CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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Gene Therapy: Design and Prospects for Craniofacial Regeneration

ABSTRACT

Gene therapy is defined as the treatment of disease by transfer of genetic material into cells. This review will explore methods available for gene transfer as well as current and potential applications for craniofacial regeneration, with emphasis on future development and design. Though non-viral gene delivery methods are limited by low gene transfer efficiency, they benefit from relative safety, low immunogenicity, ease of manufacture, and lack of DNA insert size limitation. In contrast, viral vectors are nature's gene delivery machines that can be optimized to allow for tissue-specific targeting, site-specific chromosomal integration, and efficient long-term infection of dividing and non-dividing cells. In contrast to traditional replacement gene therapy, craniofacial regeneration seeks to use genetic vectors as supplemental building blocks for tissue growth and repair. Synergistic combination of viral gene therapy with craniofacial tissue engineering will significantly enhance our ability to repair and replace tissues *in vivo*.

INTRODUCTION

Human gene therapy is defined as the treatment of disorder or disease through transfer of engineered genetic material into human cells, often by viral transduction. Since the introduction of science fiction, the popular press has toyed with the notion of viral gene delivery and its terrifying implications. One of the more recent popular works on the topic is the 2007 remake of Richard Matheson's classic 1954 novel **I Am Legend,** which details events following the discovery, release, and mutation of a genetically re-engineered measles virus that was initially hailed as the cure for cancer (Matheson, 1954; Lawrence, 2007). This adapted novel, which has been redone in three instances as a feature film, outlines the seemingly inevitable worldwide destruction that could result from viral gene therapy. With an emotionally stirring history of fictional violence and a debate that provokes both moral and medical issues, it may be surprising that, since 1990, billions of dollars have been spent on hundreds of human viral gene therapy clinical trials. Our society is in the midst of a paradigm shift that began with the discovery of viruses as dangerous infectious agents and will end with the use of viruses to cure disease and regenerate tissues.

On January 19, 1989, the director of the National Institutes of Health (NIH), Dr. James A. Wyngaarden, approved the first clinical protocol to insert a foreign gene into the immune cells of persons with cancer (Roberts, 1989). On September 14, 1990, W. French Anderson and his colleagues at the NIH performed the first approved gene therapy procedure on a four-year-old girl born with severe combined immunodeficiency (SCID) (Anderson, 1990). Despite the viral horror stories written by the popular media, this initial trial was largely a success, and the most recent report on this individual in 2004 noted that she is thriving as an 18-year-old teenager in suburban Cleveland (Springen, 2004). Over the next ten years, 300 clinical gene therapy trials were performed on about 3000 individuals (McKie, 2000). The field was then blackened with the death of an 18-year-old male four days after the introduction of 38 trillion particles of recombinant adenovirus into his liver (Somia and Verma, 2000). Despite this tragedy, we continue to move forward because of the great promise of novel genetic treatments that, when perfected, will likely outshine current methods, such as protein therapy or pharmacotherapeutics, for treatment of many diseases and defects.

We are now nearing the 20-year mark since the first gene therapy trial. Though success has been limited, the future still seems overwhelmingly promising, and we are steadily approaching an acceptable safety record. This review will explore non-viral and viral methods available for transgene introduction as well as their current and potential applications for craniofacial

regeneration and therapy, with emphasis on future development and design.

NON-VIRAL GENE DELIVERY

Though this review will focus mostly on viral methods of gene delivery, it is essential to recognize that many advances have been made in the field of non-viral gene therapy. Polymeric gene delivery is desired because of its relative safety, low immunogenicity and toxicity, ease of administration and manufacture, and lack of DNA insert size limitation (Park *et al*., 2006). The main disadvantage is insufficient gene transfer efficiency due to the need for post-uptake endosomal escape and nuclear translocation of the DNA complex (Park *et al*., 2006). In this respect, clinical efficiency and specificity standards have not yet been met.

Synthetic Polymers

The main strategy for most synthetic polymer delivery systems is to generate cationic polymers to interact electrostatically with and neutralize negatively charged DNA (Park *et al*., 2006). This facilitates properties such as protection from DNAses. If a net positive charge is maintained, the polymer/DNA complex can adhere to the cell surface glycocalyx and be internalized by endocytic mechanisms. Unfortunately, the use of endocytic uptake from the external environment perpetuates the need for endocytic escape into the cytosol. This challenge in the polymeric gene delivery field has been addressed by multiple strategies, including incorporation of fusogenic peptides for endosomal membrane binding and disruption (Cho *et al.*, 2003) and by balancing a hydrophobic cholesterol group with hydrophilic polymers to enhance escape (Mahato *et al.*, 2001).

One of the first polymers recognized for its ability to form nanoparticulate polyelectrolyte complexes with DNA was Poly L-lysine (PLL) (Laemmli, 1975). Unfortunately, this cationic material was found to have high cytotoxicity (Choi *et al*., 1998) and a tendency to aggregate and precipitate (Liu *et al*., 2001). The solution to this dilemma was found in the form of the flexible, water-soluble polymer polyethylene glycol (PEG). Covalent coupling of PEG, or 'PEGylation', of a target molecule such as PLL limits its cytotoxicity and non-specific protein adsorption (Choi *et al*., 1998). This strategy has also been used with polyethyleneimine (PEI), a cationic gene carrier with superior transfection efficiency and unique buffering properties (Boussif *et al.*, 1995), similarly to reduce the extent of inter-particular aggregation (Mishra *et al*., 2004; Quick and Anseth, 2004).

In addition to improving the bio-properties of PLL and PEI, PEGylated polymers can be conjugated to specific targeting moieties, such as sugars, antibodies, peptides, and folate (Lee and Kim, 2005). For example, peptide conjugation of the apoB-100 fragment of low-density lipoprotein can increase transfection efficiency in bovine aorta and smooth-muscle cells 150- to 180-fold (Nah *et al.*, 2002), and RGD peptides can allow for increased selection of endothelial cells (Kim *et al.*, 2005). To summarize, synthetic PEGylated polymers such as PLL and PEI are promising gene delivery molecules. Future study in this field is focused on biodegradable polycations such as poly(β-amino ester), poly(2-aminoethyl propylene phosphate), and degradable

PEI to decrease cytotoxicity and increase transfection efficiency (Akinc *et al.*, 2003).

