAV vector systems relies on co-infection of the cloned AAV vector plasmid of interest and plasmids containing the required helper genes for successful viralisation (**74**). However, a new method, referred to as "HOT," is helper virus free, optically controllable and based on transfection of only two plasmids, i.e. an AAV vector construct and a novel AAV helper plasmid (**75**). The advantages of this method are increased production simplicity and improved safety profile compared with existing methods (**75**). Regarding AAV vector preparation, either strand of the DNA can be packaged in virions as single-stranded DNA (**37;76**). To address the concerns for the risk of adenoviral contamination of the AAV vector stocks and the presence of adenoviral denatured proteins, which is unacceptable in clinical use, a three plasmid co-transfection procedure has become standard for viral AAV vector production (**74**). The three plasmids are: 1) the *p*XX2 which is composed of the genes required for capsid and replication provided by a *p*AAV packaging construct containing only the AAV *cap* and *rep* genes. This plasmid increases the *r*AAV yield 15-fold compared with the conventional packaging plasmid *p*AAV/Ad (**74**); 2) the *p*XX6, i.e. the adenovirus helper functions delivered from a plasmid, containing the essential helper genes but lacking the adenoviral structural and replication genes; 3) the cloned plasmid encoding the transgenes of interest flanked by the AAV ITRs. The combination of these plasmids ensures a vector production method providing high titre AAV vector preparations that are completely free of adenovirus and *wt*AAV. Recent protocols have emphasized a potential increase in upstream production yields by using adenoviral gene plasmids *in lieu* of active adenovirus infection of packaging cells, and by moderating the amount of long Rep (*Rep78* and *Rep68*) proteins expressed relative to short *Reps* (52/40) and capsid. Other improved production methods have included the use of stable cell lines with inducible *Rep* expression and rescuable vector genomes or the use of herpes simplex virus amplicons or recombinants (**77-80**). Recent purification strategies have sought to avoid CsCl ultracentrifugation, which seems to decrease the infectivity of vector material and instead favoured column chromatography methods (**77-81**).

Safety is critical in viral gene delivery. A non-toxic delivery vector would clearly be required for gene therapy for chronic non-lethal diseases such as osteoporosis or osteoarthritis. This excludes various viruses causing serious diseases as scaffolds for vector systems. Nonetheless, 80% of all adults have circulating antibodies against AAV the *wt*AAV is not known to cause disease in the human population (**82**). The AAV vectors have not been shown to induce liver toxicity in primate studies (**83**). Furthermore, the lifelong transgene expression obtained in other animal models after AAV-vector mediated gene transfer is, in part, due to the inability to elicit a cell-mediated immune response (**84;85**). Shedding of AAV vectors has been investigated in various *in vivo* models in relation to different methods of AAV vector administration (**II;72;73;83;86-88**). Only one extensive study of AAV vector distribution after intramuscular administration has been carried out (88). In this study, several organs in one dog (injected with 2×10^{12} viral AAV vector particles) were tested using a non-quantitative polymerase chain reaction (PCR) method, and AAV vector was found only in the draining lymph node. While no AAV could be found in the semen (**87**) or testes (**88**) of dogs injected intramuscularly with AAV vector, AAV vector has been quite con-

sistently found in germ line cells of mice and rats after intravenous administration (**87**). These authors also demonstrated a 48-h viraemia lasting 48 h in rabbits. These findings were confirmed in Macaque monkeys by Favre (**83**), and in humans by Kay (**89**). The knowledge of viral vector shedding following articular administration is very sparse. Our studies revealed that intra-articular injection of 1.5×10^{12} viral AAV vector particles into rabbit knee-joints produced infection of tissues in direct physical contact with the joint cavity, whereas presence of AAV-expressing cells outside the joint cavity or active viral AAV particles could not be demonstrated (**II**). This finding was the same in animals with partial chondral as well as full-thickness cartilage defects. Our method of analysis will, however, not detect the shedding of inactive AAV-vector particles that are not causing any protein expression, nor will it detect cells in which eGFP expression lies below detection limit (**II**).

The use of AAV vectors in early-phase clinical trials has been reported for two diseases: cystic fibrosis and haemophilia B. Three phase I trials and two phase II trials have investigated the former disease (**90-92**) and they demonstrated biological activity induced by the AAV vector delivered transgene. Interestingly, the clinical studies did not show that pre-existing anti-AAV antibodies formed a significant barrier to vector entry. However, repeated dosing and the associated rise in anti-AAV antibodies still appear to prevent efficient readministration, particularly in the respiratory tract (**93;94**). Studies in patients with haemophilia B have yielded somewhat similar results on transduction efficiency to studies of cystic fibrosis (**89;95**). AAV vector systems have not yet been applied in the treatment of bone and joint diseases.

Based on the empirical advantages of rAAV vectors for orthopedic gene therapy (**37**), and the clinical potential of this vector (**96**), our group has evaluated the effects of freeze-drying and storage at β80°C on rAAV transduction efficiency (**VIII;97**). These studies revealed that rAAV vectors possess remarkably termostability, as almost 100% of the transducing units could be routinely recovered after freeze-drying and storage (**97**). From a practical standpoint, this rAAV-coating process can be easily adaptable to standard operating procedures used by tissue banks to prepare clinical allografts and AAV vector coated orthopaedic implants (**VIII;97**).

In summary, AAV vectors have several properties that make them attractive candidates for musculoskeletal gene therapy compared with other vector systems (**37**). These properties include: 1) the absence of a host inflammatory, cytotoxic or cell-mediated immune response that clears transduced cells and threatens the host; 2) a broad cellular tropism including skeletal myocytes (**IV;VI;41;72**), osteoblasts (**VIII**), synovial cells (**84;98**) and articular chondrocytes (**I-III;68;99**); 3) the ability to infect non-dividing cells (**41;72;100;101**); 4) the ability to deliver life-long gene expression in some cell types (**102-104**); and 5) production of high titre ($>10^{13}$/mL) with facile ultra-high grade purification (**74**). However, there are also important limitations, which include paucity of cell surface receptors on some cells for the most common AAV serotype (AAV-2), the loss of site-specific integration by recombinant AAV vectors, the limited packaging constraints (<5kb), long-term gene expression in dividing cells and a laborious and technically difficult procedures of viral AAV vector production. These limitations have spurred the search for new generations of AAV vectors based on a number of alternative AAV serotypes or on designer mutants of the AAV capsid, which have been retargeted for entry into previously non-permissive cells. A deeper understanding of the mechanisms of AAV integration and persistence would also seem to make it possible to retain the best features of the native virus and allow this system to move forward into broader clinical application. AAV vectors are useful tools for *in vivo* gene transfer, particularly where long-term gene expression is required.

Adenoviral vectors

Adenovirus is a non-enveloped, medium-sized (80 nm), linear, double-stranded (36 kb of nucleotides) DNA virus (**105**). Adenovirus infections are usually associated with mild respiratory disease in humans and they are considered to be non-oncogenic (**105**). Adenoviruses, which were first isolated in the 1950s, have been developed as gene-delivery vehicles since the early 1980s (**106**).

Most recombinant adenoviral vectors are based on human adenovirus serotypes 2 (Ad-2) and 5 (Ad-5) of subgroup C. They efficiently infect and

express their genes in all cells that possess the appropriate surface receptors, including dividing and non-dividing cells. Following internalization of the adenovirus, its genome is translocated into the cell nucleus, but it remains extra-chromosomal (**105**), which minimizes the risk of insertional mutagenesis and of non-transmission to the progeny of dividing cells. Hence, the adenoviral genome exists as a free, autonomously replicating DNA sequence (episomal element). Furthermore, they are easy to manipulate using standard cloning techniques (**107;108**) and can be produced in high titres, allowing them to be used *in vivo.*

As the wild type adenoviral genome encodes approximately 20 genes, adenovirus vectors have a relatively large transgene capacity. First-generation E1-deleted Ad-2 and Ad-5 vectors can accommodate up to 7.5 kb of foreign DNA, and this capacity can be expanded by additional viral gene deletion (108). The strategy of deleting regions of the viral genome has met its ultimate realization with the so-called "gutted vectors," which retain only the ITRs and packaging signals. The gutted vectors can accommodate up to 36 kb of foreign DNA and can therefore carry large cDNAs together with appropriate regulatory elements. Compared with first-generation vectors, they have shown reduced immunogenicity and more persistent gene expression *in vivo.*

Applications demanding lifelong expression will require readministration of adenovirus vector following the eventual loss of therapeutic transgene expression. Without vector intervention or masking, the neutralizing antibody response to a previous exposure to adenoviruses either through natural infection or vector administration will preclude or significantly reduce effective readministration. However, several studies have documented effective readministration in certain limited applications. For example, an initial intramuscular administration of low-dose adenovirus vector producing low but detectable levels of transgene expression did not preclude readministration into the muscle, where systemic readministration was not effective (**109**). In a phase I/II trial for recurrent ovarian cancer where intraperitoneal readministration was used, transgene expression was measurable in 17 of 20 samples obtained after two or three cycles (**110**). Thus, it should be recognized that the ability to effectively repeat adenovirus vector administration may be dose-dependent and site-specific.

To overcome the obstacle of readministration, hybrid Ad-AAV vectors have been designed to enable chromosomal integration of adenovirus vectors capable of extending the duration of expression *in vivo* (**111**). A study was therefore performed to engineer a vector containing the first generation adenovirus vector and the ITR sequences of AAV flanking a reporter gene (**112**). The hybrid vectors could be efficiently produced as by-products of Ad-AAV amplification. The Ad-AAV vector stably transduced cell lines at comparable efficiency to AAV, but the chromosomal integration appeared to be random. In a novel approach, the surface of viral particles was coated with a multivalent copolymer based on pHPMA (**112**). These polymer-coated adenovirus particles seemed to shield against antibody recognition. This should further improve the possibilities of readministration of adenovirus to achieve improved therapeutic efficacy. Polymer coating will also permit the incorporation of tumour-specific surface antigens and biological effectors to improve tissue penetration and broaden the tropism. In another approach, aimed at diminishing T and B cell-specific immune responses to E1-deleted adenoviral vectors by modifying the capsid proteins with activated MPEGs, prolonged transgene expression after systemic administration of the E1-deleted adenovirus was achieved and readministration with native adenovirus was allowed (**113**).

The initial enthusiasm for the use of adenoviral vectors in gene therapy was tempered somewhat by their poor performance in pre-clinical and clinical studies, where adenoviral vectors have proven inadequate for long-term, stable transgene expression. A group of factors contributing to this poor performance has been identified (**114-118**): First, the persistence as nuclear episome. Second, the triggering of strong host immune responses to the vector, which makes this type of virus-mediated gene delivery unsuitable for repeated administration. Third, the immune response to viral proteins expressed from adenovirus vectors and transgene products causing clearance of transfected cells. It is likely that vector elimination is due to a combination of several, or all, of these factors. Additionally, some cell types that represent important

targets for gene transfer, express only low levels of the cellular receptors, which leads to inefficient infection.

In 1999, 3 of 18 patients being treated for partial "ornithine transcarbamylase deficiency" using adenovirus vectors became seriously ill. One patient, an 18-year old male, died (**115**). A thorough autopsy revealed that the death was the direct result of infection by the adenovirus vector itself. The reason appears to be that recombinant adenoviral vectors may behave in the host's body very differently from their natural counterparts, at least in a subset of susceptible individuals. Nevertheless, the death of one patient, although tragic, needs to be put into perspective: since 1990, many hundreds of patients have been treated with viral gene therapy vectors with no recorded deaths attributable to the vector (**119-121**).

Furthermore, the safety of recombinant adenoviral vectors has been enhanced by deletion of the E1 region of the genome, which renders the vectors replication-deficient and capable of propagation only in specially designed complementing cell lines (**122**). Although early adenovirus versions showed toxic side effects and strong immune responses, newer second- and third-generation vectors in which many of the viral genes have been deleted have demonstrated significant improvements (**123**).

The biodistribution of adenovirus vectors has been extensively studied in various experimental models. Their intravenous administration results in viraemia and general systemic dissemination (**124-128**). Adenovirus vector distribution after intramuscular injection has been studied in rabbits (**129**), where LacZ-positive cells could be verified in the liver, lung, testis and spleen. Hence, this study documents the systemic distribution of a gene transfer vector after intramuscular administration.

In summary, first generation E1-deleted adenovirus vectors have been shown to be associated with limitations. Ongoing studies are devising rational and largely successful methodological strategies based on the biology of the virus for addressing these shortcomings. These new methods may allow realization of the full potential of adenovirus vectors for gene delivery.

Retroviral vectors

Retroviruses are enveloped, single-stranded (7-11 kb of nucleotides) RNA viruses, which have been widely used in gene therapy protocols (130). Three subfamilies exist: 1) oncovirus, 2) lentivirus and 3) spumavirus. Wild type retroviruses are associated with serious diseases such as cancer, neurological disorders and "AIDS". Following attachment and receptor-mediated entry into host cells, viral reverse transcriptase and integrase enzymes mediate reverse transcription and integration of the virus genome into the host cell chromatin. Retroviruses thus offer the potential advantage of integrating genes into host chromosomes for long-term stability in dividing cells. Retrovirus comprises three genes encoding: 1) structural proteins of the virion core, 2) the virion protein coat and 3) enzymatic proteins of the virion (protease, reverse transcriptase and integrase) that are essential for replication, anchored LTRs (**131**). The LTRs define the beginning and the end of the viral genome and are required for host genome integration; they also serve as enhancer-promoter sequences. To construct a recombinant retrovirus vector, the DNA of interest is substituted for *gag, pol* and *env* (**131**).

Retroviral vectors have shown great potential for gene delivery to stem cells (**132**) and for delivery of cytotoxic genes to cancer cells (**133**). They have also been developed for constructing cDNA libaries (**134;135**).

Retroviral vectors have several limitations (**131**): 1) They cannot transduce non-dividing cells (which may, however, be overcome by using lentiviral vectors). 2) Their random retroviral insertion into the host genome may activate or inactivate genes critical to normal host cell functioning. 3) The rapid inactivation of retroviruses by human complement has lead to the design of modified retroviral vectors preventing complement activation and complement-mediated elimination. 4) Production of low titers producing poorer transduction efficiency. 5) Retroviruses are small, allowing incorporation of only 7.5 kb of foreign DNA. 6) Transcriptional shut-off may occur when the host cells recognize the foreign promoters and inactivate them by methylation. 7) Retroviruses can recombine with cellular or viral DNA or RNA producing new oncogenic viruses or replication-competent retroviruses.

Contrary to simple retroviruses, lentiviruses have evolved the ability to infect non-dividing cells (**131;136**); but the underlying mechanism has not been completely elucidated. The lentiviral genus includes the human immunodeficiency viruses, HIV-1 and HIV-2, the simian immunodeficiency virus and the various non-primate lentiviruses.

The first lentiviral vectors developed were based on HIV-1. They were found to be efficient at transducing non-dividing cells while retaining the ability of simple retrovirus vectors to integrate transgenes into the target cell genome without triggering an inflammatory response (**137-140**). Similarly to simple retrovirus vectors, the design of replication-defective HIV-1 vectors is based on the strategy of segregation of the *cis*-acting elements in the HIV-1 genome (which are required for vector RNA synthesis, packaging, reverse transcription and integration) from protein-encoding sequences (**138**). As an additional measurement of safety, envelope-encoding sequences are usually separated from the rest of the HIV-1 packaging cassette. Since the *env* gene of HIV only allows infection of cells expressing the CD4 receptor, it is substituted with *env* sequences from other viruses to modify the infection spectrum (**138**). The most commonly known lentiviral vectors are derived from HIV and the safety of these vectors must therefore be tested extensively. The risks associated with lentivirus vector-based gene therapy include the potential generation of replication competent viruses, insertional mutagenesis, vector transduction of germ cells and vector mobilization by wild-type HIV-1. Recent improvements in the lentivirus vector system include: the development SIN vectors, the minimization and splitting of the packaging cassette and the generation of packaging cell lines. These improvements have alleviated some of the concerns over the biosafety of the HIV-1 vector. However, the ability to reliably screen vector stocks for replication-competent retroviruses remains a prerequisite for using retrovirus vectors in clinical trials. Before human studies can be undertaken, these vectors must be shown to be unable to recombine or interact with another virus to produce an active HIV strain (**141**).

