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Biomolecule Delivery to Engineer the Cellular Microenvironment for Regenerative Medicine

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Abstract—To realize the potential of regenerative medicine, controlling the delivery of biomolecules in the cellular microenvironment is important as these factors control cell fate. Controlled delivery for tissue engineering and regenerative medicine often requires bioengineered materials and cells capable of spatiotemporal modulation of biomolecule release and presentation. This review discusses biomolecule delivery from the outside of the cell inwards through the delivery of soluble and insoluble biomolecules as well as from the inside of the cell outwards through gene transfer. *Ex vivo* and *in vivo* therapeutic strategies are discussed, as well as combination delivery of biomolecules, scaffolds, and cells. Various applications in regenerative medicine are highlighted including bone tissue engineering and wound healing.

Keywords—Tissue engineering, Controlled drug delivery, Biomaterial, Cell therapy, Gene delivery.

INTRODUCTION

Regenerative medicine has the potential to repair many cells and tissues to dramatically improve the quality of life of patients suffering from a myriad of diseases. In cases where a patient's own cells and tissues are used, rather than allogenic cells transplanted from another source, the complications of tissue rejection are prevented. The ability to regenerate tissue would also afford patients healthcare independent from a limited supply of allogenic cells or tissue. Although promising, regenerative medicine is still a nascent field requiring further refinement of bioengineered materials, cells, and biological microenvironmental cues, which are all necessary for integrative solutions to be found.

Tissue regeneration is not a new concept as it has been discussed for organisms such as the hydra, anurous batrachians and the midwife toad since at least 1744, 1769, and 1898, respectively.[62,75,80] Furthermore, in humans it is known that an embryo's wound healing process is faster than that of adult tissue and that it is better able to re-gain full functionality with reduced scar formation.[46] Understanding how such biological systems are capable of self-regeneration has been of great interest in the scientific community due to its translational potential.

To engineer a tissue to be a particular type and have a desired function, the microenvironment of the cells within the tissue must be controlled. Key controlling elements of cellular microenvironments or niches can be subdivided into four signaling categories which control the critical actions of cells including their cellular proliferation and death, migration, and differentiation. These signaling categories are: (1) Outside-in soluble biological factors that direct internal cell signaling; (2) Outside-in insoluble factors of the extracellular matrix (ECM) that direct cell–ECM signaling (3) Cell–cell interactions and (4) Endogenous and exogenous genetic instructions within the cell functioning from the inside-out (Fig. 1a).[8,19] In this review, we describe the current state of the art in engineering microenvironments (i.e., stem cell niches) through biomolecule delivery, highlighting biomolecule presentation to cells in soluble (i.e., autocrine/paracrine) or insoluble form, through cell–cell or cell–ECM interactions, as well as intracellular delivery of nucleic

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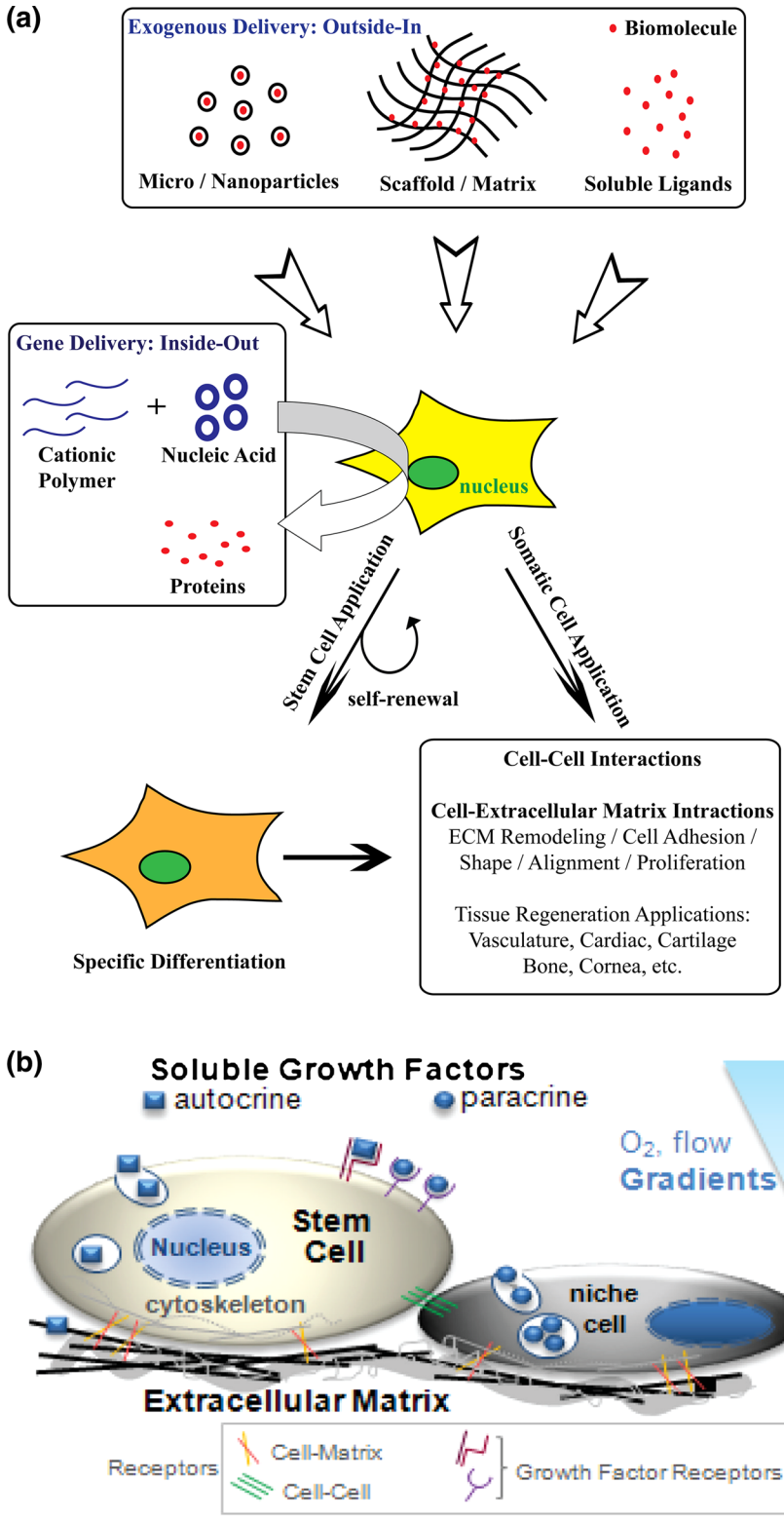