Natural Polymers

The natural polymer family contains materials such as cyclodextrin, chitosan, collagen, gelatin, and alginate. When compared with synthetic materials, natural polymers have the advantage of innate environmental responsiveness and the ability to be degraded and remodeled by cell-secreted enzymes. They are non-toxic at both low and high concentrations, are readily incorporated into oral or bolus matrix delivery systems, and can serve as tissue engineering scaffolds (Dang and Leong, 2006). The simplicity of oral delivery and mucoadhesive properties of materials such as chitosan make it an interesting potential polymer for gene delivery and vaccination (Roy *et al*., 1999). The transfection efficiency of natural polymers such as cyclodextrin, though significantly less than that of virus, is similar to that of PEI and lipofectamine (Gonzalez *et al*., 1999). Thus, while natural polymers benefit from degradation and remodeling, they still face significant transfection issues due to the requirement for endosomal escape. However, this strategy has been successfully used to increase bone regeneration with polymer 'gene activated matrix' containing DNA encoding parathyroid hormone (Bonadio *et al.*, 1999; Chen *et al.*, 2003)

VIRAL GENE DELIVERY

Viruses have undergone millions of years of evolution and are a species-conserved way of introducing DNA to cells (Dewannieux *et al.*, 2006). Scientists are now attempting to fine-tune these gene delivery vehicles for treatment of human disease and defects. Regardless of method selection, there are three universal requirements for viral gene therapy vectors. First, the delivery system must be safe and immunologically inert. Second, it must protect the genetic material from degradation. Third, the vector must encode an effective therapeutic gene that has sustained expression at a defined target site. For true commercial application, the packaged vector must also be easily produced and processed and have a reasonable shelf-life. As we near the 20-year mark from the first human gene therapy clinical trial, significant advances have been made in satisfying these three requirements. However, new objectives—such as tissue-specific targeting, site-specific chromosomal integration, and controlled infection of both dividing and non-dividing cells—have emerged. Though negative publicity has attached a significant stigma to viral gene therapy, it is indeed the most efficient method of gene transfer, and basic research and clinical trials are rapidly moving to overturn the safety concerns.

Our society is in the midst of a paradigm shift that began with the discovery of viruses as dangerous infectious agents and will end with the use of viruses to cure disease and regenerate tissues. However, safety concerns still limit the universal acceptance of this strategy. These concerns include the accidental generation of replication competent viruses during vector production and the packaging or mobilization of the engineered vector by endogenous retroviruses present in the human genome (Connolly, 2002). Either of these could lead to horizontal

dissemination of new viruses from gene therapy patients. Localized concerns include random insertion or mutagenesis of the vector leading to cancer, or germ cell alteration resulting in vertical inheritance of the acquired gene (Connolly, 2002). The need for controlled genome integration hit home when two of the 11 persons treated for X-SCID with retrovirally transduced stem cells developed leukemia due to insertion of the transgene near the oncogenic gene LMO2 (Kaiser, 2003). Site-specific chromosomal integration, conditional expression of the transgene only in target cells, and the use of self-inactivating (SIN) retroviral vectors have been proposed (Yu *et al*., 1986) and may significantly improve the safety of viral therapy. The following sections will review the use of viral vectors for *in vivo* therapy, emphasizing the construction and advantages of different viruses.

Figure 1. Retroviral vector development for increased efficiency and targeting. **(A)** Structure of a simple retroviral genome containing coding sequences for *gag*, *pro*, *pol*, and *env* for replication. **(B)** Structure of a simple γ-retroviral gene therapy vector with replication coding sequences removed and transgene inserted. Note retention of the dual long-terminal repeats (LTRs), primer binding site (PBS), signal Ψ, attachment sites (att), and polypurine tract (PPT). The U3 component of the 5′LTR is used as a promoter to drive transgene expression. A second heterologous or tissue-specific promoter (P) has been inserted to drive an ampicillin gene to facilitate *ex vivo* selection of transduced cells. **(C)** Structure of an enhanced self-inactivating retroviral gene therapy vector (note substitution of the 5′LTR U3 component). The internal promoter (P) is tissue-specific to limit transgene expression. Additional genetic elements—such as chromatin insulators (CI), chromatin structure regulators (CR), a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), scaffold or matrix attachment regions (S/MAR), or resistance genes—are incorporated to enhance site specificity and integration efficiency while limiting gene silencing.

Retrovirus

Before we can successfully manipulate retroviral vectors, the composition of their genome must be thoroughly understood. Since the discovery of retroviruses in 1910, when Peyton Rous induced malignancy in chickens by the injection of cell-free filtrates from muscle tumor (VanEpps, 2005), we have gained much insight into their mechanism of action. Three main classes of recombinant retroviruses are used as tools in gene delivery: γ-retroviruses, lentiviruses, and spumaviruses (Chang and Sadelain, 2007). Despite their negative press, exogenous retroviruses have been used in many biological studies and facilitated the discovery of proto-oncogenes (Martin, 2004), the manipulation and investigation of intracellular pathways, and successful *ex vivo* treatment of persons with hemophilia and SCID (Sumimoto and Kawakami, 2007; Chu *et al*., 2008; Scheller *et al*., 2008). Retroviruses are 80- to 100-nm enveloped viruses that contain linear, non-segmented, single-stranded RNA. Retroviruses are naturally self-replicating for viral assembly and re-infection (Kurian *et al*., 2000). Reverse transcription allows for the generation of double-stranded DNA from the transduced 7- to 12-kBp RNA and subsequent insertion into the genome. Exogenous retroviruses can be subdivided into simp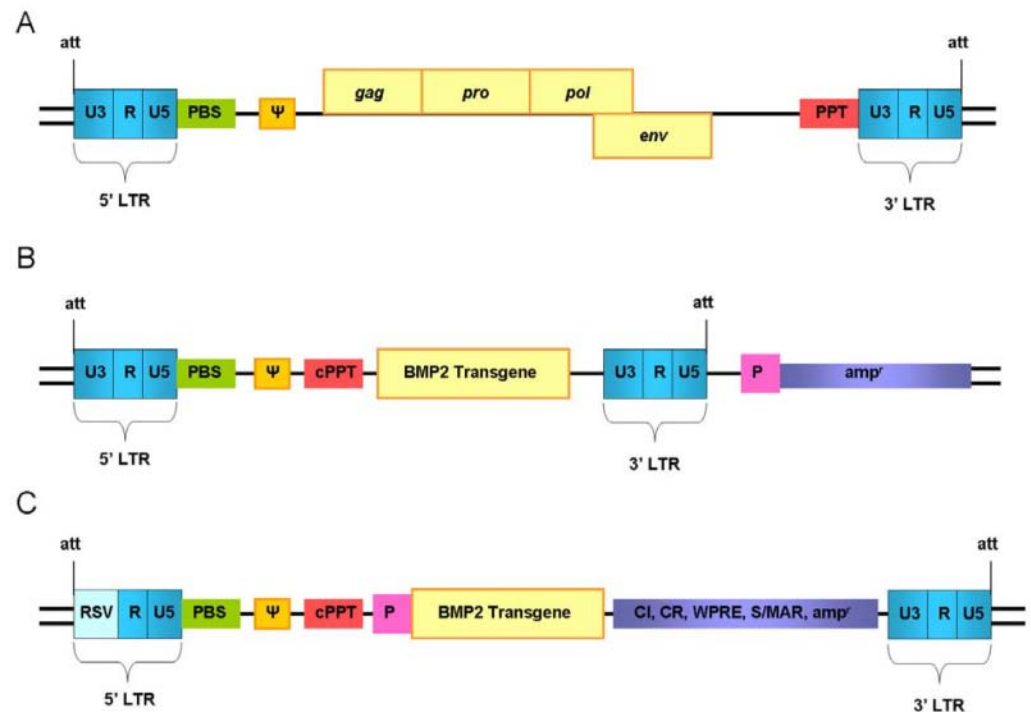le and complex categories based on the composition of their RNA vector. Simple vectors contain three basic genes—*gag*, *pol*, and *env*—which are necessary for viral replication and must be removed prior to gene therapy (Buchschacher, 2001) (Fig. 1A). Identical long terminal repeats (LTRs) are present at each end of the retroviral genome. The LTRs contain promoter, enhancer, and integration sequences which facilitate interaction with attachment sites *via* integrase (Engelman, 1999). Complex retroviruses contain up to 15 additional accessory genes, such as *tat*, the transcriptional transactivator for HIV-1, *vif, rev, nef,* etc. (Frankel and Young, 1998).