Herpes simplex viral vectors

HSV is an enveloped, double-stranded DNA virus causing cold sores and encephalitis in humans. Its genome of 156 kb nucleotides comprises more than 80 genes (**142**). Up to half of the genes are dispensable for viral replication, which allows 30 kb of nucleotides of foreign genetic material to be inserted into the virion (**142**). HSV vectors are considered potentially dangerous because of the large size of their genome, which includes many wild-type genes with unknown functions. It owes its wide host range to the binding of the viral envelope glycoproteins to the extra-cellular heparan sulphate receptor found in all cell membranes (**143;144**).

There are two types of HSV vectors, the so-called recombinant HSV vectors and the amplicon HSV vectors (**144**). The former are generated by the insertion of transcription units directly into the HSV genome. The latter are based on plasmids bearing the transcription unit of choice, an origin of replication and a packing signal. The plasmid is transferred into a cell line, which is subsequently infected by a helper virus that provides the packing and the replication functions, enabling the amplicon to be packaged into infectious HSV virions.

Although the HSV vectors enjoy the obvious advantage of a large capacity for foreign gene insertion, a wide host range and the ability to confer long-term transgene expression (**142;144**), their use for gene transfer is limited because many people have already been infected by this virus and have developed immunity to essential HSV components.

Other viral vectors

DNA vectors based on vacciniavirus and poxvirus are being considered potential candidates for vaccine generation (**145**). They have demonstrated great potential owing to their diminished cytopathic effects, high levels of protein expression and strong immunogenicity, and they are relatively safe in animals and humans (**145;146**).

SV-40, a double-stranded DNA polyomavirus under investigation as a vector for gene transfer (**147;148**), has a circular genome lacking the terminal repeats characterizing many other viral vectors, and it can efficiently transfect both quiescent and dividing cells. Nonetheless, SV-40 is oncogenic in sucking hamsters and it integrates randomly into cellular genomes (**147**). Its safety must therefore be monitored carefully before it can be used as a vector for human gene transfer trials.

Table 5. Advantages and disadvantages of gene delivery methods

Gene delivery system	Advantages	Disadvantages
Viral vectors	High transduction efficacy Suitable for systemic delivery Potential for targeting selected cell types	Complicated manufacturing process High quality-control requirement High cost Interference with pre-existing immunity Safety concerns Require low temperature storage
Chemical methods	Highly effective with cultured cells Relatively simple manufacturing for gene-based products Fewer restrictions on gene size Easy storage and quality control	Limited clinical application so far Challenge to prepare consistent formulations
Physical methods	High local transfection efficiency Not cell type-dependent Easy to standardize the process Fewer restrictions on gene size Useful for *ex vivo* application	Requires specific instruments Limited clinical applications so far

Synthetic vectors

Non-viral gene transfer using synthetic vectors may be an alternative method for gene delivery providing higher safety (Table 5). The introduction of DEAE-dextran in transfection experiments 40 years ago paved the way for the production of many synthetic compounds (**149**). The synthetic vectors enjoy a relatively low toxicity and they are simple and easy to produce. Their main problem lies in their low efficiency, especially in vivo, compared with viral vectors, but the future is expected to see more sophisticated systems. Cationic liposomes, for example, have already been used in clinical trials (**150-153**), even if the level of gene expression in these in vivo studies fell short of producing medical benefit. Significant efforts are therefore needed to improve in vivo DNA-delivery efficiency of the synthetic vectors.

Three main methods are currently used to deliver synthetic genes: 1) chemical methods, 2) polymer systems, and 3) physical methods.

Chemical methods

Chemical techniques have been developed to combine DNA with cationic polymers, such as DEAE-dextran, polybrene and the mineral calcium phosphate, in order to neutralize DNA electrostatically and allow it to be more readily taken up by cells. Chemical techniques are still considered too inefficient for clinical gene therapy.

Calcium phosphate

This method draws on the formation of small, insoluble calcium-phosphate-DNA precipitates that can be adsorbed onto the cell surface and taken up by cells through endocytosis. The procedure requires mixing of DNA with calcium ions, subsequent addition of phosphate to the mixture and presentation of the final solution to cells in culture (**154**).

The transfection efficiency can reach 50%, depending on the cell type and the size and quality of the precipitate. Variation in the composition and particle size of the calcium-phosphate-DNA precipitates produces poor reproducibility. This method does not seem to work on cells in primary culture or in animals (**154;155**).

DEAE-dextran

The use of DEAE-dextran as a chemical reagent for DNA delivery was first reported by Vaheri and Pagano in 1965 who used it for enhancing the viral infectivity of cells (**149**). Like cationic polymers, DNA and DEAE-dextran form aggregates through electrostatic interaction. A slight excess of DEAE-dextran in the mixture produces a net positive charge in the DEAE-dextran/DNA complexes formed. When added to cells, these complexes bind to the negatively charged plasma membrane and are then internalized through endocytosis. DEAE-dextran/DNA complexes have been shown to have transfection efficiencies reaching 80% (154). A

drawback of this method is the cellular toxicity of DEAE-dextran in high concentrations.

Polymer systems

Many naturally occurring or synthesized cationic lipids and cationic polymers have been used for gene transfer (**156**). They enjoy the advantage over viral gene transfer of limited immunogenicity, simple production and lack of oncogenicity. These cationic compounds, however, suffer the major limitations of inefficient transfection and cellular toxicity.

Liposomes

Liposomes are hollow spheres composed of a lipid membrane surrounding an aqueous sphere. Felgner et al pioneered the use of cationic lipids for DNA delivery almost 20 years ago (**157**).

The advantages of using liposomes for gene therapy include the lack of immunogenicity, the possibility of repeated administrations *in vivo* without adverse consequences, the ability to deliver nucleic acids of unlimited size (from single nucleotides to large mammalian artificial chromosomes), the relative ease with which large-scale DNA-liposome complexes and targeted complexes for delivery and gene expression in specific cell types may be created, and greater safety for patients due to the presence of few or no viral sequences in nucleic acids used for delivery (**158**). The disadvantages of non-viral delivery systems include the low levels of delivery and gene expression produced by ''first-generation'' complexes. However, recent advances have dramatically improved transfection efficiencies of non-viral vectors (**159;160**). Although liposomes are considered less pathogenic than some existing viral vectors, their toxicity seems to be dose-dependent, because they have been demonstrated to aggregate in the blood and can cause severe toxic reactions when administered in high concentrations (**161**).

Liposomes rest on the principle of mixing negative DNA with the positive charges at the liposome surface, which electrostatically interact with the negative charges on the phosphate backbone of the DNA to form DNA / liposome complexes (lipoplexes) (**162**). Their addition to cell cultures normally yields significant gene expression levels with a 5–90% efficiency range depending on the type of cell line used (**163**). Particularly essential among the many physicochemical properties affecting transfection activity of cationic liposomes is the cationic lipid structure. Although many complex DNA cationic liposome structures have been identified (**160;164;165**), the critical parameters affecting the transfection efficiency of cationic liposomes appear to be the particle size of the complexes (**166**) and the charge ratio (amines to DNA phosphates ratio, +/–) (**157;167-170**). Complexes with large particle size (>200 nm) and with a charge ratio slightly greater than 1 appear to be optimal for *in vitro* transfection. For intravenous transfection, however, optimal transfection activity requires a charge ratio (+/–) exceeding 12 (**171;172**).

Cationic polymers

Complex formation with DNA in protein and peptide gene transfer (polyplex formation) is mediated through electrostatic interaction between the positively charged lysine and arginine residues and the negatively charged phosphates in the DNA backbone (**173**). The hydrophilic polycations of cationic polymers and polypeptides have been utilized to condense DNA through ionic interactions and to facilitate *in vitro* cellular uptake of plasmids through non-specific adsorptive mechanisms. When polyplexes are added to cell cultures, they are endocytosed by the cells (**174**).

The potential advantages of polyplex-mediated gene transfer include an improved potential for cost-effective large-scale manufacturing, purity and homogeneity of vector stocks, the ability to target specific cell types, modular attachment of targeting ligands, absence of limitations on the size or type of nucleic acid that can be delivered, ability to tranfect *in vitro* and *in vivo,* and design of "artificial viruses" (**174-176**). The artificial viruses have several features in common with viruses such as cell targeting domains. Unlike viruses, the current versions of virus-like gene transfer systems, however, do not necessarily undergo programmed structural change (**176**). The critical step for efficient cationic polymer-mediated gene delivery is the polyplex formation, and compared with cationic lipids, the major drawback of cationic polymers is their relatively high toxicity (**177**).

Biopolymers

These compounds (i.e. polylysine, histone, chitosan) have shown relatively low transfection efficiency when used alone (**178-183**). Although they may become relatively more important in the future, they are playing no significant role at present.

Combined systems

A major increase in transfection activity can be achieved by combining synthetic systems. For instance, a significantly higher transfection activity of cationic liposomes has been reported when they were mixed with polymers (**184-187**). The mechanisms creating such a synergistic effect remain unknown, but it has been hypothesized that the structure of DNA complexes in the combined system may be more effective in escaping the endosomal degradation and/or more efficient in facilitating DNA transfer into the nucleus.

Physical methods

Viral vector systems clearly outperform efficiency of direct transfer of naked DNA. However, as naked DNA provokes no specific immune responses, techniques designed for direct gene transfer are continuously being ameliorated. These include microinjection, electroporation and gene particle bombardment.

Microinjection

Capillary microinjection into cultured somatic cells growing on a solid support has developed rapidly since its introduction (**188**). It has now become one of the most versatile, well-established methods for introducing genetic materials into living cells. Microinjection allows the use of single cells for studying complicated cellular processes, structure and function *in vitro.* It also remains a widely used method for generating transgenic animals by germ line transformation (**189;190**). An important improvement in this respect is the introduction of automation in the micromanipulation and microinjection processes as well as the control and standardization of cell preparations or the production of injection capillaries (**191;192**). The development of computer-assisted injection systems ensures improved transfection efficiencies where optimal reproducibility becomes feasible and allows for quantitative microinjection (**193**). In addition to the microinjection of nuclear DNA, mitochondrial microinjection and cytoplast fusion have been used for gene therapy against non-Mendelian genetics diseases caused by mitochondrial DNA mutations (**194**). However, with an apparent half-life of 50–90 min, mitochondrial microinjected plasmid DNA is rapidly degraded in the cytoplasm. Thus, naked DNA microinjection directly into the nucleus, bypassing cytoplasmic degradation, ensures a much higher gene expression level than injection into the cytoplasm (**195;196**). Despite its straightforward approach, microinjection is a laborious procedure. Only one cell at a time can be injected and many injections may be needed before DNA delivery is successful. This can be a problem if many cells must be injected within a limited time. With the present technology, microinjection is therefore hardly a feasible option in in vivo gene therapy.

Electroporation

Electroporation is a process whereby high-intensity electric field pulses temporarily destabilize cellular membranes. During the destabilization period, DNA molecules in the surrounding media are able to permeate the external and internal cellular membranes, entering the cytoplasm and nucleoplasm (**197**).

Electroporation provides a fast and inexpensive method of introducing exogenous DNA into cells *in vitro,* including primary cell cultures. It has not been associated with induction of transgene mutations. The main drawbacks of this technique are that specialized equipment is required, that each cell type requires fairly extensive empirical optimization and that typically only approximately 0.01% of treated cells show genomic transgene integration (**197-199**).

Electroporation has also been used in *ex vivo* gene targeting approaches, for instance to correct a defective hypoxanthine phosphoribosyltransferase gene in haematopoietic progenitor cells (**200**), and electroporation has been shown to effectively deliver exogenous genes into human haematopoetic precursor cells (**198**). This method may thus offer a means for correcting inborn genetic errors and for protecting human stem cells from the adverse effects of chemotherapy. Furthermore, efficient

gene transfer to mesenchymal stem cells has also been reported (**201**)

Electroporation may also be used for direct *in vivo* gene transfer. This method is at a very early stage of development, but empirical advances may in the future permit electroporation to be used for delivering transgenes to particular tissues or tumours. Hence, progress has been made towards intramuscular and intratumoural electrogene transfer *in vivo* (**199;202;203**). In these cases, naked plasmid DNA has been injected into the interstitial tissue spaces and the required electric pulses have been applied with needle or caliper-type electrodes. The electric pulses increased gene expression up to 1000-fold compared with needle injection alone. Direct gene transfer to articular chondrocytes *in vivo* has also been reported (**204**). Recently, the development of an electroporation device (Medpulser®) for human electroporation therapy was announced. Electroporation for *in vivo* use has yet to be fully explored. Because of the complexity of tissue architecture, parameters such as electrode configuration, pulse width and field strength need to be optimized before it can be used to augment the efficiency of DNA delivery. Tissue differences should also be thoroughly understood in order for such devices to be used for gene delivery to various organs.

Particle bombardment

In particle bombardment, DNA may be adsorbed onto spherical tungsten or gold particles (diameter approx. 4 mm) and transferred into a cell mass using a particle gun; once inside the target cells, the DNA is solubilized and may be expressed (**205**). This approach, sometimes known as biolistics, was originally developed for plant transgenesis, but has been effective for transferring transgenes into mammalian cells *in vivo* (**206;207**). It has also been used to transfect cells resistant to transfection by other means, such as multinucleated muscle fibres, mammalian neurons in primary culture and neuronal cells (**208**). The Helios™ gene gun is a hand-held device providing rapid and direct gene transfer into a range of targets *in vivo*. Delivery of less than one mg of DNA to mouse skin using the gene gun protected mice from influenza virus challenge (**209**). Both antibody and cell-mediated immunity responses have been induced in animals following nucleic acid vaccination with this technology. Thus, gene-gun delivery to skin with plasmid DNA may be a promising alternative to nucleic acid vaccination by intramuscular or intradermal inoculation. A DNA vaccine against hepatitis B virus, delivered by needle-free PowderJect™ system into skin cells, demonstrated induction of both humoral and cell-mediated immune responses in humans (**210**). At this point, experience with this type of gene delivery in humans, however, remains limited.

Biolistics may be more efficient than alternative methods such as liposome-mediated transfection and recombinant viral infection (**211**), even if current research provides insufficient data to permit definitive comparison. If biolistics does prove effective *in vivo,* tumours are the most likely targets for particle bombardment (**212**).

Delivery strategies

The ideal clinical situation for a gene therapy agent would be if a cell or tissue-targeted vector could be delivered in the least invasive manner at the lowest possible dose, permitting post-infectious control of the transgene expression, which allows regulation of the exocytosed protein levels in a manner where dosing can be adjusted as the disease evolves, and if therapy could be initiated repeatedly or terminated at will. The ideal gene therapy vector has not yet been engineered, but quick advances are being made towards this goal.

Gene delivery therapies

Genetic alteration of somatic cells may be achieved either by manipulation of cells residing naturally within the patient's body, the so-called "*in vivo*" approach; or by manipulation of cells removed from the patient's body for genetic manipulation and subsequently returned to it, the so-called "*ex vivo*" approach (Figure 4).

The advantages of the "*in vivo*" approach are that it involves only one step and if an off-the shelf technology could be developed it would be quite popular among surgeons. The disadvantages are that it is more difficult to achieve standardized and high transduction efficiencies, and that targeting specific cells only is extremely difficult in practice.