FIGURE 1. (a) Key controlling signals regulating cellular responses. (b) A depiction of niche factors which control microenvironments: soluble growth factors which could be either autocrine or paracrine acting, cellular receptors involved in binding other cells or the ECM, as well as growth factor receptors; from Discher *et al.*[15] Reprinted with permission from AAAS.

acids for regenerative medicine (Fig. 1b).[15] Although mechanical cues, such as substrate elasticity and topography, are also important and an interesting avenue of research to control cellular responses, this is outside the scope of the current review; we refer interested readers to other reviews or articles on this topic such as Higuchi or Khetan *et al.*[26,34]

BIOMOLECULE DELIVERY FOR ENGINEERING THE CELLULAR MICROENVIRONMENT

Biomolecules delivered to cells can affect cells' interactions with each other and their microenvironment, and as a result promote the repair of defective tissues. Exogenous biomolecules can be presented to the cell microenvironment through various methods including: (1) loading in microparticles, nanoparticles, or controlled release devices, (2) adsorbing or conjugating to a scaffold that may present the signal in a particular orientation, and (3) freely dissolving in a solution that is injectable and that may also contain other components such as injectable scaffolds. The box labeled "Exogenous Delivery: Outside-In" in Fig. 1a provides a schematic diagram of three methods of exogenous delivery of biomolecules. When successfully delivered, these biomolecules can trigger intracellular signaling, promote cell–cell interactions, and control cell–ECM interactions. Such events can induce healing through mechanisms of ECM remodeling or specific differentiation of stem cells towards a tissue of interest.

Biomolecule Delivery and Release of Soluble Signals

Natural ECM regulates the biological activities in a tissue through soluble, bioactive effectors such as growth factors and morphogens.[58] The ECM locally binds, stores, and releases these biomolecules to meet the needs of cells for tissue repair or remodeling. Such interactions between soluble biomolecules and the ECM provide increased concentration of signaling molecules, localized morphogenetic activity, and protection against degradation.[43] There is a need to engineer the delivery of biomolecules so that the needed factor is provided to the right cells at the right time and place to properly control cell and tissue function. Approaches to engineer biomolecule delivery systems for regenerative medicine applications have focused on mimicking the biological release dynamics of the ECM by incorporating the biomolecules into scaffolds or controlled release devices.

Several strategies have been designed to incorporate growth factors within scaffolds such as through diffusion into a porous scaffold, direct incorporation into a hydrogel for controlled release, or encapsulation in particulate delivery vehicles that are localized within a scaffold.[1,16] The simplest method of generating scaffolds that contain soluble biomolecules is by allowing the biomolecules to diffuse in and/or adsorb onto the scaffold. In order to create transplantable tissues, porous scaffolds seeded with cells *ex vivo* are incubated in bioreactors that contain flowing media with growth factors that spread through the scaffolds by convection and diffusion. For example, Davis *et al.*[12] showed that a mineralized, apatite-coated polymeric scaffold containing human mesenchymal stem cells (hMSCs) adsorbed bone morphogenetic protein-2 (BMP-2) from a BMP-2 solution and that the adsorbed BMP-2 induced osteogenic differentiation of hMSCs. In another study, new mature cartilage tissue was formed *in vivo* from a chondrocyte-collagen composite, into which basic fibroblast growth factor (bFGF) had diffused prior to implantation.[21]

An alternative approach is to incorporate soluble signals into hydrogels. A hydrogel system is composed of hydrophilic polymer(s) in a solution that solidifies into a gel upon different cues, such as a change in temperature, pH, biomolecular interactions, or UV light.[16] Hydrogels in solutions with biomolecules can be gelled *ex vivo* and implanted or injected for subsequent cross-linking *in vivo*. For example, a UV-crosslinked chitosan hydrogel was able to release entrapped fibroblast growth factor-2 (FGF-2) upon *in vivo* biodegradation of the hydrogel and thereby accelerate the wound healing process.[52] In another example, Hea Kyung *et al.*[35] used a pH/temperature-sensitive hydrogel based on a synthetic polymer. The polymer was injected into mice as a solution mixed with recombinant human bone morphogenetic protein-2 (rhBMP-2) and hMSCs, and then cross-linked *in situ* to form the hydrogel, which subsequently induced osteogenesis successfully.