The use of retrovirus as a gene delivery system necessitates re-engineering of the viral genome to block autonomous replication while maintaining integration efficiency. This is accomplished *via* the maintenance of cis-acting and removal of trans-acting factors. Five essential components for successful viral gene expression by any LTR-driven γ-retroviral vector include dual-LTRs, att site, primer binding site, signal psi, and the polypurine tract (Zhang and Godbey, 2006) (Fig. 1B). The polypurine tract aids in transport of the pre-integration complex to the nucleus and allows for internal initiation of second-strand DNA synthesis (Zennou *et al*., 2000). Most γ-retroviral

vectors rely on their LTRs to drive robust and ubiquitous trans-gene expression. Replacement of these with site-specific constitutive promoters is currently limited by expression strength and promoter silencing, but remains an active area of research that could allow for customization of transgene expression level and location. Additional vector design strategies to enhance gene expression and reduce silencing include: deletion of silencing elements (Zufferey *et al.*, 1998), incorporation of robust promoters such as U3 or PGK (Chang and Sadelain, 2007), incorporation of woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to enhance mRNA transcript stability (Loeb *et al.*, 2002), incorporation of scaffold or matrix attachment regions for anchorage of chromatin with stabilization of chromosomal loops (Agarwal *et al.*, 1998), and use of insulators such as the chicken β-globin locus control region to limit position effect variegation (West *et al.*, 2002) (Fig. 1C). Expression in bacterial plasmid form can be used for easy amplification and incorporation of drug resistance or response genes to facilitate selection *ex vivo* or expression *in vivo* (Delviks and Pathak, 1999; Jaalouk *et al.*, 2000). Addition of the gene of interest or resistance gene under the control of a tissue-specific promoter results in a dual-promoter vector designed to enhance selection and integration. However, simultaneous use of two promoters can result in significantly reduced expression of both due to promoter interference (Apperley *et al.*, 1991). SIN vectors which develop a defective promoter in the viral LTR have been used to circumvent this effect (Buchschacher, 2001) (Fig. 1C). Understanding and engineering of these vectors is rapidly advancing. For example, it has been shown that *ex vivo* transduction of hematopoietic stem cells can be improved through control of cell-cycle stage during virus delivery (Korin and Zack, 1998) and the use of proteosome inhibitors (Goff, 2004).

Retroviruses require genome integration of their vector to function, and most, excluding lentiviruses such as HIV-1, are able to infect only dividing cells (Lewis and Emerman, 1994). Stable integration of retroviral vectors is known to occur near expressed genes and appears to be non-random (Mitchell *et al.*, 2004). This allows for long-term expression of the transgene and makes retroviruses the vector of choice for *ex vivo* and *in vivo* transduction of highly replicative populations such as tumor cells and hematopoietic cells for treatment of chronic disease and genetic deficiency (Somia and Verma, 2000). Indeed, LTR-driven γ-retroviral vectors have been used in over 45 *ex vivo* clinical trials to treat diseases such as hemophilia, SCID, and leukemia (Kohn *et al.*, 2003; NIH, 2008b). To advance gene therapy with retroviral vectors, increased transduction efficiency and gene expression, site-specific chromosomal integration, and cell-specific targeting are necessary. *Ex vivo* or *in vivo* γ-retroviral transduction of rapidly dividing cells at sites of wound healing and new bone synthesis may be used in the future to enhance craniofacial tissue regeneration. Expansion of this field to include lentiviral vectors targeting non-dividing cells could allow for the long-term restoration of non-functional salivary gland tissue or repair of quiescent periodontal defects.

Adenovirus

Adenoviridae were initially isolated by Wallace Rowe in 1953 from adenoid explants as the "virus of the common cold" (Rowe *et al.*, 1957; Ginsberg, 1999). They have since become attractive tools for gene therapy, given that their infection is generally self-limiting and non-fatal (Zhang and Godbey, 2006). Adenoviruses are the largest non-enveloped virus and contain linear, double-stranded DNA. Over 50 serotypes have been identified, but the most common in nature and in adenoviral gene therapy are group C human serotypes 2 and 5 (Barnett *et al.*, 2002). The icosahedral adenoviral capsid is made up of hexon and penton proteins, knobbed fibers, and stabilizing minor cement proteins. These surround the core proteins and large 36-kBp adenoviral genome (Verma and Somia, 1997). The ends of the genome have inverted terminal repeats which flank a coding region capable of encoding more than 30 viral genes (Zhang and Godbey, 2006). These genes are termed 'early' or 'late', depending on their temporal expression. Early genes function as regulatory proteins for viral replication, while late genes encode structural proteins for new virus assembly (Zhang and Godbey, 2006). Entry of adenovirus into the cell occurs when penton base proteins bind integrins for clathrin-mediated endocytosis. Subsequent disruption of the endosome and capsid allows for viral core entry into the nucleus (Russell, 2000). Initiation of the 'immediate early' infection phase activates transcription of the E1A gene, a trans-acting transcriptional regulatory factor that is required for early gene activation (E1B, E2A, E2B, E3, E4, viron proteins) (Russell, 2000). The final 'late' infection phase activates genes L1 to L5 through complex splicing; viral particles which accumulate in the nucleus are then released *via* cell lysis (Zhang and Godbey, 2006).