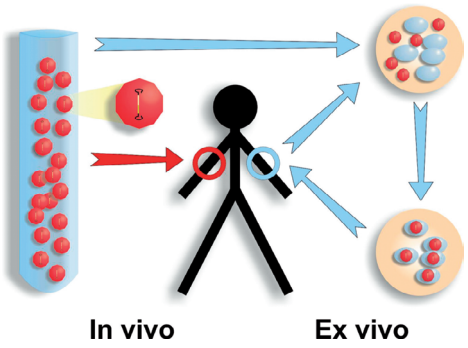

Figure 4. The *in vivo* and the *ex vivo* approaches. In the *in vivo* approach, the gene delivery system is applied directly to the patient's body. Hence, the target cells are transduced in situ where they reside. In the *ex vivo* method, the target cells are retrieved from the patient and transduction is carried out at optimized laboratory conditions before cell reimplantation.

The advantages of the "*ex vivo*" approach are that standardized and high transduction efficiencies can be achieved when gene transfer is performed in an in vitro setting. The disadvantages of this technique are that it is more laborious and may therefore not be cost-effective, and that the risk of bacterial contamination may be increased. Furthermore, the anatomy and topography of some organs may not allow capture and subsequent transplantation of genetically modified cells.

Both "*ex vivo*" and "*in vivo*" therapies are under investigation and have been attempted with respect to a wide range of conditions.

Tissue targeting

The purpose of tissue targeting of gene delivery systems is to restrict the vector tropism or transgene expression towards specific types of cells. The technologies employed include modification of receptor specificity (receptor targeting) or promoter specificity (transcriptional targeting) to tissues or diseases.

Recent years have seen attempts at retargeting viral vectors away from their primary receptors towards a desired tissue or cell-specific receptor. The aims are: 1) to restrict transduction to the organ of interest, thereby gaining the greatest benefit with the lowest dose; 2) to potentially avoid the effects of a viral vector neutralizing antibody response; and 3) to improve transduction efficiency by exchanging target receptors from the original wild type virus receptors, which may be present at low levels in some cell types that are potential targets for gene transfer (**213-215**).

Advances in the biological understanding of virus structure and virus receptor interactions have led to alterations of the major viral vectors for specific targeting. Hence, receptor targeting of AAV (**216**), adenovirus (**217-226**), retrovirus including lentivirus (**138;227**) and hybrid vector systems (**228**) has been reported.

Although most studies demonstrated promising results *in vitro*, outcomes of subsequent *in vivo* studies have generally been disappointing (**138**). Lack of in vivo stability of the engineered vectors seems to be a common problem (**229;230**). Furthermore, low transduction efficiencies in vivo could also be due to internalization of the vectors into endosomes (**82;231-234**).

The receptor targeting technology can be combined with "transcriptional targeting" approaches. Tissue or disease specificity of a gene therapy product can be achieved by incorporation of a tissue- or disease-specific promoter into the vector. This allows therapeutic gene expression only in cells expressing transcription factor proteins binding to these specific promoter sites. Some of the promoters identified and tested in animal models include the promoter of the "prostate-specific antigen" gene (**235**), the osteocalcin gene (**236;237**) and the "hypoxia response element" activated by "hypoxia inducible factors" in hypoxic/ischaemic tissues (e.g. tumours) (**238**).

Regulation of transgene expression

Gene expression control can be elegantly achieved via dose-dependent ligand binding and activation of chimeric transcription factor proteins, which then interact with DNA elements incorporated into the vector construct and regulate the level and timing of the therapeutic gene expression (**239-241**).

A pharmacological gene expression regulation system should ideally meet the following criteria: 1) Basal expression should be low and induction to high levels over a wide dose range should be possible. 2) Induction should have a positive effect (adding rather than removing a drug). 3) The use of an orally active small molecule with no pleiotropic effects in mammalian cells should be preferred. The regulatory protein should hence not interfere

with the endogenous gene expression, and it should be of human origin to minimize immunogenicity.

At the present time, four major systems have been tested *in vivo*: 1) The antibiotic tetracycline (**242**), 2) the insect steroid "ecdysone" or its analogs (**243;244**), 3) the anti-progestin mifepristone (RU486) (**244;245**), and 4) the immunosuppressant rapamycin and its analogs (dimerizer-inducible) (**244**). They all involve the small molecule-dependent recruitment of a transcriptional activation domain to a basal promoter driving the gene of interest, but differ in their recruitment mechanism (**243;244**).

Pharmacological regulation of AAV vector systems has been performed *in vivo* (**246**) using a bi-cistronic vector system inducible by the dimerizer rapamycin and expressing erythropoietin as a marker gene. During a 3-week period, proportionality between alternating concentrations of rapamycin and corresponding shifts in local marker-gene expression could be demonstrated (**246**). Incorporation of the tetracycline-inducible system into lentiviral (HIV-1) vectors has also proven its ability to control transgene expression in vivo (**247;248**). Improvements in the effectiveness and safety of the lentiviral vector regulatory systems are rapid and promising and their use in clinical trials requiring regulation of transgene expression looks increasingly feasible (**247**). Human herpes virus vectors have also used for regulation of transgene expression through the steroid analogue RU486 (**249**). Following transduction of rat hippocampus, marker gene (LacZ) expression was increased 150-fold by intraperitonal administration of RU486 (**249**).

Gene expression systems that lend themselves to induction have typically encountered limitations, like pleitropic inducer effects, basal leakiness, toxicity of inducing agents and low expression levels (**250**). But as research on this field is rapidly progressing, all of these problems seem to be relative and possible to be overcome.

AAV vector mediated gene transcription may be dramatically accelerated by factors like ionising radiation (γ) or ultraviolet light irradiation where an enhanced post-transductional gene expression using low viral vector titres is sought (**I;II;69-71**) (Figure 3). These factors accelerate formation of the double-stranded transducing AAV vector episome by activation of a host DNA repair response mediated by DNA polymerases (**67;160;251;252**). However, the induction of more subtle changes that could affect cellular metabolism needs investigation. These changes may result from tautomerization of DNA bases and the generation of reactive species like oxygen-free radicals, which are produced downstream of ultraviolet light irradiation and may produce neoplasms. The various safety issues of ultraviolet light in this application are currently being investigated at The Musculoskeletal Research Centre, University of Rochester Medical Centre, Rochester, NY, USA.

Applications and results

Healing of articular cartilage

Articular cartilage injury (**253-256**) and osteoarthritis (**257-259**) remain serious clinical problems and they are, collectively, among the most prevalent diseases affecting humans. Non-surgical therapeutic option promoting healing of articular cartilage lesions have not been found, despite numerous attempts (**260-267**). As cartilage damage and osteoarthritis affect a limited number of large, weight-bearing joints and have no major extra-articular manifestations, they are well-suited for local, intra-articular gene therapy.

Articular cartilage deterioration

A reparative response to articular cartilage damage depends mainly on the depth of the injury as only penetration of the subchondral bone elicits the three-phasic inflammatory repair responses like that observed in vascularized tissue (**268-272**). Mesenchymal stem cells originating from the underlying bone marrow proliferate into the chondral defect and undergo chondrocyte differentiation under the influence of local growth factors (TGF-β, BMPs, PDGF and IGFs) in the microenvironment (**273**). The composition of the cartilage repair tissue, however, rarely replicates the structure of normal articular cartilage (**273-275**).The hyaline cartilaginous matrix subsequently degenerates with a shift to a softer fibro-cartilaginous matrix characterized by a relatively high level type I collagen compared with type II collagen (**276**). Degenerative changes begin with surface fibrillation, followed by matrix proteoglycan loss, chondrocyte-like cell death and articular surface fissuring. Simultaneously, the remaining cells typically assume the appearance of fibroblasts as the surrounding matrix comes to consist primarily of densely packed type I collagen fibrils (**277**) that are biomechanically inferior to type II collagen in hyalin articular cartilage. The articular cartilage defects will hence progress to osteoarthritis, which is characterized by progressive erosion of articular cartilage, finally leading to exposure of subchondral bone (**278;279**). This process is accompanied by osteophyte formation and synovial inflammation (**280**).

In animal models, the first response of articular cartilage to initial damage consists of increased proliferation and matrix synthesis by chondrocytes (**281;282**). Various growth factors (TGF-β, BMPs, PDGF and IGFs) are released and their interaction triggers the metabolic response (**268**). This anabolic reaction then gives rise to an active process of matrix degradation, largely carried out by metalloproteinases of the matrix metalloproteinase and aggrecanase families (**280;283**). Inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) are likely to play a key role in established osteoarthritis, since they stimulate degradation and inhibit cartilage matrix synthesis and also activate production of inflammatory mediators like prostaglandins, leukotrienes and nitric oxide (**280;283-287**). While late osteoarthritis can be ascribed to inflammatory cytokines, the initial anabolic phase preceding cartilage degradation can be mimicked by $TGF_{\beta 1}$ (**284;288**). $TGF_{\beta 1}$ stimulates proteoglycan and collagen synthesis (**289**) and antagonizes the IL-1effects on MMP production and proteoglycan turnover (**290-292**), possibly by a decrease of IL-1 receptors (**293**). Degradation of aggrecan is mainly carried out by MMPs and aggrecanases (**294**). Intra-articular $TGF_{\beta 1}$ injection in mouse knees increased proteoglycan synthesis in cartilage (**295**). However, the effects of $TGF_{\beta 1}$ on articular cartilage could be more complex and even paradox. Thus, $TGF_{\beta 1}$ injection in rabbit knees caused a strong decline in the proteoglycan content in cartilage (**296**). Other studies have demonstrated that $TGF_{\beta 1}$ injection in mouse knees stimulated basal proteoglycan degradation, despite concomitant inhibition of IL-1-induced degradation (**297**). In a subsequent paper by the same group, TGF-β increased the proteoglycan content in the superficial layer of articular cartilage, but caused severe loss of the same component in the deep zone close to the calcified layer (**288**). Frequent large doses and direct intra-articular injections of $TGF_{\beta 1}$ induced undesired effects such as synovium inflammation

and osteophytes (**295**). Gene transduction may prove feasible in this situation because it changes the promoter area of the gene, allowing a specific therapeutic gene to be expressed in a target tissue. The use of a tissue-specific promoter for a certain collagen, e.g. type II collagen, accordingly makes it possible to limit $TGF_{\beta 1}$ expression on cartilage tissue and to reduce the severity of unwanted effects on other tissues. Additionally, gene therapy approaches may be further improved by implementation of adjustable promoters like TET (**242**).

In vitro models

For studies of vector transduction kinetics, articular chondrocyte cultures can be valuable. Such studies can be performed both on cell lines of normal human articular chondrocytes, like tsT/AC62 cells (**298**), and on primary chondrocyte cultures (**I-III**) grown in confluent monolayers. The chondrocyte culture condition is, however, considerably different from the *in vivo* condition, where chondrocytes are dispersed in a three-dimensional matrix without any direct cell-to-cell contact. The consequence of this is chondrocyte dedifferentiation and progressive slowdown of matrix synthesis as passaging proceeds (**299;300**). In order to minimize this drift, it may be recommended to limit cell proliferation by using primary cultures seeded at the highest density allowed by the number of collected cells. In our studies (**I-III**), the articular chondrocyte maturity was confirmed by RT-PCR for markers of articular chondrocyte maturity (aggrecan, type I, II and X collagen) before and after experiments. Furthermore, our monolayer culture experiments did not exceed a maximum of 8 days (**I-III**).

In some osteoarthritic cartilage cultures, reduced responses from growth factor stimulation may be due, among others, to decreased receptor sensitivity because of previous long-term exposure to the cytokine in joints at a late stage of disease (**280;283-285**).

The bioactivity of viral vectors needs to be confirmed *in vitro* before application on biological systems. In our studies (**III**), the AAV-$TGF_{\beta 1}$ vector was tested on a mink lung cell assay (CLL-64, ATCC) in which bioactive $TGF_{\beta 1}$ inhibited the proliferative response of CCL-64 to serum (**301;302**).

In vivo models

Small animal models are useful for gaining knowledge of the efficiency of direct *in vivo* gene transfer to articular cartilage. Species like rat (**303**), mouse (**304**) and rabbit (**II;305**) are commonly used. It should be borne in mind, however, that these small animal models cannot be used for meaningful studies of cartilage repair and regeneration due to important differences in both gross anatomy and histology between these animals and man: In the human knee, the layer of hyaline articular cartilage is approximately 2 to 3 mm thick on the medial femoral condyle (**306**). In the mature rabbit, it has a depth of only approximately 400 μm in the corresponding location (**307**), which is 1/7 to 1/5 of the thickness of its human counterpart. The sizes of chondrocytes within human and rabbit articular cartilage do not differ significantly from one another. However, the matrix domain sustained and remodeled by an individual cellular unit is, in the human, approximately 8 to 10 times larger than that in the rabbit; the difference being most striking in the radial zone. The overall cell volume density in human articular cartilage is approximately 1.7% in the medial femoral condyle as opposed to 12.2% in the adult rabbit (**307**). The corresponding numerical density is 1800 per mm^3 of tissue in humans and 7500 per mm^3 in rabbits (**307**). These examples show that the quantitative structural organization of articular cartilage in humans is fundamentally different from that in rabbits.

On the other hand, small animal models enjoy important advantages such as large genetically homogenity, ease of handling, involvement of large study groups and lower demands for viral titres for transduction studies. Moreover, as for the rabbit knee model, both isolated chondral lesions and osteochondral injuries can be introduced in a fairly standardized way (**II**).

Gene therapy results

The chondrocyte is the native cell of articular cartilage, and chondrocytes would therefore seem a logical target for genetic modification. They possess characteristics such as long cellular life span that are making them attractive as gene delivery targets in chronic disorders. Chondrocytes cultured in monolayer are receptive to transduction using the more common viral vectors, including

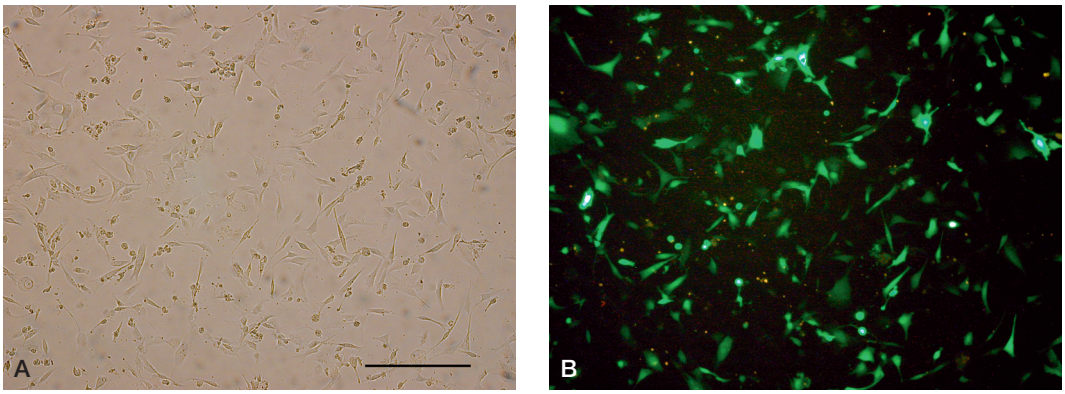

Figure 5. Monolayer cultures of primary human articular chondrocytes transduced with the AAV-eGFP vector. Identical AAV-eGFP tranductions have been performed in cultures of normal (I) and osteoarthritic human articular chondrocytes (III). In order to confirm transduction and eGFp expression, bright field microscopy (A) and ultraviolet microscopy for green-fluorescence (B) were performed. Dimensions are given by scale bar (100 μm).

adenovirus (**308-310**), retroviruses (**266**) and AAV (**I;III;99;309**) (Figure 5). Although they have been somewhat resistant to transfection with plasmid DNA, formulation with certain commercially available lipid-based reagents like FuGENE6 (**327,328**) and Lipofectin was found to enhance the efficiency of DNA uptake, as was pre-treatment of the cells with hyaluronidase (**311**) and mild detergent (**312**). Furthermore, electroporation seems to be a reliable and inexpensive technique with the ability to target the chondrocytes despite the barrier effect of the extra-cellular matrix and without using viral vectors (**313**).