In an effort to better mimic physiologically relevant environments, growth factors have been incorporated into degradable matrices as well by means of cleavable covalent and separable non-covalent interactions.[60] Chemical conjugation or enzymatic cross-linking techniques are used to covalently bind growth factors to the backbone polymers of matrices. Lorentz *et al.*[42] demonstrated that fibrin matrix cross-linked with α2-plasmin inhibitor-fused insulin-like growth factor-1 at bladder lesion sites *in vivo* induced significant increase in smooth muscle cell proliferation. Non-covalent binding occurs *via* growth factors' specific interactions with ECM components. For example, bone morphogenetic protein-2 with high heparin-binding affinity added to collagen/heparin matrix significantly improved bone formation *in vivo*.[31] Also, in many situations, physiological "on-demand release" is mimicked *via* proteolytic degradation by proteases, a key step

during tissue remodeling. Natural and synthetic hydrogels with specific protease cleavage domains can release their cross-linked growth factors upon physiological demand *in situ*. Lutolf *et al.*[44] showed that poly(ethylene glycol) hydrogel with matrix metalloproteinase as linkers efficiently delivered human bone morphogenetic protein-2, recruited primary human fibroblasts, and remodeled bony tissue in rat crania *in situ*.

An often-used delivery strategy is to utilize a particulate delivery system for controlled release. Micro- and nanoparticles are constructed from biomaterials, such as polymers, and incorporate therapeutic agents either throughout the particle or concentrated at the core. The particles can protect sensitive biological cargo from quick degradation and clearance by the body, thereby significantly extending activity of the biomolecule.[49] The particles may be further surface-modified to render them hydrophilic and/or neutral in charge in order to evade non-specific protein adsorption, minimize immune response and clearance, and prolong circulation time. Some of the conventional methods include coating particles with polyethylene glycol, polyacrylamide, polysaccharides such as dextran, albumin, and transferrin.[37,39,53,74,79] Another primary advantage of using a particulate delivery system is the ability for controlled release of the encapsulated biomolecules. Particle-based release can enable constant release of a desired drug or biological factor to maintain a gradient or an effective concentration at a target site over time. Particulate delivery systems can be combined to enable programmed temporal release of multiple factors simultaneously or sequentially such as the release of BMP-2 and BMP-7 from particles to promote MSC differentiation and osteogenic activity.[91] Polymeric particle release kinetics can be controlled and modeled by the design of a material's physicochemical characteristics such as chemical bond degradation rate and diffusivity of water through the polymer as well as the length scale of the particle.[64,83]

Microparticles and nanoparticles can be either injected alone or incorporated into a scaffold. Seshadri *et al.*[70] demonstrated that the direct injection of superoxide dismutase encapsulated in polyketal microparticles in the myocardium following myocardial infarction significantly reduced myocyte apoptosis and improved cardiac function. In an application related to bone tissue engineering, Park *et al.*[56] showed that rabbit bone marrow mesenchymal stem cells encapsulated in an oligo(poly(ethylene glycol) fumarate) hydrogel were able to differentiate into a chondrogenic lineage upon exposure to transforming growth factor-β1 (TGF) released from co-encapsulated gelatin microparticles. The authors of this study demonstrated that only the hydrogel containing TGF-β1 loaded microparticles induced stem cells to express chondrocyte-specific type II collagen and aggrecan in a dose-dependent manner. Particles can also be combined into hydrogels to facilitate sequential delivery of multiple growth factors, such as a system composed of alginate hydrogels and poly(lactic-co-glycolic acid) (PLGA) microparticles for sequential release of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) to promote angiogenesis.[76]

As multiple soluble factors can have synergistic function for regenerative medicine and this activity is often dependent on kinetics, the temporally controlled sequential delivery of multiple biomolecules can be critical.[61] A single delivery system may be used to achieve multiple stages of release by incorporating drugs at the core and the surface.[69] On the other hand, combining more than one system may also provide temporal control of the release of multiple biomolecules. In one study to engineer a vascularized bone tissue, Kempen *et al.*[32] formulated a composite with PLGA microparticles encapsulating BMP-2 embedded within a poly(propylene) scaffold which was in turn surrounded by a gelatin hydrogel loaded with VEGF. Figure 2 shows enhanced vasculature and bone formation in the scaffold loaded with both BMP-2 and VEGF after 8 weeks of subcutaneous implantation.[32]

Insoluble Biomolecule Delivery Affecting Cell–Cell and Cell–ECM Interactions

Soluble factors in the previous section act by triggering cells' intracellular signaling to affect cell fate and promote tissue regeneration. However, cells in native tissue are surrounded by neighboring cells as well as ECM that also influence the decisions that cells make through insoluble signals. Much research has focused on delivering exogenous biomolecules that support desired cell–cell and cell–ECM interactions.