During the engineering of adenoviral vectors for gene delivery, up to 30 kbp of the 36-kbp genome can be replaced with foreign DNA (Smith, 1995). Multiple strategies have been used to produce replication-defective, transforming adenoviral vectors. In first-generation adenoviral vectors, E1 and E3 genes are deleted to allow for a 6.5-kbp insertion. However, cell-line endogenous expression of E1 can lead to E2 expression and viral replication at low levels (Russell, 2000). Other first-generation vectors have used deletion of E2 and E4 regions to allow for an insert of greater than 6.5 kbp (Lusky *et al.*, 1998). These vectors are impaired by limited expression and a robust inflammatory response (Khan *et al.*, 2003). Second-generation 'gutless' vectors appear to be the most promising. Gutless vectors retain only the inverted terminal repeats and packaging sequence around the transgene (Russell, 2000). This results in prolonged transgene expression, increased insert size allowance, and reduced immune response (Fleury *et al.*, 2004). Studies have shown that adenoviral gene expression occurs *via* episome formation, and only 1 in 1000 infectious units can integrate into the genome (Tenenbaum *et al.*, 2003). Though this decreases the risk of insertional mutagenesis, it also limits adenoviral application to high-level transient transgene expression, because the gene is often lost 5 to 20 days post-transduction (Dai *et al.*, 1995). Adenoviruses have the significant advantage of being able to infect both dividing and non-dividing cells (Verma and Somia, 1997). This makes

them specially suited for applications involving brain, eye, lung, pancreas, hepatocytes, neurons, and monocytes (Blomer *et al.*, 1997; Kafri *et al.*, 1997). PEGylation and expression of targeting ligands on the viral capsid are being investigated to decrease the immune response and enhance targeting of adenoviral vectors (Eto *et al.*, 2008). A clinical trial with adenovirus to repair salivary gland tissue post-radiation therapy is ongoing (Baum *et al.*, 2006; NIH, 2008b).

Adeno-associated Virus

Adeno-associated virus (AAV) is a parvovirus of the Dependovirus genus that was discovered in 1965 as a co-infecting agent of adenovirus preparations (Carter, 2005). The first infectious clone of AAV serotype 2 to be used for human gene therapy was generated in 1982 (Samulski *et al.*, 1982). Since that time, AAV serotypes 1-12 and over 100 AAV variants have been isolated (Wu *et al.*, 2006). AAV is a non-enveloped DNA virus with a 22-nm icosahedral capsid containing a 4.7-kBp linear single-stranded DNA genome. Coding capacity is limited to 4.5 kBp, but may be extended by splitting the sequence between 2 viruses that can later concatamerize after transduction (Nakai *et al.*, 2000). The genome contains 2 unique open reading frames (ORFs) which encode 4 replication proteins and 3 capsid proteins, respectively (Ding *et al.*, 2005). Inverted GC-rich self-complementary terminal repeats flank the ORFs and are the only cis-acting factors required for genome replication and packaging (Ding *et al.*, 2005). Two ORF-encoded trans-acting proteins required for viral replication are *rep,* which controls viral replication and integration, and *cap,* which encodes structural components of the capsid. Though site-specific integration on chromosome 19 occurs if *rep* is maintained (Kotin *et al.*, 1990), it is generally removed from rAAV-engineered vectors. Advantages of AAVs include low immunogenicity, lack of pathogenicity, a wide range of infectivity with potential cell-/tissue-specific targeting, and the ability to establish long-term latent transgene expression in both dividing and non-dividing cells.

AAVs are naturally replication-deficient and require a helper virus for replication and dissemination (Zhang and Godbey, 2006). Self-limiting infection, coupled with their ability to stably infect dividing and non-dividing cells, makes them an excellent target for *in vivo* gene therapy and craniofacial applications. It is generally accepted that AAV vectors persist as non-integrated circular episomal concatemers, and research shows an integration frequency of less than 1 in 30 million particles in studies of AAV delivery to muscle in rabbits (Schnepp *et al.*, 2003; Schultz and Chamberlain, 2008). Infection occurs through binding of viral proteins to charged heparin sulfate proteoglycans (Summerford and Samulski, 1998) and is potentially enhanced by interactions with alpha-V-beta-5 integrins (Summerford *et al.*, 1999) and human fibroblast growth factor receptor-1 (Qing *et al.*, 1999). After clathrin-mediated endocytosis, endosomal escape, and nuclear translocation, AAV can produce latent long-term infection *via* episome formation in which the transgene reaches maximum expression levels after an incubation period of 4 to 8 wks and remains stable for up to 2-3 yrs in animal models (Thomas *et al.*, 2004; Manno *et al.*, 2006). Though it is becoming less of an issue, one of the challenges facing viral

engineers is the large-scale amplification of AAVs. Baculovirus expression systems for rAAV2 vector production in SF9 cells show promise for large-scale production (Urabe *et al.*, 2002). However, current clinical trials are limited by their reliance on transient production systems and still require complete elimination of helper virus during production (Kay *et al.*, 2000).