Following genetic modification, chondrocytes in culture are capable of sustained expression of certain transgene products at biologically relevant levels. Delivery of the genes for $TGF_{\beta 1}$ (**III;314;315**), IGF-1 (**315;316**), BMP-2 (**315**) or BMP-7 (**317**) to monolayer cultures of chondrocytes isolated from animals and humans has been shown to stimulate expression of cartilage matrix genes, resulting in increased synthesis of proteoglycan and collagen type II. The delivery of genes whose products enable maintenance of the phenotype of hyaline cartilage without hypertrophy of the chondrocytes would be highly desirable, as it would enable culture proliferation for *ex vivo* approaches. In this regard, IGF-1 gene transfer has been demonstrated to render chondrocyte cultures resistant to dedifferentiation in monolayer, enabling maintenance of the chondrocytic phenotype for at least 28 days in culture (**318**).

Genetically altered chondrocytes retain their ability to attach to and colonize cartilage explants in culture (**308**). Following engraftment to the explant surface, they are capable of expressing a transgene product. Initial studies by Baragi *et al* (**308**) showed that in this context, chondrocytes adenovirally transduced to overexpress IL-1 receptor antagonist were resistant to IL-1-induced proteoglycan degradation. Madry *et al* (**319**) showed that transplantation of chondrocytes transfected with plasmid DNA encoding IGF-1 onto articular cartilage explants caused significant resurfacing. The IGF-1-expressing chondrocytes were observed to generate tissue that was thicker than that produced by similarly transplanted control cells and was rich in type II collagen and proteoglycan.

However, because of the dense cartilaginous extra-cellular matrix surrounding the inhabitant cells, they have typically been unavailable for genetic modification by direct intra-articular injection of most recombinant vectors (**320-323**). The reported AAV vector mediated transduction of chondrocytes in cartilage explants in culture (**I;68;324**) has been attributed to the smaller size of the AAV particle relative to other viral systems (Figure 6). Using an AAV vector, we have shown high transduction efficiency of chondrocytes in the intermediate and basal layers in the periphery compared with the centre of articular cartilage explants, even in the absence of ultraviolet light irradiation (Figure 7). This indicates that under passive diffusion conditions (absence of fluid force), the AAV

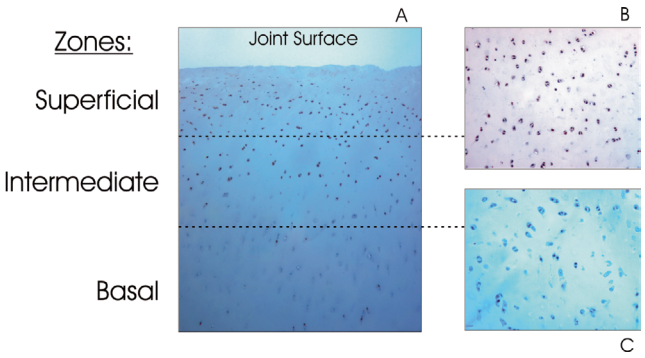

Figure 6. Immunohistochemistry of AAV-eGFP transduced human articular chondrocytes in cartilage explants. Surgically retrieved human articular cartilage was prepared into explant cultures and infected with AAV-eGFP (I). After 8 days of culture the explants were processed for immunohistochemistry. Representative micrographs taken at 10× (A) or 40× of the superficial zone (B) or the intermediate zone (C) at the centre of the transduced cartilage are shown. The dark brown staining indicates positive eGFP immunoreactivity.

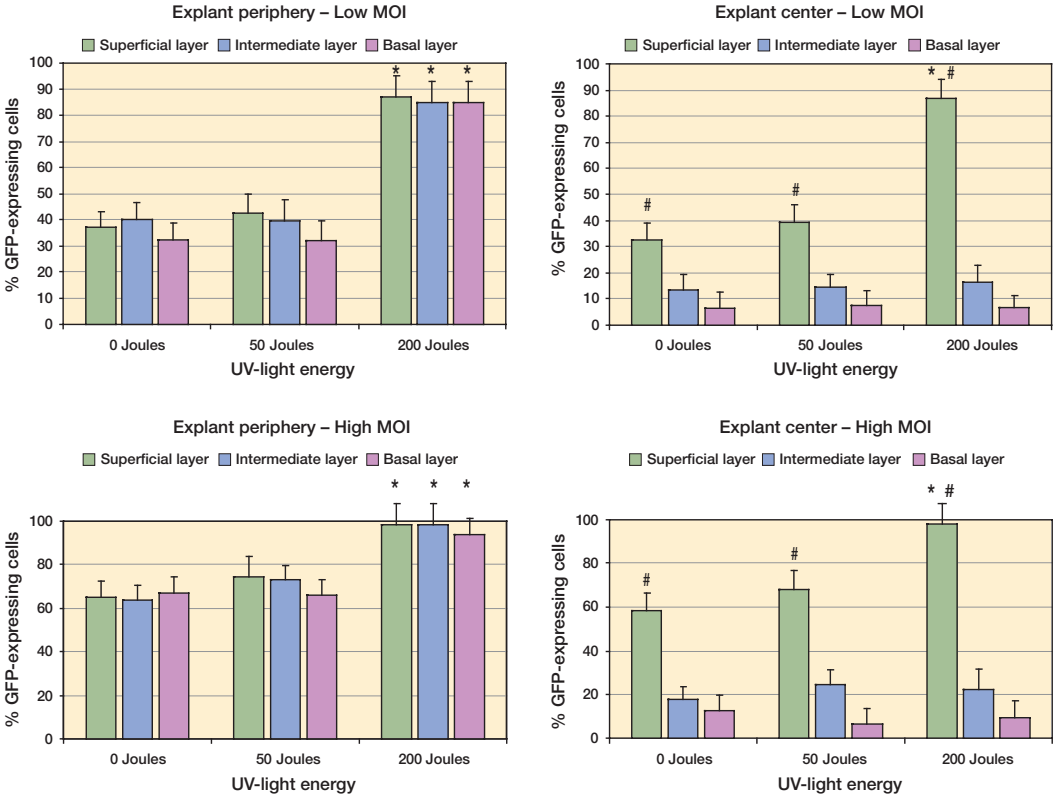

Figure 7. Ultraviolet light enhancement of transduction efficiency and gene expression in AAV-eGFP-transduced chondrocytes in human cartilage explants (mean ± SEM). Human articular cartilage explants were cultured, exposed to the indicated doses of ultraviolet light irradiation and infected with AAV-eGFP at either low (3.5×10^6 vp/mL) or high (1.1×10^7 vp/mL) concentration (I). After 8 days of culture, the explants were processed for analysis of eGFP by immunohistochemistry, and the percentages of eGFP-immunostained chondrocytes in the superficial, intermediate and basal zones were determined at the periphery or the centre of the explants. * p-value < 0.05 (exposed versus unexposed controls) (N = 6).

vector can effectively penetrate the extra-cellular matrix of superficial cartilage but may have difficulty penetrating the intermediate and basal zones matrixes. The chondrocytes in these zones may also be transduced as efficiently as those in the superficial zone in regions of cartilage degeneration and surgical debridement. *In vivo* experiments demonstrated a significant reduction in the number

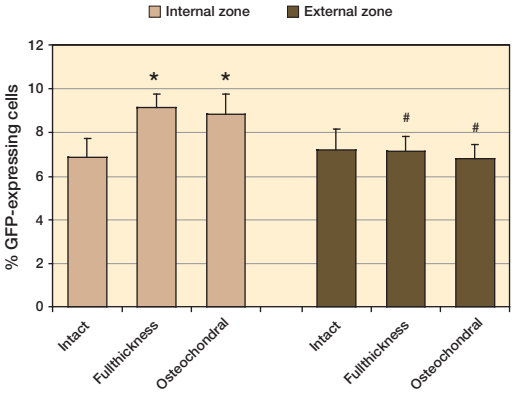

Figure 8. Direct *in vivo* transduction of articular chondrocytes at cartilage defects (mean ± SEM). Full thickness chondral defects or deep osteochondral defects were created in rabbit knees. Subsequently, 1.5×10^{12} vp of AAV–eGFP were injected intra-articularly. After 3 weeks, the articular cartilage from the internal zones and from the external zones was harvested. The chondrocytes in the two zones were isolated and prepared for flowcytometry (II). * p-value < 0.05 (intact cartilage versus full thickness or osteochondral defects). # p-value < 0.05 (internal zone versus external zone) (N = 6).

of AAV-transduced chondrocytes in relation to the distance from an acute articular cartilage defect (Figure 8) (II).

Various research groups have focused their attention on the *ex vivo* approach. Although laborious, it satisfies several important criteria: 1) it provides a means for increasing local cellularity; 2) it targets transgenic expression specifically to the site of repair; 3) free vector particles are not administered to the subject; 4) it enables analysis of cells post-modification for levels of transgenic expression and potential safety issues prior to delivery; and 5) the transduction efficiency can be optimized. Furthermore, the emergence of studies of *ex vivo* gene delivery to chondrocytes has largely coincided with the advent of ACI as a surgical approach to cartilage repair (**325**). Thus, the potential benefit of *ex vivo* gene transfer using chondrocytes lies in the technical ease of isolation, culturing and handling of these cells. A large-scale production of chondrocytes for clinical application has therefore already been established. Finally, the *ex vivo* chondrocyte transplantation technique may obviously be combined with the use and manipulation of bone marrow derived mesenchymal stem cells or embryonic stem cells. Hence, studies confirm the feasibility of using chondrocytes as a means of *ex vivo* gene transfer to cartilage lesions and the feasibility of prolonged transgenic *in vivo* expression (i.e. 2 weeks) using commonly available vectors and three-dimensional support scaffolds (**267;324;326**). *Ex vivo* gene therapeutic manipulation of fibroblasts over-expressing $TGF_{\beta 1}$ has been performed in combination with autologous chondrocyte transplantation in a rabbit model with stimulatory effects on collagen type II formation and tissue integration (**327**), and *ex vivo* gene transfer to cartilage lesions in the horse using allergenic chondrocytes, adenovirally transduced to express equine IGF-1 or BMP-7, has demonstrated accelerated cartilage repair in osteochondral lesions (**328**).

Recent research has identified TNF-α, ILs, collagenase, aggrecanase and other matrix metalloproteinases and their signal transduction pathways as important pathobiologic targets in osteoarthritis (**21;329;330**). Gene therapy could serve as a system for drug delivery of secreted anti-arthritic agents to circumvent the rapid clearance of these proteins when administered orally or by injection (**331**). Two strategies have been used in arthritis: local therapy transferring genes within individual affected joints and systemic therapy in which the gene products are secreted giving them access to the systemic circulation (**332**).

Genes showing efficacy in intra-articularly administered gene therapy targeting osteoarthritic chondrocytes include "IL-1 receptor antagonist", $TGF_{\beta 1}$, IL-10 and IL-13 (**333-336**). IL-1 is implicated to be a key mediator of cartilage loss in osteoarthrosis, and the therapeutic effects of IL-1Ra gene transfer were confirmed in three different experimental models of osteoarthrosis (**12;337;338**). $TGF_{\beta 1}$, IGF-1 and BMP-2 overexpression was also found to rescue proteoglycan synthesis following pre-treatment of the chondrocytes with IL-1, a potent inhibitor of matrix synthesis (**315**). Furthermore, it has been demonstrated that adenovirus-mediated over-expression of FADD into rheumatoid arthritis synoviocytes enhanced apoptosis of these cells both *in vitro* and *in vivo* in mice, and that the induced apoptosis was limited to the inflammatory cells of the synovium tissue and had no effect on the chondrocytes. The adenovirus thereby mediated FADD, serving as a potential novel therapy for rheumatoid arthritis (**339;340**).

Our research on the AAV vector mediated $TGF_{\beta 1}$ gene transfer to human articular chondrocytes has revealed that the AAV-$TGF_{\beta 1}$ vector efficiently transduces both normal and osteoarthritic human chondrocytes, and it would seem that normal articular chondrocytes and osteoarthritic chondrocytes are equally responsive to AAV vector mediated gene transduction (**III**). Furthermore, the AAV vector mediated $TGF_{\beta 1}$-overexpression results in elevated RNA expression levels of type II collagen and aggrecan, whereas the RNA expression of matrix metalloproteinase 3 is reduced (**III**).

Future directions

No natural repair mechanism can heal damaged or diseased cartilage. Existing pharmacologic, surgical and cell-based treatment may offer temporary relief, but is incapable of restoring damaged cartilage to its normal phenotype. Gene transfer offers an opportunity to achieve sustained, localized presentation of bioactive proteins or gene products to sites of tissue damage. Various cDNAs have been cloned that may be used to stimulate biological processes that could improve cartilage healing by 1) inducing mitosis and the synthesis and deposition of cartilage extra-cellular matrix components by chondrocytes, 2) inducing chondrogenesis by mesenchymal progenitor cells, or 3) inhibiting cellular responses to inflammatory stimuli. The challenge is to adapt this technology to a useful clinical treatment modality. Using different marker genes, the principle of gene delivery to synovium, chondrocytes and mesenchymal progenitor cells has been convincingly demonstrated. An articular cartilage progenitor cell population from the surface zone of articular cartilage has recently been identified that may be an attractive gene transfer candidate for stimulation of cartilage neogenesis (**341**). The potential offered by this discovery lies in the better understanding of both cartilage growth and maintenance and the novel solutions for articular cartilage repair.

Research within this field seems to move towards functional studies. This involves the identification of appropriate genes or gene combinations, incorporation of these cDNAs into appropriate vectors and delivery to specific target cells within the proper biological context to achieve a meaningful therapeutic response. Methods currently explored range from simple direct delivery of a vector to a cartilage defect to the complex synthesis of cartilaginous implants through gene-enhanced tissue engineering. Data from recent efficacy studies provide optimism that gene delivery can be harnessed to guide biological processes toward both accelerated and improved articular cartilage repair (**342-344**).

Further studies should provide a clearer picture of the functional boundaries of gene transfer techniques and the parameters critical to success. An area that has received little attention is that of regulated transgenic expression, presumably because it has yet to be determined how much expression is required and for how long. Once this has been more clearly established, steps can be taken to finetune the process through the incorporation of sophisticated promoter systems.

In the process of preclinical evaluation of experimental methods for cartilage treatment and repair, a major challenge is the choice of an animal model of genuine relevance to human disease. Smaller mammals like rats and rabbits enjoy the advantage of large sample size, but the chondral layer in these animals is quite thin and they have a tendency to self-repair. Given the elective nature of medical treatment for cartilage repair and the risks associated with gene delivery (perceived or otherwise), veterinary medicine may, indeed, be a prudent pathway to take before clinical studies in man. Large animals like caprines have joints proportionally similar to those of humans, with articular cartilage of more comparable depth. These animals also develop actual clinical disease analogous to that found in humans. Since many of the gene products implicated for therapeutic use may potentially generate significant side effects, it may be best to first establish a sustained track record of safety and long-term clinical efficacy in animal models prior to human testing.