Cells are in close proximity with homotypic or heterotypic cells in a living tissue. These cells need to communicate with each other through gap, adherens, and tight junctions as part of their cell–cell interactions in order to support tissue function and structure.[10] Due to the important roles that cell–cell interaction plays in tissue function, research in tissue engineering has aimed to mimic such architecture within an engineered construct.[33] A widely employed technique in this area of research is the patterned coculture of cells by utilizing a three-dimensional microfluidic system,[9] a molded hydrogel,[78] or thermally responsive cell sheets using poly(*N*-isopropylacrylamide) (PIPAAm).[86] Also, a layer-by-layer deposition method can be used to coculture cells together, such as fibroblasts and hepatocytes. This method involves sequential coating of different biomolecules to a substrate, utilizing ionic

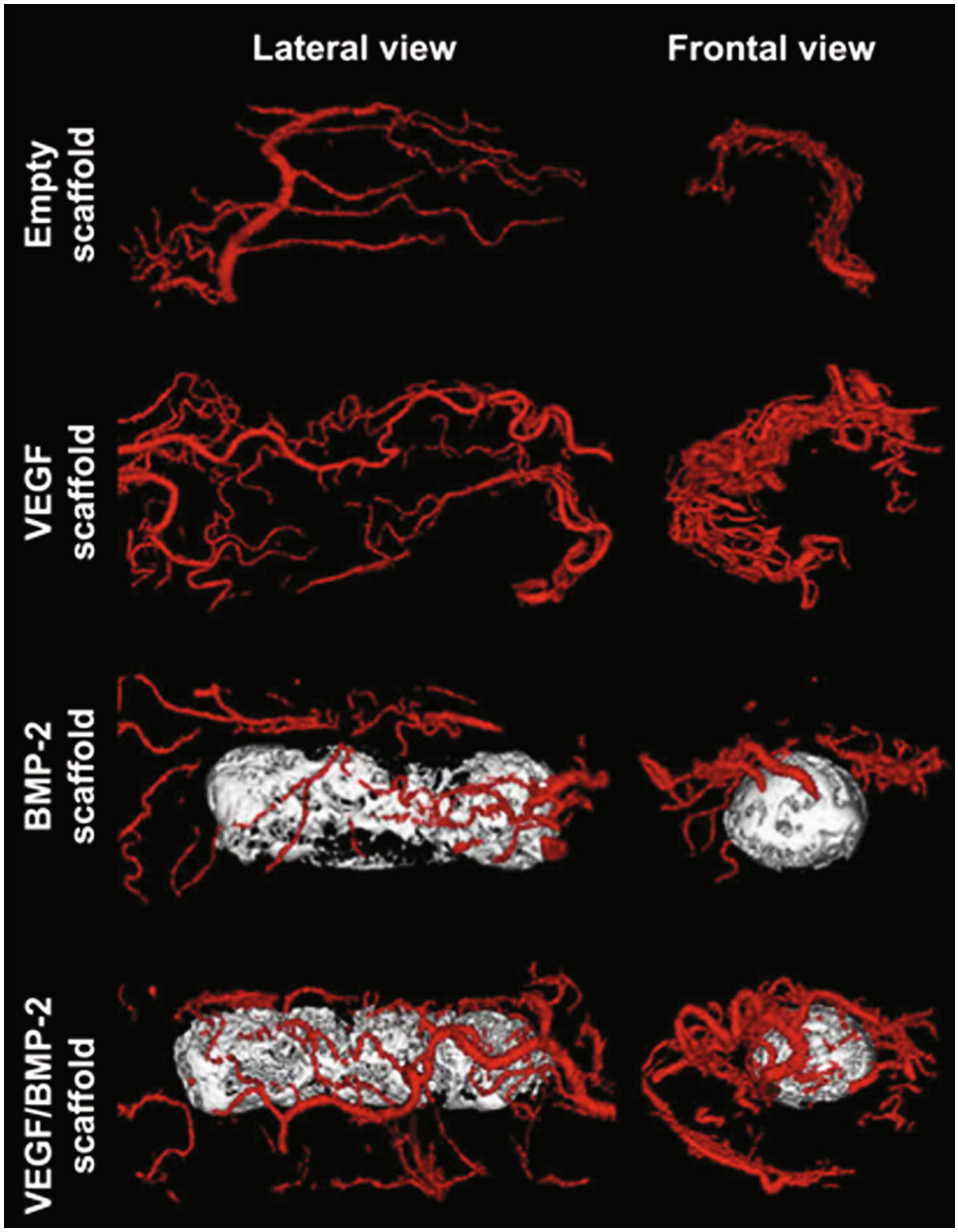

FIGURE 2. A depiction of enhanced vasculature and bone formation in the scaffold loaded with both BMP-2 and VEGF after 8 weeks of subcutaneous implantation. Reprinted from Kempen *et al.*[32] with permission from Elsevier.

adsorption of charged polyelectrolytes such as hyaluronic acid (HA) and poly-L-lysine.[6] The function of hepatocytes can be modulated by the degree of heterotypic interaction with fibroblasts and by the extent of homotypic interaction between two fibroblasts. Such artificial tissue constructs have potential applications in not only the regeneration of the liver, but also other tissues with specific architecture for cell–cell interaction.

The ECM is a three-dimensional support for cells that provides biomolecular as well as mechanical cues and guides tissue formation and regeneration processes. Because of the similar viscoelastic and diffusive properties between natural ECM and hydrogels, many types of artificial scaffolds used in tissue engineering are synthetic hydrogels.[43] Other types of hydrogels composed of more hydrophobic constituents are aimed at providing stronger mechanical architecture.[16] Scaffold designs often integrate biologically important molecules which mimic structural and functional aspects of natural, tissue-specific microenvironments. In this manner key insoluble biomolecules can be delivered and presented in a biomimetic manner. Examples include presentation of receptor-binding ligands for cell adhesion as well as

proteolytic degradability for cell migration and ECM remodeling.[27,72]