The primary goal for rAAV engineering is to improve transduction efficiency to decrease vector loading while increasing target specificity. An initial successful effort to improve transduction was a switch from single-strand to self-complementary recombinant AAV vectors, to bypass the rate-limiting second-strand DNA synthesis step (McCarty *et al.*, 2001). Because of high variation among capsids, AAV vectors have inherent tissue-targeting abilities that can be enhanced with capsid re-engineering. For example, AAV6 demonstrates increased transduction efficiency in skeletal muscle (Gao *et al.,* 2002), and AAV4 shows preference for the CNS (Davidson *et al.*, 2000). DNA shuffling and cloning technologies are currently being used to generate extensive libraries of recombinant AAVs that display diverse tissue specificity and potential to evade host-neutralizing antibodies (Perabo *et al.*, 2006; Li *et al.*, 2008). The crystal structure of the AAV2 capsid was solved in 2002 (Xie *et al.*, 2002). AAV virons have icosahedral capsids made of 60 copies of VP1, VP2, and VP3 proteins encoded by the second genomic ORF in a variable predicted ratio of 1:1:18 (Muzyczka and Warrington, 2005). VP1 and VP2 are variable between AAV serotypes (Wu *et al.*, 2006). Mosaic vectors (capsid structure derived from subunits of different serotypes) or chimeric vectors (capsid proteins modified by domain or amino acid swapping between serotypes) have been generated through trans-capsidation or marker-rescue/domain-swapping (Wu *et al.*, 2006) (Fig. 2A) to enable the infection of tissues refractory to transduction by naturally occurring AAV vectors or to limit AAV infection to specific tissues (Wu *et al.*, 2006). Insertion of peptide ligands, conjugate-based targeting, and presentation of large protein ligands on the AAV capsid are additional strategies that have been used to enhance targeting and transduction of rAAVs (Muzyczka and Warrington, 2005) (Fig. 2B). Insertion sites for peptide-encoding DNA sequences are limited to maintain infectivity of the viron. A 14-residue core RGD peptide motif insertion is possible in VP3 at residues 261, 381, 447, 459, 573, 584, 587, and 588 (Girod *et al.*, 1999; Shi and Bartlett, 2003) (Fig. 2B). Integrin-RGD interactions could be exploited by craniofacial tissue engineers to enhance infection of endothelial cells and localization of rAAV to matrix-laden sites such as bone and tooth.

CLINICAL GENE THERAPY

State of the Field

There are three main strategies for gene delivery: *in vivo, in vitro,* and *ex vivo*. Though the most direct method is *in vivo* injection, this approach lacks the improved patient safety of *in vitro* and *ex vivo* methods. Systemic delivery is desirable if the target tissue is not directly accessible. However, this method often results in low specificity of gene expression, risks of toxicity due to the high vector concentration required, and potential damage to the function of healthy tissues (Zhang and Godbey, 2006). Alternatively, matrix-based
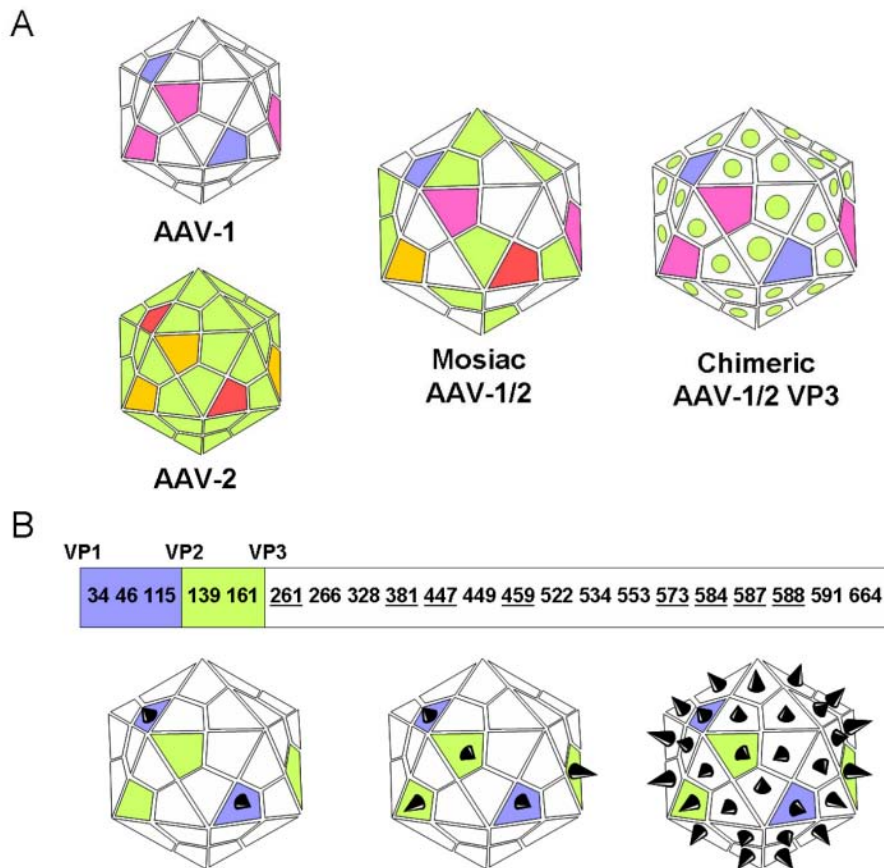
Figure 2. AAV capsid engineering for enhanced transduction and tissue-specific targeting. **(A)** Mosaic vectors (capsid structure derived from subunits of different serotypes) or chimeric vectors (capsid proteins modified by domain or amino acid swapping between serotypes) have been generated through trans-capsidation or marker-rescue/domain-swapping (Wu *et al.*, 2006). Seemingly limitless engineered combinations of the 12 identified AAV serotypes and over 100 AAV variants can be generated to enhance tissue targeting and transduction. **(B)** Insertion of peptide ligands and their presentation on the AAV capsid is a strategy that has been used to enhance targeting and transduction by rAAVs (Muzyczka and Warrington, 2005). The AAV capsid is made up of 3 subunits—VP1 (white), VP2 (green), and VP3 (blue)—in a variable ratio of 1:1:18. Each of these subunits shares a conserved VP3 sequence, with VP2 building upon VP3, and VP1 building upon VP2, as shown. These similarities can be exploited to regulate surface expression of an incorporated peptide. For example, if the peptide sequence is incorporated into the coding section unique to VP1, the peptide will be expressed only by that capsid protein. There is a limited number of sites that can support peptide insertion while maintaining viron infectivity. A 14-residue core RGD peptide motif insertion is possible in VP3 at residues 261, 381, 447, 459, 573, 584, 587, and 588 (Girod *et al.*, 1999; Shi and Bartlett, 2003). Integrin-RGD interactions could be exploited by craniofacial tissue engineers to enhance infection of endothelial cells and localization of rAAV to matrix-laden sites such as bones and teeth. Panel B adapted from Muzyczka and Warrington, 2005.

delivery allows for tissue-specific gene delivery, higher localized loading of DNA or virus, and increased control over the structural microenvironment (Dang and Leong, 2006). Thus far, human *in vivo* clinical trials have introduced adenovirus, AAV, retrovirus, and herpes simplex virus by intravenous (IV) injection, intra-tissue injection, or lung aerosol (Kemeny *et al.*, 2006). In contrast, *ex vivo* trials have focused on stable retroviral transduction of rapidly dividing populations such as CD8+ T-cells, hematopoietic stem cells, hepatocytes, and fibroblasts, followed by IV or local re-introduction. At the time of this publication, a search of the NIH Genetic Modification Clinical Research Information System

(GeMCRIS) revealed 908 total gene therapy clinical trial entries in the database (NIH, 2008a). At clinicaltrials.gov, a search for interventions with "gene transfer" OR "gene therapy" returned 174 studies, of which 145 are viral-based, with 84 active, 48 completed, and 7 terminated. This cross-section of results translates to 1605 persons who have participated in this subset of completed gene therapy trials and nearly 5000 total active or anticipated participants, based on each study's documented enrollment since 1990. The following sections will briefly review the progress of gene therapy since 1990.