Wear debris-induced osteolysis

Although total joint replacement surgery is one of the most successful clinical procedures performed today, patholocial osteolysis around hip and knee implants, resulting in aseptic loosening of the prosthesis, remains a major problem. It is estimated that 20% of all prosthetic implants will demon-

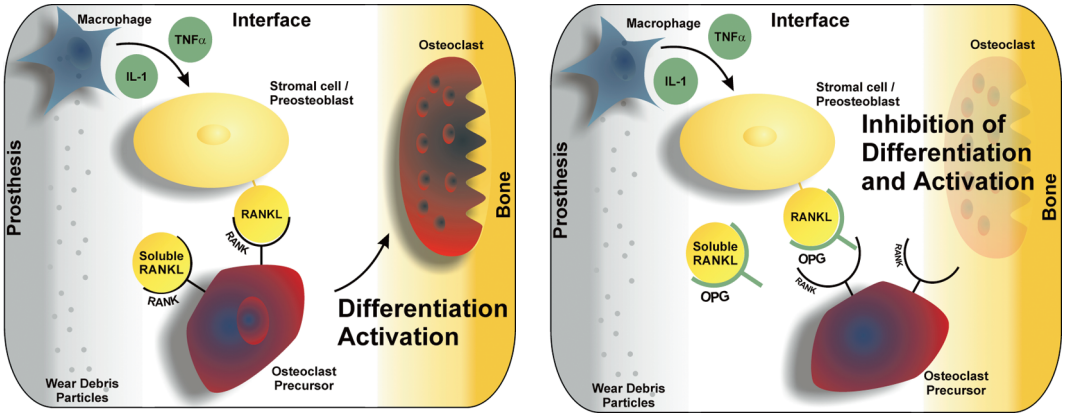

Figure 9. Role of the RANK / RANKL / OPG system on long-term aseptic periprosthetic osteolysis (IV). RANKL expression and OPG production are modulated by various cytokines, hormones, drugs and mechanical strains. In bone, osteoblasts and their precursor cells (stromal cells) express RANKL in a cellular form and a truncated form generated by the protease, TNFα converting enzyme. RANKL stimulates the receptor RANK on osteoclast precursors and mature osteoclasts and activates intracellular pathways to promote osteoclast differentiation and activation (NF-κB and c-Jun *N*-terminal kinase pathways), and cytoskeletal reorganization and survival (PKB/Akt pathway) that increase bone resorption and bone loss (left). Stromal cells and osteoblasts secrete osteoprotegerin (OPG), which acts as a decoy receptor and blocks RANKL (right).

strate findings of aseptic loosening (**345-348**), often requiring surgical revision. Aseptic loosening *per se* cannot be prevented or treated by existing non-surgical methods. Gene therapy, however, offers novel possibilities.

Pathogenesis of aseptic loosening

The current paradigm to explain periprosthetic osteolysis is that micro-particles of polyethylene, metal and acrylic cement (wear debris particles) generated by wear of the prosthesis are phagocytosed by local macrophages and foreign-body giant cells (**349-351**). Repeated phagocytosis of the wear debris particles, which are impervious to enzymatic destruction, results in activated inflammatory cells that secrete pro-inflammatory cytokines and proteolytic enzymes, which activates a periprosthetic osteolytic cascade (Figure 9A). In particular, IL-1 and TNFα are potent mediators of bone resorption (**352-356**), and the immune-activating cytokines PDGF and IL-11 were recently identified also within the periprosthetic tissue (**357;358**). These cytokines provide activation signals to lymphocytes (**359**), and, in turn, the lymphocyte-derived cytokines IL-2, IL-6 and IFN-γ may influence osteoclastic activity and bone remodelling (**360**). Additionally, macrophages induced to phagocytose biomaterials (UHMWPE, PMMA and orthopaedic alloys) may potentially differentiate into osteoclastic cells capable of extensive lacunar bone resorption during bone culture (**361;362**) and they may also express high levels of the osteoclast activating cytokine M-CSF (**358**).

This inflammatory process leads to the overexpression of an essential osteoclast differentiation factor, RANKL (**363-365**), by marrow stromal cells, osteoblasts and activated T-cells. This directly stimulates osteoclastogenesis and bone resorption by binding to the RANKL receptor on osteoclasts and their precursors, RANK, which is a member of TNF receptor superfamily (**366-370**). Dougall *et al* (**371**) determined the essential role of RANK in regulating osteoclastogenesis in RANK gene knockout ($RANK^{-/-}$) mice. Li *et al* (**372**) reported that osteoclastogenic effects of RANKL could not be reproduced in $RANK^{-/-}$ mice, suggesting that RANK serves as the sole osteoclast receptor for RANKL and controls osteoclast development and activation (**373**). In addition, failure of RANK $^{-/-}$ (knock-out) mice to mount a significant osteoclastic response in the presence of experimental inflammatory arthritis further supports the concept that RANK plays a key role in controlling osteoclastogenesis (**374**).

The wear debris-induced cascade is negatively regulated by OPG (**377**), which is a natural

decoy TNF receptor-related protein that binds to RANKL, blocks its interaction with RANK and inhibits osteoclast development and activation (**367**) (Figure 9B). Data indicate that OPG and RANKL interact as key regulators of osteoclastogenesis and bone resorption (**364;365**). RANK blockade with OPG or the recombinant receptor antagonist RANK:Fc dominates osteoclastogenesis and bone resorption inhibition. The clinical potential of this observation has been convincingly demonstrated in a variety of animal models (**375**) for conditions such as wear debris-induced osteolysis (**368;376**), osteoporosis (**377**), erosive arthritis (**374**), humoral hypercalcemia of malignancy and experimental bone metastasis (**378;379**).

As periprosthetic osteolysis is a chronic inflammatory process, gene therapy offers the possibility of lifelong delivery of therapeutic agents such as OPG.

In vitro models

In order to explore the effect of transgene OPG on osteoclastogenesis and activation, we developed a bi-cistronic recombinant AAV-OPG-IRES-eGFP vector for *in vivo* delivery of OPG (**IV**). The efficiency and bioactivity of the vector was tested in a series of *in vitro* experiments to determine the bioactivity of trans-gene OPG (**IV;380**). The *in vitro* conditions provided separate studies of the transgene OPG effects on osteoclastogenesis and osteoclast-mediated osteolysis (**IV;380**).

Osteoclastogenesis can be assessed in cultures of murine splenocytes stimulated with RANKL and M-CSF (**381**). Using transwell membranes, uninfected fibroblast-like synoviocytes or fibroblast-like synoviocytes expressing transgene OPG or LacZ were co-cultured with murine splenocytes. After six days in co-culture, osteoclast formation could be quantified by counting the number of multinucleated "tartrate-resistant acid phosphatase" positive cells (**382**).

The osteoclast bone resorption activity can be studied *in vitro* by adding neonatal rat calvarial bone cells to bovine cortical bone wafers in the presence of parathyroid hormone to induce osteoclastic resorption (**380**). The activity of the transgene OPG was determined by adding culture supernatants from the AAV-OPG-IRES-eGFP transduced 293-cells (**IV**).

These *in vitro* models providing separate pieces of information about anti-osteoclast gene delivery and osteoclast development and activation are unique.

In vivo models

Clinical aseptic loosening of orthopaedic implants usually occurs more than 5 years after operation with successful primary fixation and integration. Similar implant survival duration may be observed in animal models mimicking aseptic loosening (**383-385**). We studied the biology of wear debris-induced osteolysis independent of the critical biomechanical components in aseptic loosening (**IV**). We used a murine calvaria model with surgical implantation of wear debris particles on top of the skull. The wear debris induces an intense inflammatory reaction that leads to osteoclastic formation and osteolysis within 1 week (**382;386;387**). The calvaria mouse model permits the use of exquisite molecular reagents and genetically defined strains together with highly quantitative outcome measures of osteoclast formation and bone resorption. The use of mice genetically defective in TNF (**388**) and RANK (**368**) signalling produced the first *in vivo* evidence in support of the proinflammatory osteolysis as the cause of aseptic loosening. Furthermore, similar findings from studies in which wild-type mice were treated with TNFR:Fc (**380**) and RANK:Fc (**368**) demonstrated the potential of a therapeutic agent or gene therapy for aseptic loosening.

Gene therapy results

Pharmacological intervention targeted towards the macrophage may reduce the response to wear debris (**389**). However, delivering adequate levels of cell-specific therapy to the site of periprosthetic inflammation, without causing undesirable systemic effects, represents a considerable challenge. Local cytokine inhibition is a potential therapy that may reduce inflammation in the periprosthetic tissue, and several biological mediators have been identified as useful for clinical application. In particular, the IL-1 receptor antagonist protein has been successful in reducing inflammation (**390;391**), and the anti-inflammatory cytokine IL-10 appears to possess the capacity to reduce cell-mediated inflammatory reactions (**392-394**). Still, the delivery of appropriate doses of proinflammatory cytokine

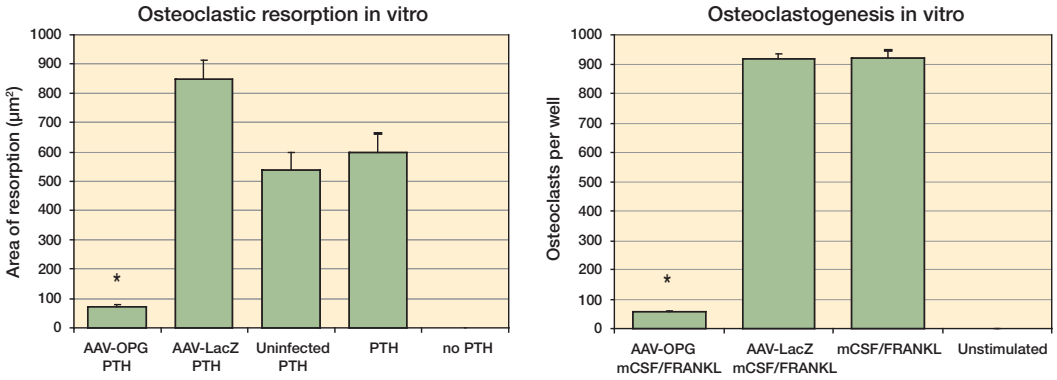

Figure 10. AAV vector mediated OPG inhibits osteoclastic resorption and osteoclastogenesis *in vitro*. Culture media from either AAV-transduced (AAV-OPG or AAV-LacZ) or uninfected human embryonic kidney 293 cells was added to parathyroid hormone stimulated bone wafer cultures over 10 days. The resorbed area on the surface of the bone wafers was determine from a 40x digital image using Osteometrics® software (Atlanta, GA) (mean ± SEM) (IV).

Murine splenocytes stimulated with M-CSF and RANKL to differentiate into osteoclasts were co-cultured with transduced or uninfected fibroblast-like synoviocytes. After six days of culture, the cells were fixed and stained for tartrate-resistant acid phosphatase and the number of positive, multinucleated cells (osteoclasts) in each well was counted (mean ± SEM) (IV). * p-value < 0.05 (N = 6 and N = 5, respectively).

inhibitors to the periprosthetic tissue remains a problem. Recent advances in gene therapy techniques (**395;396**), however, suggest that viral vectors may be capable of delivering anti-inflammatory cytokine genes to the periprosthetic tissues, which could control the local reaction and extend the life of the prosthesis.

Studies have been performed to evaluate the effects of anti-TNFα gene therapy in the calvaria model using a recombinant adenovirus expressing a soluble TNF receptor (**397**). The experiments demonstrated the predicted antiresorptive effects in nude mice, but it also, surprisingly, produced two additional findings. First, local gene delivery was not significantly more efficacious than systemic gene therapy, and inhibition of bone resorption correlated with the TNFR:Fc concentration in serum; second, and more importantly, the production of a tremendous inflammatory reaction from the adenoviral vector alone, which induced an osteolytic response that significantly exceeded that of the wear debris when placed directly on the calvaria of wild-type mice. This finding is consistent with many previous studies regarding the immunogenicity of adenoviral vectors and may still warn against their clinical use (**398;399**).

To investigate the potential of the RANKL system as a therapeutic target in aseptic loosening, we investigated whether OPG gene transfer using an AAV vector protected against orthopaedic wear debris-induced bone loss (**IV**). *In vitro* experiments demonstrated that fibroblast-like synoviocytes transduced with the AAV-OPG-IRES-eGFP vector produced much higher OPG levels (135 ng/mL) (**IV**) than fibroblast-like synoviocytes, which were stably transfected with a retroviral vector expressing OPG (0.3 ng/mL) (**376**), as determined by ELISA. These higher levels may be attributed to multiple copies of the episomal rAAV compared with the single copy of the integrated retroviral vector. Furthermore, this OPG was bioactive as determined in osteoclastogenesis and in bone wafer resorption ***in vitro*** assays (Figure 10) (**IV**).

The effects of AAV-OPG-IRES-eGFP gene therapy on osteoclastogenesis and wear debris-induced bone resorption *in vivo* were examined using a well-characterized murine calvaria model of debris-induced bone resorption (**386;387**). The rapid onset and quantifiable measures available with this model make it attractive for screening the effectiveness of various agents designed to prevent osteolysis. Long-term expression from a single vector administration is a future therapeutic goal, and previous successful experiments with AAV vectors therefore made the quadriceps muscle a natural choice as a candidate for direct injection of the AAV vector encoding human OPG gene or the eGFP marker gene (**37**). Successful transgene expression was confirmed by the detection of elevated OPG concentrations in serum and positive

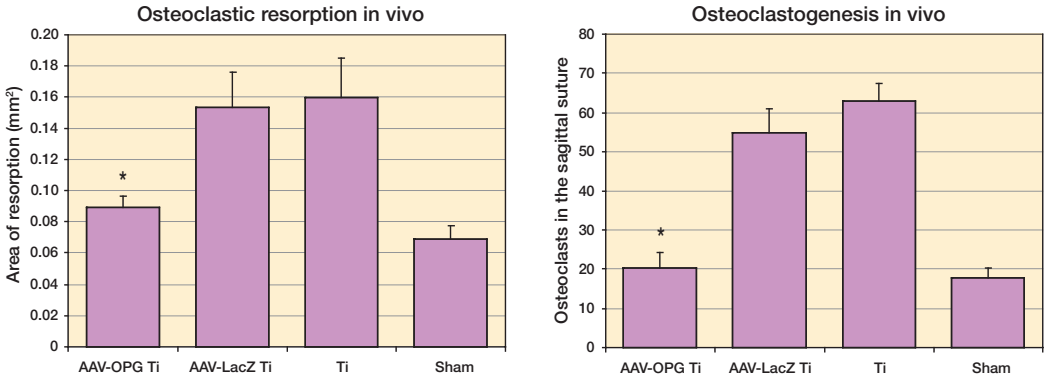

Figure 11. AAV-OPG gene therapy inhibits wear debris-induced osteoclastogenesis and osteolysis *in vivo*. The effects of AAV-OPG gene therapy on wear debris-induced osteolysis and osteoclastogenesis were evaluated in the mouse calvaria model (mean ± SEM) (IV). The resorption and the number of osteoclasts in the sagittal suture area were significantly lower in the AAV-OPG treated mice than in the positive controls (AAV-LacZ Ti and Ti groups). * p-value < 0.05 (N = 5).

eGFP fluorescence of the myocytes. The serum OPG levels were significantly increased two days after intramuscular injection of AAV-OPG-IRES-eGFP, peaked after six days and then progressively decreased until a steady concentration within the therapeutic interval was reached on day 8 (**IV**). In other studies involving a single intramuscular injection of AAV-OPG-IRES-eGFP, we found that the serum OPG levels can be maintained for over two months (**VI**). The AAV vector expression kinetics were consistent with those reported in other studies, showing that high serum levels of trans-gene OPG can be achieved within days after transduction (**400**) and that intramuscular AAV vector administration can maintain long-term (years) transgene expression (**37**). Equally important to the success of this transgene delivery by the AAV-OPG-IRES-eGFP vector is the absence of an immune response to either the vector or the gene product. Furthermore, our investigations demonstrated complete inhibition of osteolysis in animals receiving this therapy (Figure 11). Again, these results are similar to those obtained in an air pouch micro-particle model (**401**) and they support the hypothesis that RANKL is the final effector of osteoclastogenesis and bone resorption.

In conclusion, our studies have demonstrated elimination of wear debris-induced osteolysis by means of *in vivo* gene therapy with AAV transferring OPG. In so doing, we overcame the limitations of the *ex vivo* approach (**376**) and the host response problems associated with adenovirally mediated gene therapy (**397**). As such, our studies represent proof-of-principle that *in vivo* targeting of somatic tissue with a single administration of an AAV-OPG vector at the time of surgery could be sufficient for lifelong protection against aseptic loosening.

Future directions

Significant evidence now exists indicating that aseptic loosening of orthopaedic implants is caused by wear debris-induced inflammation, osteoclastogenesis and subsequent osteolysis around the prosthesis. Our preliminary results have provided encouraging preclinical data for OPG-mediated therapy in aseptic loosening, and, overall, the data suggest that gene therapy may be an appropriate technique for boosting local anti-bone resorptive activity at the prosthesis–bone interface. Novel studies in our laboratories using AAV vector coated implants are showing promising results in terms of increased implant-bone integration (**402**). Future studies designed to assess safety and efficacy and biomechnical analysis in large animal models with an actual prosthesis or prosthesis dummies will be useful for evaluating the potential of this gene therapy for human use.