Various ligands of small oligopeptide sequence promoting cell adhesion have been identified and are incorporated into tissue scaffolds.[72] One of the most commonly used motifs is the Arginine–Glycine–Aspartate (RGD) sequence that specifically recognizes and binds to integrin receptors. Many studies have tested the effectiveness of RGD peptide coated scaffolds for cell adhesion and their influence on cell behavior with respect to tissue engineering.[25] For example, Yang *et al.*[88] studied the growth and differentiation of human osteoprogenitor cells with RGD containing scaffolds. Human bone marrow cells were able to adhere, grow, migrate and undergo osteogenesis on a three-dimensional PLGA scaffold modified with GRGDS peptides. In a more recent study, Wang *et al.*[84] showed that differential RGD nanospacing on a poly(ethylene glycol) (PEG) hydrogel directed preferential lineage commitment of mesenchymal stem cells (MSCs). Adipogenic/osteogenic co-induction of MSCs on large RGD nanospacings resulted in a more robust differentiation into osteoblasts. These studies reinforce that defined and controlled presentation of insoluble cell-adhesion ligands on scaffolds is a critical parameter for engineering cells and tissues.

Other biomolecules are used to promote scaffold interaction with cell surface glycosaminoglycans (GAGs). Peptide sequences in this category are mostly derived from extracellular matrix proteins, such as laminin, fibronectin and vitronectin. For example, a vitronectin-derived GAG-binding peptide GKKQRFRHRNRKG was linked to a polyacrylamide hydrogel, into which human pluripotent stem cells were seeded. This hydrogel, which was unable to adhere nor self-renew stem cells prior to modification, was able to control self-renewal and maintain pluripotency in its peptide modified form.[50] Silva *et al.*[73] investigated cell–ECM interactions in inducing specific differentiation of neural progenitor cells (NPC) into neurons but not astrocytes. They showed that NPCs increased expression of β-tubulin (neuronal marker) when cultured in a three-dimensional nanofiber matrix in the presence of bioactive epitope IKVAV of laminin, which is known to promote adhesion of neurons, further demonstrating how delivery and presentation of insoluble factors is key to control cell fate.

Cell migration through natural extracellular matrices is one of the key processes in tissue development, maintenance and regeneration.[43] Cells in synthetic three-dimensional scaffolds can migrate in two different ways. In the first case, cells can migrate in matrices with macroscopic pores of size larger than the cell diameter.[5] Secondly, cells can actively pave their migration path by utilizing proteases, such as matrix metalloproteinases (MMP), collagenase, serine proteases, and hyaluronidases that degrade extracellular proteins and proteoglycans.[20,41] In one example, smooth muscle cells (SMC) were able to migrate through PEG hydrogels functionalized with an RGD sequence and a polyalanine peptide sequence by secreting elastase which degraded the polyalanine peptide sequences.[23,45] The degradation, migration and formation of void space in a scaffold can allow the natural process of tissue formation by allowing cellular synthesis and deposition of biomolecules such as collagen.

INTRACELLULAR NUCLEIC ACID DELIVERY FOR ENGINEERING THE CELLULAR MICROENVIRONMENT

In contrast to engineering cells from the outside-in *via* soluble or insoluble factors, nucleic acid delivery approaches allow the engineering of the cell from the inside-out through promoting or inhibiting protein expression as a result of delivering nucleic acid. Intracellular nucleic acid delivery to the cytoplasm or nucleus is more challenging than delivery to the extracellular space, but can enable novel regeneration modalities by turning on and off exogenous and endogenous genes. Figure 3, taken from Sunshine *et al.*,[77] depicts barriers of gene delivery which must be overcome for successful gene modulation that include: (1) Complexation or condensation of the nucleic acids, nanoparticle formation, and protection against nucleases; (2) Cellular uptake (i.e., *via* endocytosis); (3) Endosomal escape of the particle to the cytosol; (4) Release of the cargo from the gene carrier into the cytosol, which is the target location of short interfering RNA (siRNA); (5) Degradation of the gene carrier to minimize cytotoxicity; (6) Nuclear import for the case of DNA and short hairpin RNA (shRNA) plasmids.[77]

Nucleic acids are delivered using either viral or non-viral vectors. Viral methods can be effective, but the efficacy can diminish with repeated administration due to an adaptive immune response against the viral vector. Furthermore, viral vectors can have delivery limitations such as small cargo capacity and safety limitations such as insertional mutagenesis. Despite these complications, viral vectors are the most commonly used modality for gene transfer in clinical trials. Non-viral methods, albeit typically less effective in comparison to viral vectors, have a higher cargo capacity, can be more easily functionalized for tissue targeting, more easily manufactured, are less immunogenic, and can be engineered to be relatively non-toxic.

However, both viral and non-viral gene delivery vectors have difficulty delivering their cargo intracellularly