Treatment of Disease

Gene therapy is specially suited for long-term delivery of a transgene to persons with a single genetic deficiency that is not amenable to protein or pharmacokinetic therapy. This was the premise of the first successful gene therapy clinical trials that inserted genes *ex vivo* into CD34+ cells to treat persons with SCID (Anderson, 1990; Blaese *et al.*, 1993). Amazingly, persistence of the adenosine deaminase (ADA) transgene was noted in peripheral blood leukocytes 12 yrs post-therapy without adverse events (Muul *et al.*, 2003). Since 1990, clinical treatment of genetic diseases—including cystic fibrosis, hemophilia, Leber congenital amaurosis, muscular dystrophy, ornithine transcarbamylase deficiency, Pompe disease, and Gaucher's disease—has been attempted, with promising documented success (Aiuti *et al.*, 2007; Alexander *et al.*, 2007). Following the SCID trials, treatment of cystic fibrosis by re-introduction of the cystic fibrosis transmembrane regulator (CFTR) chloride ion channel to lung epithelial cells was highly targeted and was the first use of rAAV in humans (Flotte *et al.*, 2003). However, like many other *in vivo* and *ex vivo* clinical trials, transduction efficiency was generally insufficient to improve clinical parameters significantly. Apart from SCID, the most promising documented results for genetic deficiency correction have been the replacement of factor IX (F-IX) in hemophilia. Studies by Avigen Inc. have examined rAAV2-mediated F-IX delivery to the liver. In dogs, therapeutic levels of F-IX were achieved for multiple years following vector treatment (Manno *et al.*, 2006). In humans, delivery of rAAV2.F-IX through the hepatic artery achieved therapeutic levels of F-IX expression for approximately 8 wks (Aiuti *et al.*, 2007). It appears that cell-mediated immunity to the rAAV2 capsid limits expression

in humans. Thus, immunomodulation and capsid engineering may make F-IX therapy a near-future reality (Krebsbach *et al*., 2003; Manno *et al*., 2006). Gene therapy is also highly desired for the treatment of neurologic and other chronic disease. Clinical trials have been implemented and/or completed for the treatment of HIV/AIDS, arthritis, angina pectoris, solid tumors, Parkinson's disease, Huntington's disease, Alzheimer's disease, Batten disease, Canavan disease, and familial hypercholesterolemia (Aiuti *et al*., 2007; Alexander *et al*., 2007; NIH, 2008a,b). Despite the many hurdles, most clinical trials are progressing steadily, with treatments for angina pectoris (Henry *et al.*, 2007), prostate cancer (Freytag *et al*., 2007), non-small-cell lung cancer, and head and neck cancer now entering phase III clinical trials (NIH, 2008b).

GENE THERAPY FOR CRANIOFACIAL REGENERATION

More than 85% of the United States population requires repair or replacement of a craniofacial structure, including bone, tooth, temporomandibular joint, salivary gland, and mucosa. Regeneration of oral and craniofacial tissues presents a formidable challenge that requires synthesis of basic science, clinical science, and engineering technology. Identification of appropriate scaffolds, cell sources, and spatial and temporal signals are necessary to optimize development of a single tissue, hybrid organs consisting of multiple tissues, or tissue interface. In contrast to traditional replacement gene therapy, craniofacial regeneration *via* gene therapy seeks to use genetic vectors as supplemental building blocks for tissue growth and repair. Synergistic combination of viral gene therapy with craniofacial tissue engineering will significantly enhance our ability to repair and regenerate tissues *in vivo*.

Head and Neck Squamous Cell Carcinoma (HNSCC)

Though the treatment of HNSCC does not directly fall in the category of craniofacial regeneration, it is the most well-developed use of gene therapy in the craniofacial region. There are three main strategies to target any solid tumor with gene therapy. First, immunomodulatory therapy seeks to increase the visibility of the tumor cells to the immune system *in vivo* or to modify the effector cells *ex vivo* to increase targeting of the tumor *via* the introduction of specific gene expression. In 2007, the dendric cell vaccine 'Provenge' was deemed safe and preliminarily approved by the FDA advisory panel in a 13 to 4 vote for the treatment of prostate cancer. However, it was later denied final approval and is currently being re-evaluated (Moyad, 2007). Second, oncolytic viruses have been developed that can selectively target, multiply in, and destroy cancer cells (Dambach *et al.*, 2006). A phase II clinical trial of OncoVex (GM-CSF), with combined chemoradiotherapy in locally advanced head and neck cancers, is ongoing (Aiuti *et al.,* 2007). In addition, the 'H101' oncolytic adenovirus has undergone phase I-III clinical trials for treating head and neck cancer and is now approved for use in China (Yu and Fang, 2007). Third, suicide genes such as herpes simplex thymidine kinase can be introduced to cancer cells to increase their susceptibility to anti-viral drugs such as acyclovir (Niculescu-Duvaz and Springer, 2005). As

mentioned above, application of these methods for treatment of various cancers comprises the majority of the current phase III clinical gene therapy trials (NIH, 2008b). Additional strategies of interest for specific targeting of HNSCC include local viral introduction of genes encoding p53 (Clayman *et al.*, 1999; Yoo *et al.*, 2004), endostatin (Lin *et al.*, 2007), and non-viral IL-2/IL-12 (O'Malley *et al.*, 2005).