Fracture healing in relation to osteoporosis

Osteoporosis is characterized by a relative increase in the bone resorption to bone formation ratio. Reducing the speed of bone resorption may be an attractive option for therapeutic intervention. As the osteoporotic patient is highly susceptible

to bone fracture, the effect of anti-osteoporosis treatment on fracture healing is essential. Medical non-compliance of the osteoporotic patient is a well-known phenomenon critical to the efficiency of traditional medical therapy. Gene therapy may provide an effective long-term treatment alternative in this situation.

Biology of fracture healing and osteoporosis

Bone healing can be divided into four stages of regeneration: 1) generation of haematoma, 2) proliferation of osteogenetic cells, 3) callus formation, and 4) consolidation. The initial phases are mainly characterized by callus tissue formation, whereas the later stages are characterized by callus ossification and remodelling. Various growth factors, cytokines and hormones conduct these complex processes. GH (**403;404**), PTH (**405-407**), TGF-β (**408**) and BMPs (**VIII;409**) are important contributors to callus expansion and new bone formation mediated by chondrocytes and osteoblasts. Subsequently, the role of osteoclastic activity increases as callus is being remodelled. The RANK / RANKL / OPG receptor-ligand complex (see "Pathogenesis of aseptic loosening") is the key factor in regulating osteoclast formation, apoptosis and osteoclast activity. Several of the above growth substances converse in mediating their function via the RANK / RANKL / OPG complex. Cellular expression of OPG and RANKL in murine callus tissue has been demonstrated to be tightly coupled during fracture healing and to be involved in the regulation of both endochondral resorption and remodelling (**410**). However, our studies were the first to investigate the effects of OPG on fracture healing (**V-VII**).

An elevated skeletal RANKL / OPG ratio apparently promotes bone loss (**411**). This implicates the RANK / RANKL / OPG system in the pathogenesis of postmenopausal osteoporosis, which is characterized by increased bone resorption due to estrogen deficiency. Postmenopausal women also express higher RANKL levels in marrow stromal cells or lymphocytes than premenopausal women or postmenopausal women on estrogen therapy (**412**). In addition, RANKL expression correlates inversely with serum levels of 17β-estradiol and positively with bone resorption markers, and some studies have documented that polymorphisms in the promoter region of the OPG gene may involve an increased risk of developing postmenopausal osteoporosis (**412**). Several lines of estrogens and the selective estrogen receptor modulator raloxifene have been demonstrated to stimulate OPG production in osteoblasts (**365;413**). An analysis of 180 women with postmenopausal osteoporosis revealed that OPG serum levels were positively correlated with 17β-estradiol serum levels and BMD and negatively with biochemical markers of bone metabolism (**414**).

Systemic glucocorticoid therapy lowers OPG serum levels, which may contribute to glucocorticoid-induced osteoporosis (**415**). Restoring a balanced RANKL / OPG ratio or blunting RANK responsiveness may, accordingly, be an effective method for preventing pathological osteoclast activation causing bone resorption (**377**). Different strategies may be used for therapeutic purposes: RANKL effects can be blocked by synthetic OPG fusion proteins (**377;416-419**), soluble RANK fusion proteins (**370;379**) or RANKL antibodies. RANK activation can be suppressed by peptidomimetics preventing RANKL binding to RANK (**420**) or by blocking post-receptor signalling (**421**). OPG production can be stimulated by 17β-estradiol (**422**), raloxifene (**413**) and bisphosphonates *in vitro*, and by mechanical strain (**423**) and selective small molecule stimulators *in vivo* (**424**).

The attributes of endogenous OPG, which are shared by recombinant OPG, are likely to account for the unique ability of recombinant OPG treatment to consistently and dramatically reduce osteoclast numbers in animal studies (**378;425-428**). A single injection of recombinant human OPG-Fc into intact disease-free rats caused a rapid (12 h) reduction in osteoclast surfaces, which was maintained for 30 days (**429**).

Therapeutic experience on OPG and RANK-Fc fusion proteins rests mainly on studies of osteoporosis performed *in vitro* and in animal studies. On this basis two randomized controlled trials were performed to evaluate the short-term effects of a single dose of human OPG-Fc fusion protein on biochemical bone markers in humans. In the first of these studies, OPG treatment caused the rapid (12 h) and sustained (30 days) suppression of bone resorption (urinary excretion of deoxypyridinoline levels –80%) and bone formation markers (serum levels of osteocalcin –20%) in 52 postmenopausal

women with osteoporosis who were followed for 85 days (**430**). The second study compared the effects of a single dose of OPG versus pamidronate in patients with myeloma bone disease (n = 28) and in women with skeletal metastases due to advanced breast cancer (n = 26). These women were followed for 56 days (**431**). Urinary excretion of the bone resorption marker ***N***-telopeptide was reduced by OPG treatment by 74% (breast cancer) and 47% (myeloma), which was comparable to the pamidronate effects (**431**). While these short-term studies were not intended to assess clinical end points (BMD, fractures, hypocalcaemia), they represent proof of principle that RANKL blockade is an efficient modality for treating altered bone metabolism in humans (**430;431**). Treatment with OPG-Fc fusion protein in humans has produced mild, asymptomatic hypocalcaemia, and, in rare cases, the development of anti-OPG antibodies. No such effects have been observed using RANKL antibodies (**432**), which appear to outperform OPG-Fc fusion protein because they specifically bind RANKL, but no other TNF ligands, for example TRAIL.

To this end, Amgen® has developed a fully human monoclonal antibody (AMG 162) that specifically targets RANKL. Early clinical data seem to show that AMG 162 is well-tolerated by patients and Amgen® has announced interim data from a phase II clinical study showing the clinical effects of AMG 162 on bone endpoints. Phase III clinical studies with AMG 162 were subsequently initiated in 2004 (**433**). In addition to its potential use in osteoporosis, the ability of AMG 162 to suppress bone loss in metastatic bone disease is being studied in cancer patients (phase II clinical studies were initiated in 2004).

As osteoporosis is a disease characterized by persistent reduction of bone mass without alteration in bone composition, eventually leading to fractures, it is important to know how anti-resorptive treatment like OPG affects fracture healing.

In vivo models

Bone healing studies have been carried out in various animal models: dog (**434;435**), sheep (**436**), rabbit (**408**), rat (**403-405;407**) and mouse (**437**). Rat and mouse, in particular, have been widely used for studies of bone healing and much knowledge is therefore available on both intact and fractured bones. Homogenous populations of rats and mice are readily available and the relatively simple experimental procedures needed for fracture and fixation in rats are well-tolerated by the animals. Bone biology in rat and mouse is very similar. The greater size of rat bones facilitates operative procedures in comparison to mouse bones. An advantage of the mouse model is the availability of transgene strings (genetically knock-in and knock-out mice) (**VII**).

Rat and mouse bone healing models are, however, associated with several problems. Laboratory animals fed ad libitum generally continue to gain body weight for most of their life span (**438**). The growth of the rat tibia seems to be rapid until 6 month of age and then it declines (**439**). In old rats there is no longer evidence of osteogenesis in the growth plate (**439;440**). Rats and mice do not have Haverian osteoms and, hence, not the same pattern of bone remodelling as humans. But intracortical bone remodelling is seen in old rats and after prostaglandin E2 stimulation (**441**). Cortical bone in rats and mice therefore has a latent remodelling capacity, which makes it likely that the basic mechanisms of human bone turnover also exist in rats (**442**).

The bone repair process in rats and mice is usually classified as non-osteonal according to the classification proposed by Chao *et al* (**443**), but it features the same stages and morphological components as secondary fracture repair in higher animals, including cartilage formation (**444-448**). The stereology and architecture of bone collagen in rodents are also similar to that of higher animals. Differences in the fracture repair process among species are, however, quite distinct. Thus the relative amount of cartilaginous callus formed during fracture healing in lower animals is high (**444**), and the bone-inductive potential of bone matrix from higher animals is lower than that of bone matrix from rodents (**449**).

Several fracture models have been proposed for rats and mice: Manually produced fractures of the tibia (**445;450;451**) or femur (**452**) without fixation, fractures of the fibula inherently stable through a tibia (**453**) and fractures or osteotomies of the femur or tibia immobilized with an intramedullary nail (**454-456**). Femoral and tibial fractures stabi-

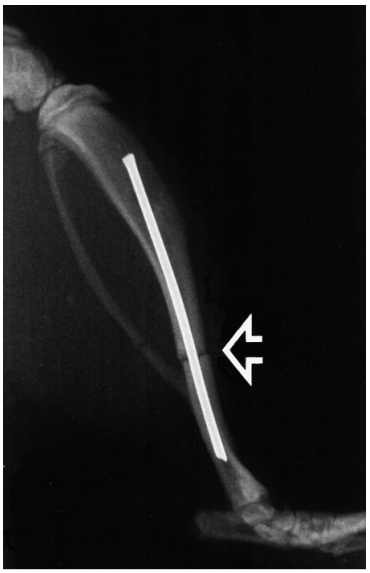

Figure 12. Using an external three-point bending procedure and closed intra-medullar nailing, uniform cross-sectional fractures could be produced without damage to the soft tissue component (V-VII).

lized with intramedullary nailing are properbly the most commonly used models, and the healing fractures are usually compared with the contralateral non-fractured bone. For the purpose of producing a fracture, the rat and mouse tibia offers the advantage of being only partly covered with muscles. A closed fracture can therefore be produced manually, or by the means of a three-point bending device (**V-VII;456;457**) (Figure 12). The latter procedure ensures standardization of the fracture level (2-4 mm proximal to the talocrural joint) and the soft tissue damage, which is important in experimental studies (**V-VII;458**). Furthermore, the tibiofibular bone is loaded in the same direction as in the subsequent mechanical testing. Animals with comminute fractures, fractures outside the specified fracture level, or with displaced nails should be excluded from further analysis (**V-VII**). The rat tibia fracture model is characterized by ossification of cartilaginous callus at 3 weeks of healing and by a substantial remodelling of osseous callus at 8 weeks of healing (**V;VI**). In the mouse tibia fracture model the corresponding time pint are at 2 weeks of healing and 4 weeks of heeling (**VII**).

The mechanical conditions during fracture healing, i.e. the frequency and degree of inter-fragmentary motion, determine the type of fracture repair, the callus tissue developed and the extra-cellular matrices produced during healing (**443;459;460**). Functional activities of the fractured extremity (**454;461**) as well as induced micro-movements (**462**) have been found to speed fracture healing. Both excessive flexibility and rigid fixation inhibit fracture healing (**463**) and the optimal conditions have yet to be determined.

As rotational instability impedes fracture healing (**464**), we chose to fixate the experimental fracture with an intra-medullary pin (**V-VII**). An open medullary nailing procedure is associated with delayed healing (**465**), and we therefore used closed medullary nailing (**V-VII**). No reaming of the medullary cavity was performed. The size of the intramedullary nails was determined by the largest dimension of Kierschner wire (rat) or insect pin (mouse) that could be introduced into the marrow cavity without reaming. Since the intramedullary nail filled the medullary canal, formation of endosteal callus might have been compromised. Intramedullary nailing also impedes the endosteal blood supply, causing ischaemic necrosis (**466;467**). Thus, the method of fracture fixation used might have delayed fracture healing.

Gene therapy results

Osteoporotic patients' non-compliance seriously impairs the efficiency of traditional medical therapy, affecting, in particular, the risk of future fractures (**468**). Compliance is known to be linked to frequent dosing, which for some osteoporosis therapies drops by 50% within a year of initiation (**469**). The minimum dosing frequency among currently approved osteoporosis therapies is once per week (**470**), but efforts are underway to test once-per-year intravenous dosing of a bisphosphonate (**471**). OPG is a protein and the potential therefore exists for using gene therapy delivery to facilitate even longer treatment intervals. A proof-of-concept study recently demonstrated that OPG could be effectively delivered in mice using gene therapy. A single injection with an adenovirus vector containing human OPG-Fc produced systemic exposure reaching therapeutic OPG levels for at least 18 months (**472**). This Ad-OPG treatment successfully prevented development of osteopenia in ovariectomized mice. Prior reports demonstrated that OPG

Table 6. Histological analyses of fractured tibiae after 3 and 8 weeks of healing in AAV-OPG treated rats (VI)

	AAV	AAV-OPG	p-value
3 weeks of healing			
Number	19	19	
Osteoclasts [a]	66.4 (2.4)	22.0 (2.0)	<0.001
Resorption pits [b]	13	7	0.1
Woven bone formation [c]	2	0	0.5
8 weeks of healing			
Number	17	17	
Osteoclasts [a]	61.6 (2.6)	19.9 (2.3)	<0.001
Resorption pits [b]	17	8	<0.001
Woven bone formation [c]	17	7	<0.001

[a] Number of osteoclasts per mm^2 in the callus, mean and (SEM).
[b] Number of animals with resorption pits in the fracture line of the original cortical bone.
[c] Number of animals with formation of woven bone in the fracture line of the original cortical bone.

can be consistently delivered at therapeutic levels using an AAV vector (**IV;400;401**). In these reports, a single AAV-OPG injection generated persistent hyperexpression of biologically active human OPG, which resulted in stable levels at 100–300 ng of circulating human OPG per mL serum. Of particular interest is that at these concentrations human OPG by means of AAV-OPG therapy was capable of fully reversing established osteopenia in ovarietomized mice (**400**). The success of future OPG treatment of osteoporosis is highly dependent on the effect of OPG on fracture healing and remodelling.

Our study (**VI**) demonstrated that AAV-OPG gene therapy, resulting in equivalent OPG serum concentrations, did not conflict with normal bone healing. However, the histological findings indicated that osteoclast numbers were depleted and that AAV-OPG therapy depressed remodelling and integration of the genuine cortical bone at the fracture line (Table 6). These findings are in agreement with data from repeated administration of high dosages of both human OPG (intravenous) (**V**) and RANK:Fc (intraperitoneal) (**VII**) on healing fractures (Figure 13). In the intravenous OPG study (**V**), OPG was administered at dosages of 10 mg/kg twice weekly as previous experiments have demonstrated that this dose of *h*OPG ameliorated bone loss in rats after ovariectomy (**400**). Moreover, a single intravenous *h*OPG (5mg/kg) injection caused an approximately 95% reduction in osteoclast surface/bone surface after 10 days (**429**). Likewise, our intravenous OPG study (**V**) showed that callus tissue seemed to respond in a similar way to OPG treatment as the number of osteoclasts was reduced by approximately 93%. In the RANK:Fc and OPG treatment studies, the results indicated that administration of RANK signaling inhibitors sufficient to reduce bone resorption were not detrimental to fracture healing (**V;VII**). In the RANK:Fc study, withdrawal of the agent restored osteoclast numbers and activity during the remodelling phase of fracture healing (**VII**). We also found that RANK

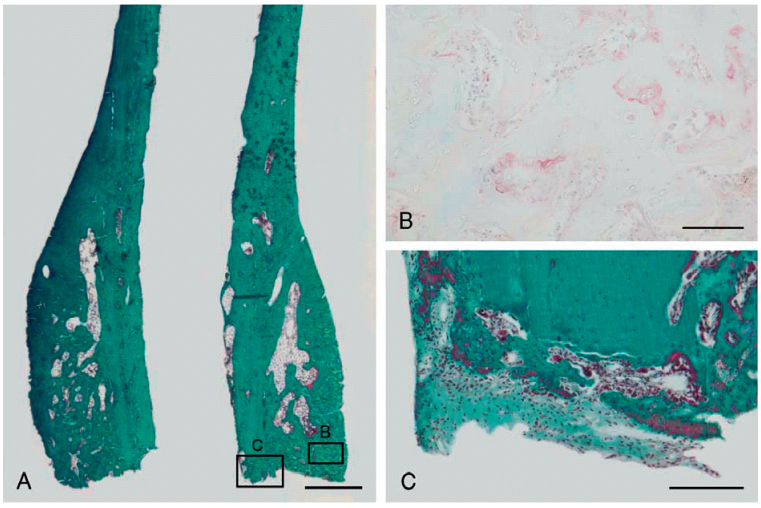

Figure 13. Frontal tibia section including proximal fracture line, callus, and original cortical bone (A). Frame B refers to location of pictures B and frame C refers to location of pictures C. TRAP-staining of external callus (B). Osteoclast cytoplasma is stained in red. Goldener-Trichrome staining of original cortical bone fracture line with resorptions (C). Dimensions are given by bars (A: 1 mm; B: 100 μm; C: 200 μm) (V).

signaling was not required for the early phase of fracture healing as evidenced by the repair (callus formation) observed in RANK knock-out (RANK -/-) mice (**VII**).