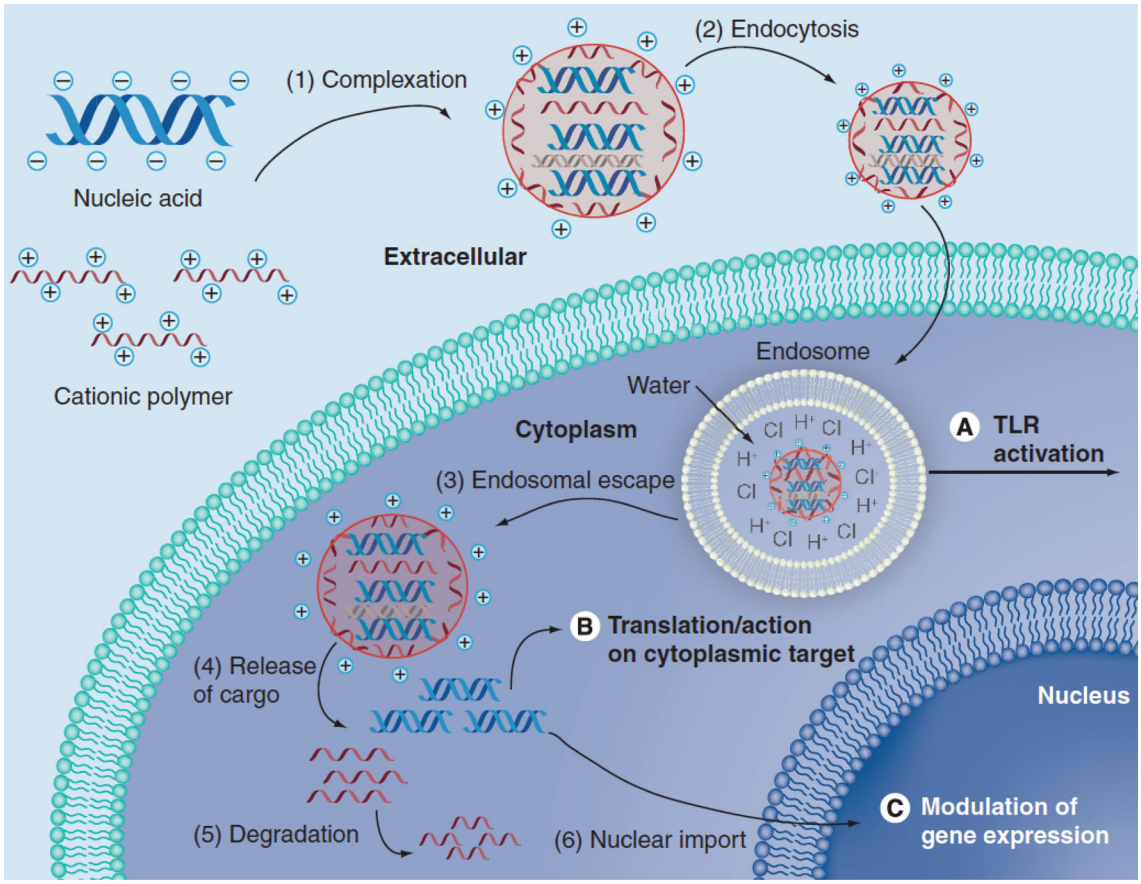

FIGURE 3. Barriers of nucleic acid delivery for gene modulation. Reprinted from Sunshine *et al.*[77] with permission from Future Science Ltd.

in vivo. To bypass *in vivo* nucleic acid delivery challenges, researchers often genetically manipulate autologous or allogenic cells *ex vivo* then deliver the modified cells for the specific regenerative medicine application. Caution is still warranted however as transplanting cells can be potentially associated with graft vs. host disease, immunosuppression requirements, tumor formation, and unregulated protein synthesis.[51]

Viral Vectors for Ex Vivo Cell Engineering

Many researchers have utilized viruses to *ex vivo* engineer cells that are subsequently injected into animal models. In one example to aid cardiac regeneration after an ischemic heart attack, Haider *et al.*[24] supplied a source of myoblasts as well as growth factors to promote angiogenesis. Haider *et al.* created $VEGF_{165}$ expressing human skeletal myoblasts which were transduced by adenoviral vectors carrying human VEGF ($hVEGF_{165}$) and a *lac-z* reporter gene. 3×10^8 transduced cells were injected at 20 different intramyocardial sites in Yorkshire swine, which were used as a model for chronic infarction. The authors found this angiomyogenesis regeneration method safe and able to result in improved perfusion, myocardial contractility and overall performance.

To promote vascularization in a rat myocardial infarct model, human embryonic stem cells (hESC) were transduced by an adenovirus to express $VEGF_{165}$ to enhance the stem cells' differentiation down the endothelial cell line.[65] The obtained endothelial progenitor cells were then transplanted intramyocardially into the infarcted and peri-infarcted regions in the rat model; the progenitor cells were able to survive in the infarct model and aided infarcted myocardium regeneration (size and mature blood vessel density).

For osteogenic applications, Blum *et al.*[7] transfected and transduced rat marrow stromal cells to overexpress human bone morphogenetic protein 2 (hBMP-2) *via* Lipofectamine® Plus™ and an adenovirus or retrovirus and found that the adenovirus was the only vector capable of expressing detectable hBMP-2. The authors were able to significantly increase endogenous alkaline phosphatase activity which indicates successful osteogenic differentiation. 4×10^5 stromal cells were then seeded onto Ti-mesh scaffolds for *in vivo* osteogenic application assessment in an orthotopic, critically-sized, rat cranium. The authors found there

was a small, statistically significant improvement in osteogenesis when using the adenoviral vector.[7]

To repair large nonunion bone defects, Wojtowicz *et al.*[85] retrovirally transduced bone marrow stromal cells (BMSC) to overexpress Runx2, a transcription factor regulating osteoblast differentiation. These modified BMSCs were delivered in rats, which had a critical-sized femur defect, on polycaprolactone scaffolds with type I collagen mesh. The authors observed accelerated healing in the large bone defects compared to unmodified BMSCs.[85]

Cerebral vascular diseases of an occlusive nature lead to brain ischemia, causing neuropathological complications. Zhao *et al.*,[92] endeavored to induce marrow stromal cells to express hepatocyte growth factor (HGF) using a herpes simplex virus type-1 vector. Marrow stromal cells themselves are capable of releasing cytokines and growth factors and are able to migrate towards damaged areas, improving functional recovery after cerebrovascular accidents. HGF has been associated with anti-apoptosis, angiogenesis, increased neurite growth, and neuroprotective properties post-ischemia and Zhao *et al.*[92] observed a significant neurological recovery when they intracerebrally transplanted transduced stromal cells into a rat occluded artery model.