Mineralized Tissues

Animal-model-based gene therapy and engineering of individual craniofacial structures such as bone and cartilage have firmly established a productive relationship, and novel approaches to regeneration of complex mineralized tissues such as tooth (Nakashima *et al*., 2006) and TMJ (Rabie *et al.*, 2007) are just beginning to emerge. Clinical protein delivery of PDGF-B or bone morphogenetic proteins (BMPs) at periodontal defect sites is well-known to enhance repair and healing of bone and gingiva (Kaigler *et al.*, 2006). Gene delivery can allow for localized sustained protein expression at therapeutic levels and can overcome recombinant protein delivery issues such as cost, half-life, supra-physiologic dosing, and poor retention. In support of this, studies have shown that use of adenovirus expressing PDGF-B for treatment of periodontal defects demonstrates better results than continuous protein therapy (Jin *et al*., 2004; Franceschi, 2005) (Table). Adenoviral-, retroviral-, and AAV-mediated delivery of osteogenic genes has been demonstrated to enhance fracture repair and intramembranous or endochondral bone formation *in vivo* in animal models (Table). To meet clinical needs, gene delivery must be safe, simple, and cost-effective. Thus, focus on *in vivo* strategies which avoid primary cell isolation and long-term culture is ideal. An "expedited *ex vivo*" bone regeneration strategy has recently been proposed in which explants of adipose tissue or muscle directly transduced with Ad.BMP-2 without culture can be re-implanted at defect sites to enhance regeneration of critical-sized rat femoral defects (Betz *et al.*, 2008) (Fig. 3A). In addition, studies to release virus directly from biomaterials have been effective for bone regeneration in animal models (Hu *et al.,* 2007)

Inducible vector systems, use of rAAV, and transduction of novel osteogenic factors have outstanding potential for mineralized tissue regeneration. In addition to vector design and capsid engineering for cell-specific transduction, we must now consider the use of systemic drug-inducible vector components. For example, an early study using a retroviral vector demonstrated dexamethasone-inducible GFP expression from transduced BMSCs *in vitro* (Jaalouk *et al*., 2000). Researchers have gone on to explore doxycycline-inducible 'tetON' promoter systems. In these systems, selective induction of BMP-2 or BMP-4 expression achieved by administration of oral doxycycline can allow for localized induction of bone formation only at vector-containing sites *in vivo* (Gafni *et al*., 2004; Peng *et al.*, 2004) (Fig. 3B). The field of rAAV-mediated bone repair is rapidly advancing and promises superior safety, tissue targeting, and high *in vivo* transduction efficiency of non-dividing cells. In the past 5 years, proof-of-principle studies have been completed and have shown positive results for AAV transduction of bone-forming cells and enhanced healing of osseous defects from *in vivo* application of rAAV expressing constitutively

Table. Virally Transduced Genes for Regeneration of Craniofacial Tissues

Gene	Application	Virus	References
BMP-4	Bone repair	Retrovirus, Adenovirus	Shen 2004, Wright 2002, Lin 2006
BMP-6	Bone repair	Adenovirus	Jane 2002
BMP-9	Bone repair	Adenovirus	Alden 2000
Shh	Bone repair	Retrovirus	Edwards 2005
caAlk2	Bone repair	AAV	Ulrich-Vinther 2007
VEGF	Bone repair, Condylar growth	Retrovirus, Adenovirus, AAV	Tarkka 2003, Rabie 2007, Jiang 2008
BMP-2	Bone repair, Chondrogenesis	Retrovirus, Adenovirus	Lee 2002, Chang 2003, Hu 2007, Smith 2000
BMP-7	Bone repair, Chondrogenesis	Retrovirus, Adenovirus	Jin 2003, Krebsbach 2000, Hidaka 2001
TGF-B1	Chondrogenesis	Retrovirus, Adenovirus, AAV	Lee 2001, Smith 2000, Zhao 2002, Pagnotto 2007
SOX9	Chondrogenesis	Retrovirus, Adenovirus, AAV	Li 2004, Cucchiarini 2007
IGF-1	Chondrogenesis	Adenovirus, AAV	Smith 2000, Izal 2008
GDF5	Chondrogenesis	Adenovirus	Feng 2008
AQP-1	Salivary gland repair	Adenovirus, AAV	Baum 2006
PDGF-B	Wound healing, Bone repair	Retrovirus, Adenovirus	Breitbart 2001, Jin 2004

BMP, bone morphogenetic protein; Shh, sonic hedgehog; caAlk2, constitutively active activin-like kinase 2; TGF-β1, transforming growth factor beta 1; IGF, insulin-like growth factor; GDF, growth and differentiation factor; AQP, aquaporin; PDGF, platelet-derived growth factor.
(Alden *et al.*, 2000; Baum *et al.*, 2006; Breitbart *et al.*, 2001; Chang *et al.*, 2003; Cucchiarini *et al.*, 2007; Edwards *et al.*, 2005; Feng *et al.*, 2008; Hidaka *et al.*, 2001; Hu *et al.*, 2007; Izal *et al.*, 2008; Jane *et al.*, 2002; Jiang *et al.*, 2008; Jin *et al.*, 2004; Jin *et al.*, 2003; Krebsbach *et al.*, 2000; Lee *et al.*, 2002; Lee *et al.*, 2001; Li *et al.*, 2004; Lin *et al.*, 2006; Pagnotto *et al.*, 2007; Rabie *et al.*, 2007; Shen *et al.*, 2004; Smith *et al.*, 2000; Tarkka *et al.*, 2003; Ulrich-Vinther, 2007; Wright *et al.*, 2002; Zhao *et al.*, 2002)
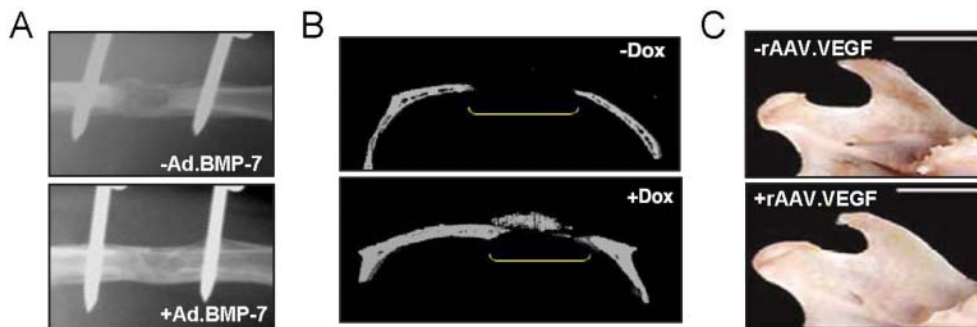

Figure 3. Gene therapy for bone regeneration. **(A)** An "expedited *ex vivo*" bone regeneration strategy has recently been proposed in which explants of adipose tissue or muscle can be directly transduced with Ad.BMP-2 without culture. This has shown promising results in the regeneration of critical-sized rat femoral defects (Betz *et al.*, 2008). **(B)** A vector for rAAV-based BMP-2 gene delivery regulated by the tetracycline-sensitive promoter (TetON) has been generated (Gafni *et al.*, 2004). Calvarial defect bone formation was noted in mice only after the administration of Doxycycline *via* the drinking water to induce BMP-2 expression. This represents a novel strategy for localized inducible gene expression. **(C)** Local injection of rAAV-VEGF to the mandibular condyle of rats results in increased condylar growth after 60 days, as demonstrated by increased condyle width and length (Rabie *et al.*, 2007).

active activin receptor-like kinase-2 (caAlk2), VEGF/RANKL, and *ex vivo* BMP-7 (Kang *et al.*, 2007; Ulrich-Vinther, 2007) (Table). The use of caAlk2, a receptor that mediates BMP signaling, is emerging as an interesting gene therapy target, because of its low required therapeutic expression level and inability to be blocked by native BMP antagonist noggin and chordin (Zhang *et al.*, 2003; Koefoed *et al.*, 2005; Ulrich-Vinther, 2007).