The normal remodelling processes of healing fractures substantially enhance material properties in the callus tissue (**405**). In contrast to high dosage intravenous OPG treatment, the AAV-OPG gene therapy data showed that AAV-OPG did not impair the enhancement in structural strength or ultimate stress of the fractures seen at 8 weeks of healing (Figure 14). Again, this may due to the dose-dependent pharmacokinetics of OPG. This is, however, not tantamount to claiming that OPG inhibits fracture healing and delays the sequences of callus remodelling in the long term.

Essential to the success of stable transgene delivery by rAAV vectors is the absence of an immune response to either the vector or the gene product. Thus, AAV vector-mediated gene delivery of OPG to mice with micro-particle-induced osteolysis reduced both osteoclasteogenesis and bone resorption (**IV;401**). Systemic vector-related toxicity was addressed in a recent work by Kostenuik (**400**), who revealed that AAV-OPG seemed to have an acceptable safety profile and safety margin. Even though these safety-efficiency results need to be evaluated in more advanced models, the results may be proof of an AAV-OPG resorptive bone disorders or osteoporosis gene therapy concept.

The effects of various bisphosphonates on fracture healing in different animal models have been studied in a number of studies which collectively report an augmented callus size (**473-482**). The increased callus dimension has been shown to persist in rat fractures even after 49 weeks of healing (**480**). Some investigations found enhanced mechanical strength of the fractures following bisphosphonate treatment (**478;480;481**), whereas other studies indicated no effects or even decreased callus strength (**476;477;482;483**). Our study indicates concordance between OPG and bisphosphonate treatment as far as callus dimensions are concerned. The pronounced reduction in callus material properties after high dosage intravenous OPG treatment was not found with bisphosphonate (**480**), possibly because OPG severely reduces the number of osteoclasts in callus tissue, whereas bisphosphonate treatment elicits a more modest reduction (**479**).

RANKL inhibitors may remain an attractive alternative to bisphosphonates in the treatment of chronic bone resorptive disorders like osteoporosis (**V-VII;484-486**). Although direct comparisons are not available, OPG seems to be a potent osteoclast inhibitor and its effects on fracture healing seem to be dose-dependent (**V-VII**).

Future directions

The use of pharmaceutical agents for treatment of osteoporosis has now become widespread, and the demand for new therapies is expected to rise dramatically. Anabolic agents represent an important new advance in osteoporosis therapy. PTH is the most promising of the anabolics currently available (**487**), but important questions remain to be answered about PTH, which also modifies the RANK / RANKL / OPG complex. An anabolic effect on cortical bone must be further documented, for instance in studies combining anabolic and anti-resorptive treatment. An interesting anti-resorptive factor of the future will be the direct RANKL blockade by novel drugs such as the RANKL antibody, AMG 162 (**432**). All these agents act in various ways on bone remodelling and repair processes via the osteoclast and their use can therefore affect the progress of fracture healing, fixation of implants and treatment of osteomyelitis.

Structural bone allograft healing

Bone grafting is commonly used in orthopaedic reconstruction surgeries such as spinal fusion, revision of failed joint arthroplasty and repair of skeletal defects following trauma or removal of tumor. Both experimental and clinical studies have shown that fresh autogenous grafts are vastly superior to allograft bone in graft repair and remodeling (**488;489**). However, due to the size limitations of autogenous bone grafts, problems with chronic pain at the donor site, and also complications of the procedures, processed allograft remains an attractive substitute for bone grafting (**490**). Extensive research has shown that the critical difference between autograft and allograft healing is the participation of the grafted cells (**491**). Gene therapy may offer a solution to provide bone inductive and

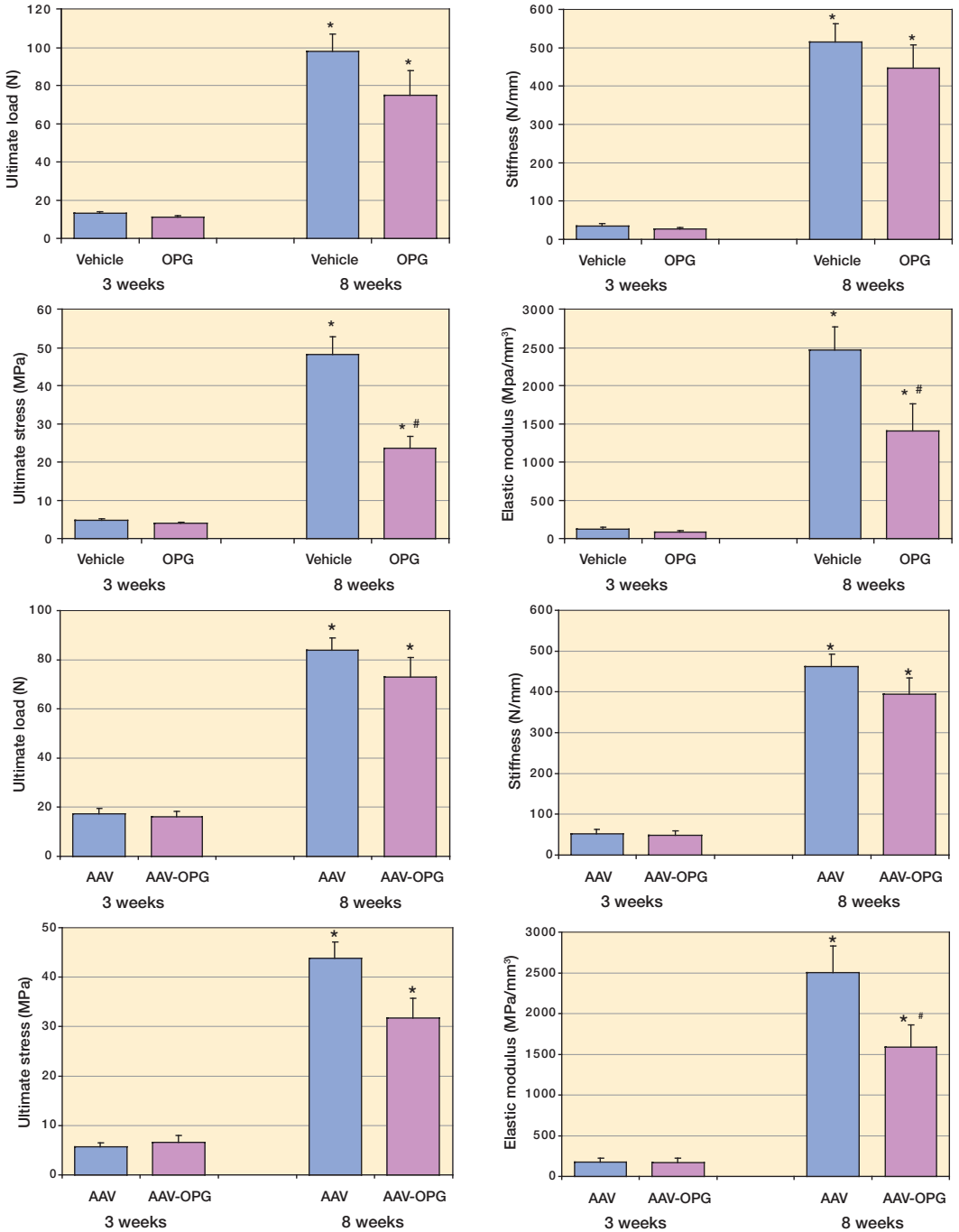

Figure 14.The mechanical properties of healing fractures after 3 and 8 weeks of healing. The influences of intravenous OPG treatment (A) (V) and AAV-OPG therapy (B) (VI) are displayed. Ultimate load and stiffness increased from week 3 to week 8 in all groups. No differences in either ultimate load or stiffness were found between vehicle and OPG groups or AAV-eGFP and AAV-OPG groups at 3 or 8 weeks. Ultimate stress and elastic modulus increased from week 3 to week 8 in both all groups. The increase was less pronounced in the OPG-treated animals, and therefore ultimate stress and elastic modulus were lower in the group treated with OPG intravenously after 8 weeks of healing. In the AAV-OPG group, only elastic modulus was significantly lower after 8 weeks of healing. *: Significantly different from corresponding group value at 3 weeks of healing ($p<0.05$). **#:** Significantly different from control animals at corresponding time-points ($p<0.05$).

conductive signal proteins to the cells surrounding the allograft *in vivo*.

Biology of structural bone allograft healing

The repair and incorporation of bone graft is a regulated process that is very similar to fracture healing as described above (see "Biology of fracture healing and osteoporosis"). The initial phase is characterized by inflammation and vascular invasion from the host bed, which facilitates recruitment of osteoblasts and mesenchymal stem cells that will differentiate into the bone-forming cells (**492**). In the case of bone autografts, both graft and host bones contribute these osteogenic cells (**493**). In contrast, since bone allograft does not contain any live cells, healing relies upon invasion of the graft by host cells and tissues. While the later phases of graft healing are characterized by remodeling, allografts remodel very slowly, and in the case of large structural allografts, remodeling along the allograft is very limited (**494;495**). The limited bone forming and remodeling of structural allografts is associated directly with the 25 to 35% failure rate due to nonunion and fracture (**494;495**). Thus, a major challenge to the field of bone grafting is to elucidate the central factors that govern autograft healing and devise a method to transfer them to processed allograft such that it will have similar healing properties.

There are two conceivable approaches by which osteoinductive and remodeling properties can be conferred onto processed allograft. The first is to engraft bone forming cells to promote bone formation from the graft. While several groups have demonstrated the efficacy of this approach (**496**), many issues remain regarding its clinical potential, including the source of the cells, reproducible engraftment of cells onto the graft, and added cost and complexity. The other approach is to introduce the critical factor(s) onto the allograft directly. In the case of cancellous grafts and bone graft substitutes, BMP has been approved by the FDA for this approach (**497;498**). Unfortunately, high required dose and short protein half-life limit this strategy for large structural grafts. Gene therapy may offer a cost-effective solution to these problems.

Transient transduction of bone marrow stromal cells with adenoviral constructs containing BMP has demonstrated efficacy for the enhancement of bone regeneration in a number of animal models (**7;499**). More recently, recombinant adeno-associated viruses (rAAV) expressing BMP have been utilized in combination with cultured MSC for *ex vivo* and *in vivo* models of bone healing (**500-502**). However, an effective *in vivo* gene therapy approach to heal a large bone defect without the addition of exogenous cells has yet to be demonstrated.

Since BMP gene therapy requires a high level of gene expression for efficacy, we have focused on the caALK2) (**VIII**). The specificity of caALK2 to BMP signaling was illustrated by Chen *et al*. (**503**). Recently, it has been shown that caAlk2 can potently induce mesenchymal cell differentiation *in vitro* and *in vivo* and that injection of a caAlk2-expressing retrovirus into chick limbs dramatically induced chondrogenesis and endochondral bone formation (**504**).

Based on these findings, a functional rAAV-caAlk2 vector has been developed (**VIII**) and a method to immobilize rAAV onto the cortical surface of allografts via freeze-drying has been established (**VIII;97**). Combining these features, the osteogenic and remodeling properties of rAAV-caAlk2-coated allografts could be investigated in a murine femur model (**VIII**).

In vivo models

In our studies, we have used a murine segmental femoral allograft model as described by Tiyapatanaputi *et al*. (**505**). This murine segmental bone-grafting model mimics the healing of cortical bone grafts in humans (**505**). Hence, autografts can be rapidly incorporated and underwent extensive remodelling within a short period of time (2–3 weeks) (**505**). In contrast, incorporation of frozen allografts depended upon host repair activity and occurred only at the allograft–host junction. The rest of the allograft remained inert with minimal evidence of periosteal bone formation or remodelling. These findings are in accord with extensive observations in other experimental animal models as well as in humans (**491;506-508**).

The major advantage of this murine graft model over the established ones is the potential to use transgenic or knockout mice to characterize the molecular mechanisms of graft repair and remodelling.

Mechanical stability has been demonstrated as one of the most important factors for cortical–cortical bone junction healing (**491;509**) as it is believed to facilitate ingrowth of vascular tissues and host bone (**510**). However, a recent study showed that the traditional concept of fixation for allografts to achieve maximum rigidity of the construct may not necessarily promote the most appropriate host healing response in all situations. The authors found that a less rigid fixation method produced more callus resulting in superior torsional and bending properties (**511**). In the present murine model of femoral graft healing, a fairly rigid fixation to prevent graft migration and fracture has been aimed to be achieved with an intramedullary pin. This method has been adopted from the widely used murine model of fracture healing (**512**).

Gene therapy results

The lack of an effective treatment to repair large structural defects remains a major orthopedic problem. While the commercial development of BMP as an adjuvant for spinal fusion and fracture healing has formally demonstrated its clinical utility, the high doses (milligrams) that must be used to observe efficacy and the short half-life (hours) limit its utility for large structural grafts (**497;498**). Although gene therapy offers a potential solution to these obstacles, a safe, effective, practical method to deliver the therapeutic gene and allograft during surgery remains elusive.

The first attempt to combine an osteoconductive bone substitute with *in vivo* gene therapy was performed by Bonadio *et al.,* who developed the gene-activated matrix (GAM) (**513**). In this approach the investigators evaluated the potency of plasmid gene delivery from genes physically entrapped in a polymer matrix using bone regeneration in a canine critical defect as the endpoint. While this study demonstrated target gene expression for 6 weeks, and the induction of centimeters of normal new bone in a stable, reproducible, dose- and time-dependent manner, GAM *in vivo* transduction efficiency has never been reported. Unfortunately, others and we have been unable to achieve effective transduction efficiencies in our models using GAMs and have turned to viral-mediated gene transfer approaches. Based on the empirical advantages of rAAV vectors for orthopedic gene therapy (**37**), and the clinical potential of this vector (**96**), we evaluated the effects of freeze-drying and storage at −80°C on rAAV transduction efficiency (**97**). These studies revealed that rAAV vectors are remarkably durable, as we routinely recover ~100% of the transducing units after freeze-drying and storage. From a practical standpoint, this rAAV-coating process can be easily adaptable to standard operating procedures used by tissue banks to prepare clinical allografts.

To evaluate the efficacy of transferring BMP signals to the cortical surface of processed allografts we coated femoral allografts with rAAV-LacZ (control) or rAAV-caAlk2 (experimental) and evaluated healing responses in our mouse allograft model at 2, 4, and 6-weeks (**VIII**). Our choice to use a constitutively active BMP receptor as the target gene, instead of the cytokine, was based on the low level of *in vivo* expression required to induce significant endochondral bone formation (**504**) and the fact that caAlk2 signals cannot be blocked by the endogenous BMP antagonists noggin and chordin.

Histology demonstrated several remarkable features of rAAV-caAlk2-coated allograft healing including: 1) the absence of a foreign body reaction that normally encases the allograft in inflammatory tissue, 2) endochondral bone formation directly on the allograft surface, 3) vascularization of the cartilage over the graft, 4) a new bone collar that extends the entire length of the allograft, 5) live bone marrow within the allograft, and 6) osteoclastic resorption of the allograft (**VIII**).