Viral Vectors for In Vivo Regeneration

While *in vivo* gene therapy is more challenging than *ex vivo* gene therapy, several studies suggest that may be a promising avenue for regenerative medicine. As there is limited osseointegration for allografts used in critical bone defects, Yazici *et al.*[89] endeavored to coat allografts in 10^{10} self-complementary adeno-associated virus (AAV) serotype 2.5 vector, delivering the BMP-2 gene. The coated allograft was able to form cortical shells that were indistinguishable from those formed by live autografts; furthermore, there was reduced bone resorption, which led to increased bone volume than the autograft which rendered superior biomechanical properties.[89]

Gelse *et al.*[22] investigated complementary DNA (cDNA) delivery using adenoviral vectors encoding for bone morphogenetic protein or insulin-like growth factor 1 for an articular cartilage repair application. Hyaline repair cartilage in the defect was produced in most partial thickness lesions in the rat model that was used. However, cells that failed to be transduced did not fill the defect or were associated with type I collagen.[22]

To assess spinal cord injury regeneration, Shea *et al.*[82] used a rat spinal cord hemisection injury model using PLGA multichannel bridges. Lentiviruses encoding for brain derived neurotrophic factor and neurotrophin 3 transduced a number of cells including, astrocytes, macrophages, fibroblasts, and Schwann cells for at least 4 weeks, resulting in a significant induction of myelinated axons into the bridge in comparison to bridges with lentiviruses encoding for β-galactosidase.

In a final example, Bainbridge *et al.*[2] used recombinant adeno-associated viral vectors carrying a gene encoding for retinal pigment epithelium (RPE65) using the RPE65 promoter for three patients suffering from Leber's congenital amaurosis which is associated with infantile-onset rod-cone dystrophy. There were no adverse events due to the subretinal vector delivery, nor changes in visual acuity, peripheral visual fields, and retinal responses to electroretinography, indicating the vision was stabilized.[2] Thus, *in vivo* viral gene therapy may be a promising approach for certain regenerative medicine applications.

Non-viral Vectors for Ex Vivo Cell Engineering

Numerous non-viral approaches for *ex vivo* gene transfer for regenerative medicine have been described. Autologous bone grafts from the iliac crest are commonly used for spinal arthrodeses for stabilizing adjacent disks and minimizing back pain. However, to avoid donor site morbidity complications Sheyn *et al.*[71] non-virally nucleofected primary porcine adipose tissue-derived stem cells to over express recombinant hBMP-6 which is capable of osteogenesis induction. Post-transfection, 5×10^6 cells were injected into the lumbar paravertebral muscle of mice. The authors found this gene transfer method to be safe and the mice exhibited temporary overexpression of BMP-6. Enough bone was formed in the lumbar region to fuse two to four vertebrae of the spine.[71]

Post-myocardial ischemia, overexpression of VEGF may result in the formation of angioma. Lei *et al.* complexed a hypoxia-regulated VEGF plasmid with the cationic polymer, polyethyleneimine (PEI), to form particles and delivered them to rabbit skeletal myoblasts and then transplanted the transfected myoblasts to an acute myocardial infarcted rabbit model.[90] 1×10^7 transfected cells were intramyocardially injected into the infarcted and peri-infarcted areas. The authors found that the ability to repair the infarcted tissue, as well as the global left ventricular function was improved.

With the purpose of increasing neovascularization for myocardial ischemia and peripheral vascular disease, human umbilical cord blood-derived progenitor cells were used in conjunction with polyethersulfone (PES) electrospun nanofibers by Das *et al.*[11] The purpose of the nanofibers were to expand the stem/progenitor cells many fold without differentiation in an *ex vivo* environment; the progenitor cells were able to retain their phenotype prior to *in vivo* delivery. The

progenitor cells were transfected using Nucleofector® technology to overexpress the VEGF-A_{164} and PDGF-BB growth factors. 5×10^5 cells were delivered per immunodeficient NOD/SCID mouse, in a hind limb vascular injury animal model. The growth factor-overexpressing cells were able to promote angiogenesis more effectively compared to non-expressing progenitor cells.[11]

Non-viral Vectors for In Vivo Regeneration

Several studies aimed at *in vivo* regeneration through the use of non-viral gene delivery have shown promise. Large bone implant osseointegration presents healing challenges even while using autologous bone grafts. Park *et al.*[55] investigated whether liposomal vectors delivering BMP-2 cDNA mixed with crushed bone and delivered onto the implant surface as well as the peri-implant defect could transfect trabecular-lined cells and induce bone—graft osseointegration in pig calvariae. The authors found that the liposomal vector was able to induce abundant BMP-2 protein production throughout the defect, enhance bone formation and caused the particulate bone to become trabecular, in contrast to the control groups.[55]

As there are still no optimal solutions for nonunion bone defects, Kimelman-Bleich *et al.*[36] endeavored to recruit host progenitor cells to nonunion radius bone defect sites in mice using a collagen sponge. A plasmid encoding BMP-9 was injected into the radial defects and electroporation was accomplished 10 days after the defect was created. The authors found that the gene expression was localized to the site of the defect and that bone formation bridged the nonunion gap; whereas the gaps remained in the control groups.[36]

Trentin *et al.*[81] demonstrated that they were able to achieve upregulation of VEGF-A_{165} in full-thickness dermal wounds in a mouse model with vessel maturity by delivering a mutated hypoxia-inducible factor (HIF)-1α gene lacking the oxygen sensor so that the gene was constitutively on. The gene was delivered using an intracellularly reducible disulfide-containing, cysteine-flanked lysine peptide in a fibrin matrix.[81]

Shea *et al.*[63] investigated the efficacy of delivering of FGF-2-encoding plasmids using a porous PLGA scaffold formed *via* a gas foaming method in the intraperitoneal fat of C57BL/6 male mice. They found the expression peaked after the first couple of days and subsided over the following week or two and that the vascular volume fraction increased 40% in comparison to the controls at week two.