Successful engineering of teeth and the TMJ is challenging and requires the generation of functional interfaces. The introduction of BMPs *in vivo* to exposed pulp tissue has been proposed as a novel strategy for odontoblast transduction to enhance dentin regeneration and repair (Nakashima *et al.,* 2006). However, gene therapy has not yet been applied to the field of total tooth engineering (Young *et al.*, 2005; Hu *et al.*, 2006). Engineering of the TMJ requires the creation

of functional bone and cartilage with an appropriate transition zone. Investigators have generated such osteochondral grafts by seeding differentiated pig chondrocytes and Ad.BMP7-transduced human gingival fibroblasts onto biphasic PLLA/hydroxyapatite composite scaffolds and implanting them subcutaneously into N: Nih-bg-nu-xid immunocompromised mice (Schek *et al.*, 2004, 2005). Marrow-containing vascularized bone, mature cartilage, and a defined mineralized interface can be generated within 4 wks of implantation (Schek *et al.*, 2004, 2005). A second approach to TMJ repair is the *in vivo* introduction of therapeutic genes to the mandibular condyle. Recent work has demonstrated successful rAAV2-mediated transduction of VEGF to condylar tissue *in vivo* that subsequently enhanced mandibular condylar growth (Rabie *et al.*, 2007) (Fig. 3C). These pioneering studies provide proof-of-principle evidence for the fabrication of a physiologic osteochondral graft and direct TMJ transduction that may be developed to treat persons with TMJ disorders or developmental deformities.

Salivary Gland

Loss of salivary gland function can result as a pharmacologic side-effect, from radiation therapy, or as a consequence of autoimmune diseases such as Sjögren's syndrome. In addition to direct repair of

non-functional glandular tissue, researchers are working to develop an engineered salivary gland substitute that could be implanted in place of the parotid gland (Aframian and Palmon, 2008). Unlike acinar cells, ductal epithelial cells are incapable of fluid secretion. Because researchers have been unable to isolate and expand acinar cells *in vitro,* identification and localization of membrane proteins required for ionic gradient formation and fluid flow in acinar cells have informed efforts to modify ductal cell populations by gene transfer. Acinar cells require 4 membrane proteins to generate an osmotic gradient for unidirectional fluid movement: (1) the N^+K^+-ATPase, used to maintain membrane potential; (2) a Ca^{2+}-activated K^+ channel; (3) the secretory isoform of the $Na^+/K^+/2Cl^-$ co-transporter; and (4) the apical membrane-bound Ca^{2+}-activated Cl^- channel (Melvin *et al.*, 2005; Aframian and Palmon, 2008). Salivation occurs in response to agonists that generate an increase in intracellular Ca^{2+} concentration and is facilitated by osmotic gradient-directed fluid movement through water channels in the apical membrane, known as aquaporins (AQP) (Melvin *et al.*, 2005). It is now recognized that isolated ductal epithelial cells lack expression of AQP and, as such, cannot mediate fluid movement (Tran *et al.*, 2006). Re-introduction of transient AQP expression by adenoviral transduction has been successful in rhesus monkey parotid duct cells *in vitro* (Tran *et al.*, 2005) and rat and mini-pig salivary gland tissue *in vivo* (Baum *et al.*, 2006). Indeed, attempts to restore salivary flow by *in vivo* transduction of adenovirus encoding AQP1 into remaining glandular tissue of persons treated with radiation for head and neck cancer is the *first* human craniofacial repair gene therapy clinical trial and is currently ongoing (Baum *et al.*, 2006; NIH, 2008b).

Wound Healing/Mucosa

Engineering of skin and mucosal equivalents is essential for the esthetic reconstruction of individuals disfigured by trauma, resective surgery, or severe burns. Skin is composed of layered dermis and epidermis in a configuration that must be preserved for optimum regeneration. The first attempts to repair damaged skin and mucosa with an engineered graft did not occur until the 1980s (Madden *et al.*, 1986). Skin with both dermal and epidermal components, such as Dermagraft™ (Purdue *et al.*, 1997) and Apligraf™, used for coverage of burns and acute wounds (Eaglstein *et al.*, 1995), was the first FDA-approved tissue-engineered construct that has been put into clinical practice. Clinically, a product known as gene-activated matrix (GAM) has been developed as an enhanced skin graft substitute. GAM for wound-specific delivery of adenovirus vector encoding PDGF-B to improve healing of diabetic ulcers is currently in Phase II clinical trials (Gu *et al.*, 2004; NIH, 2008b). It is reasonably expected that these developments could be expanded to enhance wound healing and tissue repair in the craniofacial region (Jin *et al.*, 2004).

CONCLUSION: PROSPECTS AND CHALLENGES

Since the beginning of human gene therapy in 1990, nearly 1000 clinical trials have been initiated. Patient follow-up for as much as 18 yrs post-gene transfer has been generally positive, with isolated tragedy (Muul *et al.*, 2003). It is encouraging that many gene therapy trials for single-gene and complex disorders are now complete, vector selection and design strategies have significantly improved, and a safety profile is nearly established, as evidenced by the many current phase III clinical trials. Though strategies such as *ex vivo* transduction of cells with integrating retrovirus are promising, and early success led to high hopes, it is essential to keep our expectations of gene therapy realistic, because future development will require slow, stepwise progress. As we near the 20-year mark for gene therapy and begin its integration with craniofacial engineering, our focus must evolve to include expansion of placebo-controlled clinical trials, development of targeted vectors to increase transduction efficiency and to overcome the immune response, and consideration of the concept of 'genotoxicity' testing as a fundamental feature of gene therapy research.

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