Using reconstructed micro-CT images, difference in new bone generation between rAAV-LacZ- and rAAV-caAlk2-coated allografts was demonstrated (0.67 ± 0.21 vs 2.49 ± 0.40 mm^3; $P < 0.005$) (Figure 15). Visualization of 3D images showed that the new bone formation around rAAV-caAlk2-coated allografts is nonuniform and has a highly variable form, making it difficult to measure accurately by conventional 2D histomorphometry (**VIII**). The lack of new bone formation around the rAAV-LacZ-coated allografts was the same as that seen in the uncoated allografts, indicating the innocuous effects of the vector (**VIII**).

In conclusion, we have found that the efficacy of the AAV-caAlk2 coating may be derived from four effects that are never observed in uncoated or AAV-LacZ-coated allografts: osteogenesis, inhibition of the foreign-body reaction, angiogenesis, and osteo-

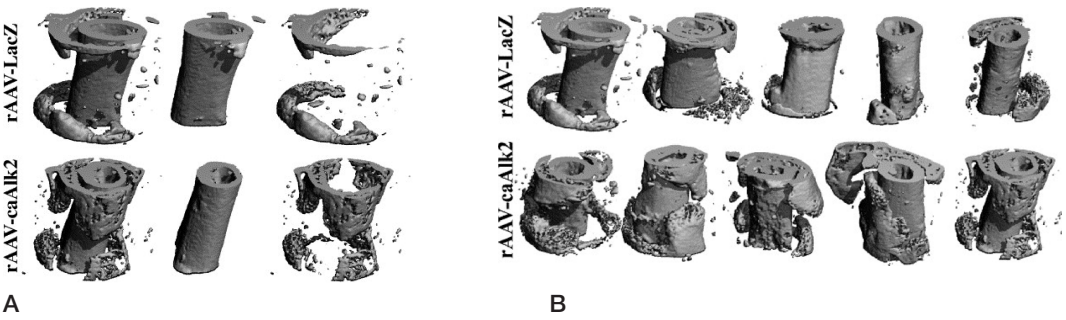

Figure 15. Volumetric quantitation of new bone formation by micro-CT (A). Femurs were scanned and imaged by micro-CT (VIII). To quantify new bone formation surrounding the allografts a region of interest was defined extending from the proximal to the distal end of the defect region. For each image, total bone volume was calculated, including both the implanted allograft and the surrounding new bone formation (left). Following manual segmentation, a second evaluation was performed to calculate bone volume of the allograft alone (center). The difference between the two volumes defined the volume of new bone formation surrounding the allograft (right).

rAAV-caAlk2-coated allografts produce a new bone collar *in vivo* (B). Reconstructed images of the rAAV-coated allografts demonstrate the lack of new bone around the rAAV-LacZ-coated allografts and reflects the variability in size and distribution of the new bone that forms around the rAAV-caAlk2-coated allografts (VIII).

Volume of new bone: rAAV-LacZ 0.67 ± 0.21 mm^3 and rAAV-caAlk2 2.49 ± 0.40 mm^3. rAAV-caAlk2-coated allografts significantly different from rAAV-LacZ-coated allografts ($p<0.05$).

clastic resorption of the allograft (**VIII**). While the induction of orthotopic bone formation on the cortical surface can be readily explained by the caAlk2 transduction of mesenchymal stem cells, the molecular mechanism by which this gene therapy prevents the formation of fibrotic tissue around the allograft, promotes blood vessel ingrowth, and stimulates osteoclastogenesis remains to be formally proven.

Future directions

Although our results demonstrate the potential of AAV vector coating as a method to revitalize structural allografts (**VIII**), there are some additional advances that are needed to further this technology for human use. The first is to improve the connectivity of the new bone that forms around the host bone and the allograft, as a junction-to-junction union of new cortical bone is the primary goal. This could be done by using corrugated allografts or a bone graft substitute that allows a uniform distribution of the AAV before freeze-drying. Another necessary advance is the establishment of technology and protocols for *in vivo* 3D imaging of new bone formation and vascular ingrowth of allografts with metal screws and plates for large-animal preclinical and clinical trials. Recently there has been new technology developed in this area that may serve this purpose (**514;515**). Finally, since the primary function of structural bone is to support *in vivo* loads, the biomechanical properties of AAV-coated allografts must be determined and correlated with volumetric and morphometric parameters determined by micro-CT in auto- and allografts after various healing periods. Success in these areas will be necessary to demonstrate the efficacy of AAV-caAlk2 coating therapy, which may result in the first remodeling allograft for large bone defects.

Perspectives

Genes

The orthopaedic genome is a constellation of thousands of genes directly involved in the genesis and maintenance of the musculoskeletal system via intracellular and extra-cellular signalling pathways involved in the autocrine, paracrine and endocrine control of skeletal metabolism. Notable discoveries in the study of the orthopaedic genome during the last decade have included the identification of parathyroid hormone-related protein (PTHrP) (**516**), BMPs (**517**), the hedgehog family of molecules (**518**) and the RANK/RANKL/OPG receptor interactions (**370;377;519-521**). A deeper insight and understanding of the regulation and control of intracellular signalling pathways and transcription factors is predicted to have great impact on future gene therapy and tissue engineering, because it will aid in the development of powerful and specific pharmacological agents and will increase our capacity to treat an even greater spectrum of human diseases. Drug design will be enhanced by the identification of specific gene targets, and pharmacological agents will be synthesized to target the activity of a specific gene or protein. Gene therapy will become a reality in the future, and while it will undoubtedly be used for the treatment of diseases arising from genetic mutation, it will probably also be used for the treatment of developmental, degenerative and traumatic conditions as well as cancer.

Gene transfer systems

Gene transfer vectors based on AAV-2 have been developed and tested in pre-clinical studies for almost 20 years, and they are currently being evaluated in clinical trials. These studies have so far provided evidence that AAV-2 vectors possess many properties that make them very attractive for therapeutic gene delivery to humans, for instance lack of pathogenicity or toxicity and the ability to confer long-term gene expression. There is some concern, though, that the clinical use of AAV-2 vectors in humans may be limited; first, because they are rather inefficient at transducing some cells of therapeutic interest, e.g. liver cells; second, because neutralizing anti-AAV-2 antibodies, which are highly prevalent in the human population, may hamper gene transfer. Strategies attempting to overcome these limitations include the cross-packaging of an AAV-2 vector genome into the capsids of the other AAV serotypes, resulting in a new generation of "pseudotyped" AAV vectors. *In vitro* and *in vivo*, these novel vectors have been shown to have a host range different from AAV-2, and to escape the anti-AAV-2 immune response, thus underscoring the great potential of this approach (**522;523**).

Rapid progress during the past few years clearly demonstrates the great potential of electroporation-based gene therapy technology. This technique allows genes to be transferred to chondrocytes in articular cartilage, myocytes in muscles, dendritic cell in CNS and bone marrow derived mesenchymal stem cells. However, both *in vitro* and *in vivo* applications are facing challenges, which include the transfection efficiency, the duration of gene expression, the survival rate for *in vitro* cell transfection and the biocompatibility *in vivo* (**33**).

Gene therapy is conventionally carried out by transferring genetic material to the target cell where the exogenous gene is expressed using the endogenous transcription and translation machinery in parallel with the target cell genome. A new paradigm of gene therapy modifies the genetic repertoire at the pre-mRNA level (trans-splicing) with a view to treating genetic and acquired disorders (**524**).

Achieving efficient regulation and tissue-specific gene expression is one of the major goals for gene therapists. A number of inducible systems and tissue-specific promoters are currently available. The regulators, controlling the timing and levels of gene expression, must be non-toxic and non-immunogenic to the recipients. Such an ideal system has still to be developed.

Bioengineering

Tissue engineering was introduced by Langer and Vacanti in 1993 as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function"(**525**). Three key elements are involved: 1) cells, 2) tissue inducing substances, and 3) cell-containing scaffolds.

Indeed, tissue-engineering strategies offer several potential advantages in regeneration of damaged or diseased tissues (**526;527**). First, lost tissue substance, even when extensive, can be replaced. Second, preservation of the cellular phenotype, or differentiation of bone marrow derived mesenchymal stem cells into mature cells of the desired linkage, depend on the cellular environment. Three-dimensional cellular scaffolds promote diffenrentiation of the cellular phenotype. Third, tissue engineered constructs may involve immediate therapeutic benefit as they do not necessarily depend on the relatively slow recreation of the tissue substance. A major limitation of conventional tissue engineering approaches in repairing tissue like articular cartilage lies in the limited intrinsic mitotic and matrix synthetic activity of the cellular component of the engineered constructs (**528**). By combining the respective benefits of gene transfer and tissue engineering, we may reasonably speculate that a more efficacious treatment may be achieved than with either method alone (**526**). In particular, gene transfer to mesenchymal stem cells may be attractive with a view to achieving the required cellular differentiation and function.

Safety

Great hope was founded on rapid breakthrough, but progress has been slower than anticipated. The first major gene therapy success was the retrovirus-based treatment of infants suffering from SCID-X1, which demonstrated the real potential of long-term or even permanent cure of hereditary disease (**529**). However, the field has suffered some setbacks, like the unfortunate, fatal case of adenovirus-based treatment of a non-life-threatening disease, OTCD (**530**), and the recent discovery that one of the SCID-X1-treated patients developed a leukaemia-like condition (**531**) that might have been caused by vector-induced incisional mutagenesis. Still, this led to improved control of study design and monitoring, and has directly influenced vector development and the engineering of alternative delivery vehicles (**121**). Five factors directly pertaining in particular to the safety of gene therapy vectors have so far hampered progress towards the ideal delivery system: 1) Inefficient delivery: although many non-viral and viral vectors demonstrated high gene delivery efficacy in cell lines, their potency *in vivo* has been disappointingly modest. Hence, high levels of vectors must be applied in order to achieve biological effect. 2) Targeting of transgene expression: specific targeting to the cells or tissue of interest is extremely important to avoid expression of therapeutic and, specifically in cancer therapy applications, toxic gene products in healthy tissue. 3) Duration of expression: poor replication and stability of episomal vectors and inefficient or inappropriate vector integration into the host genome have hampered the establishment of long-term expression. 4) Rescue of viral vectors and random integration into recipient genome: vectors that are replication-deficient and for which the potential probability of homologous recombination is reduced to theoretically acceptable levels need to be engineered. Moreover, positioned genome integration is not sufficiently accurate and cytotoxicity and immunogenicity still pose problems. 5) Toxicity: the toxicity associated with the use of viral vectors is extremely complex, involving both the innate and adaptive immune responses. It seems to be dose-dependent, occurs in phases, is related to route of administration, is dependent on tissue and cell type targeted and varies with species.

Overall, the past few years have seen major improvements in all these aspects of gene delivery vector development and gene expression targeting. However, it remains obvious that there are no universally applicable ideal viral vector systems available.

As a reflection of the importance of adenovirus vector-induced toxicity, significant changes have been proposed to modify the guidelines of gene therapy trials by the US National Institutes of Health Recombinant DNA Advisory Committee (**530**). The new guidelines aim to unify 1) standards

of quantification, 2) end points, 3) patient evaluation and monitoring, 4) administration routes and biodistribution, 5) vector quality control, 6) control studies, 7) preclinical data evaluation, and 8) the use of autopsy data to ensure comparable safe clinical trials using this new promising technology.

Bioethical concepts of gene therapy

The care and safety of the patient are of particularly relevance to the medical doctor. Recent advances in genetic research will hopefully allow the use of gene transfer for treating diseases associated with genetic mutations (e.g. osteogenesis imperfecta) and a variety of other musculoskeletal problems, including rheumatoid arthritis, bone loss associated with fracture, non-union, or revision total joint arthroplasty and cartilage injury (**37;399;532-535**). Although investigators in this field are enthusiastic about the potential of using gene therapy for treating difficult clinical problems, the tragic episode (**115**) in an experimental human gene-transfer trial at the University of Pennsylvania demonstrates the inherent risk associated with the development of new treatment regimens.

The last 5 years have witnessed high-profile reports of adverse events in trials with recombinant adenovirus and retrovirus vectors, and many have jumped to the conclusion that these immune response findings have dire consequences for yet another vector system. The association of some of these cases with a general lack of protection of human subjects and the question of a financial conflict of interest highlights the potential ethical problems associated with the use of new technology (**536**).

Somehow lost in these perspectives is the fact that the past years have seen numerous positive findings in gene therapy as well. Retroviral gene therapies have produced long-term benefits for approximately 15 patients with different types of SCID, disorders that are uniformly fatal without bone marrow transplantation, and the two patients with leukaemia-like syndromes as a complication of gene therapy are in complete remission and still demonstrating therapeutic benefit (**537**). Furthermore, an AAV-2-CFTR vector has been safely administered to more than 175 patients with cystic fibrosis without any serious vector-related adverse effects (**91;93**). Other ongoing trials in patients with Parkinson's disease, Canavan's disease and α1-antitrypsin deficiency appear to be moving forward without incidents. Notably, all these disorders are eventually fatal and lack any definitive therapy. In this context, investigators should be wary of patients' unrealistic expectations about the efficacy of new therapeutic regimens, particularly when treating patients with musculoskeletal conditions, many of whom are young and otherwise healthy. In most cases, the proposed therapy will not be life-saving and the goal will be to improve quality of life. In this situation safety clearly overrules efficacy.

Abstract

Background: Gene therapy is a technique that draws on the introduction of new genes into cells for the purpose of treating disease by restoring or adding gene expression. Numerous growth factors and other proteins with the ability to promote the regeneration of tissues in the locomotive system have been identified, but their clinical use is often hindered by delivery problems. In principle, these problems can be overcome by delivering the relevant genes, as the therapeutic substances thereby can be persistently produced directly by local cells at the site of diseases.

Healing of articular cartilage: Articular chondrocytes are receptive to transduction using various gene delivery methods. Following genetic modification, they are capable of sustained expression of transgene products at biologically relevant levels. Our research has proved the AAV vector to be an effective tool for gene delivery to articular chondrocytes *in vitro* as well as *in vivo*. To this end, we have demonstrated that the AAV vector mediated $TGF_{\beta 1}$-overexpression stimulates cartilage anabolism.

Wear debris-induced osteolysis: The RANKL system may be a key therapeutic target in treatment of aseptic periprosthetic loosening. We investigated whether gene transfer of OPG using an AAV vector has protective effects against orthopaedic wear debris-induced bone loss. In osteoclastogenesis and in bone wafer resorption assays, the bioactivity of the transgene OPG was proven by depletion of osteoclastogenesis and reduced bone resorption. Using an *in vivo* model of debris-induced bone resorption, we demonstrated complete inhibition of osteolysis in animals receiving AAV-OPG gene therapy.

Fracture healing in relation to osteoporosis: The success of future OPG treatment of osteoporosis is highly dependent on its effects on fracture healing and remodelling. Using an *in vivo* fracture healing model, our studies demonstrated that AAV-OPG gene therapy did not conflict with normal bone healing, in contrast to high-dosage intravenous treatment with OPG. However, AAV-OPG therapy depressed remodelling and integration of the genuine cortical bone at the fracture line.

Structural bone allograft healing: Structural bone allografts often fracture due to their lack of osteogenic and remodelling potiential. To overcome these limitations, we utilized allografts coated with AAV-caALK2 vector that mediated *in vivo* gene transfer. We showed that the AAV vector was capable of transducing adjacent inflammatory cells and osteoblasts in the fracture callus and that BMP signals delivered via AAV-caALK2 coating induced bone formation directly on the cortical surface of the allograft.

Conclusion: The presented research may be seen as initial steps towards development of gene therapeutic treatment options for complex orthopaedic diseases. As such, our studies represent proof-of-principle that the rAAV vector promotes efficient gene transfer *in vitro* to a spectrum of cells with orthopaedic relevance, and that *in vivo* targeting of somatic tissue with a single administration of a rAAV vector at the time of surgery could be sufficient for long-term expression of therapeutic proteins.

Essential to the future success of transgene delivery by rAAV vectors is the absence of an immune response to either the vector or the gene product. Furthermore, development of rAAV vectors with regulatory gene expression needs further attention in future research.

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