Park *et al.*[54] demonstrated the ability to significantly increase vascular endothelial growth factor and stromal cell-derived factor-1α chemokine in mice with full-thickness dermal wounds by intradermally delivering the sonic hedgehog gene using poly(β-amino ester)

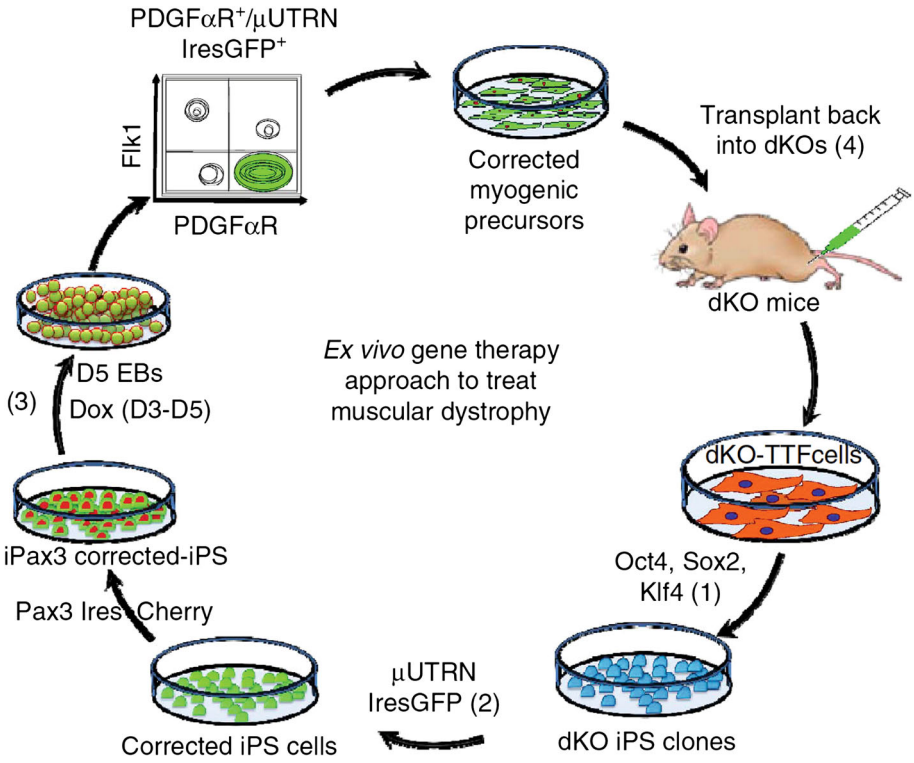

FIGURE 4. ***Ex vivo*** **gene therapy approach to treat muscular dystrophy.[18] Reprinted by permission from Macmillan Publishers Ltd: Nature Communications, Copyright (2013).[18]**

polymers. By varying the type of small molecule end-cap, they were able to optimize toxicity and achieve higher transfection of the morphogen than commercially available reagents such as Lipofectamine® 2000 *in vitro*.[54] Thus, emerging non-viral approaches are able to achieve efficacy *in vivo* for regenerative medicine challenges, while also reducing potential safety concerns as compared to viruses.

COMBINATION BIOMOLECULE AND CELL THERAPY FOR REGENERATIVE MEDICINE

There have been several biomolecule and cell-based combination therapies used for regenerative medicine. One of particular interest involves the treatment of Duchenne muscular dystrophy (DMD). Filareto *et al.*[18] investigated an autologous cell-based combination therapy in a DMD mouse model which involved the following procedures: the extraction of fibroblasts and their induction of pluripotency; gene delivery to correct for the missing micro-utrophin gene in the fibroblasts; the promotion of myogenic progenitor cells using Pax3; and the eventual re-implantation of the corrected myogenic progenitor cells into a DMD mouse model.

More specifically, Filareto *et al.*[18] retrovirally transduced dystrophin/utrophin-double knockout (dKO) tail-tip fibroblast cells with Oct4, Sox2, and Klf4 to obtain the dystrophic induced pluripotent stem cells (iPSC), which were then expanded (Fig. 4). The dystrophic iPSCs were corrected by delivering the micro-utrophin gene *via* the Sleeping Beauty transposon system to allow for precise excision and relocation of a DNA segment. Pax3 was then used to promote skeletal muscle stem/progenitors cells. Flk-1 and PDGFα receptor expression were shown to establish pluripotency using differentiated embryoid bodies. The micro-utrophin-corrected myogenic precursors were then autologously re-implanted back into the same dKO mice. The muscles of such mice had improved contractility and muscle regeneration *in vivo*.[18]

CURRENT LIMITATIONS

The development of thick complex tissues requires multiple cell types and involves microenvironments

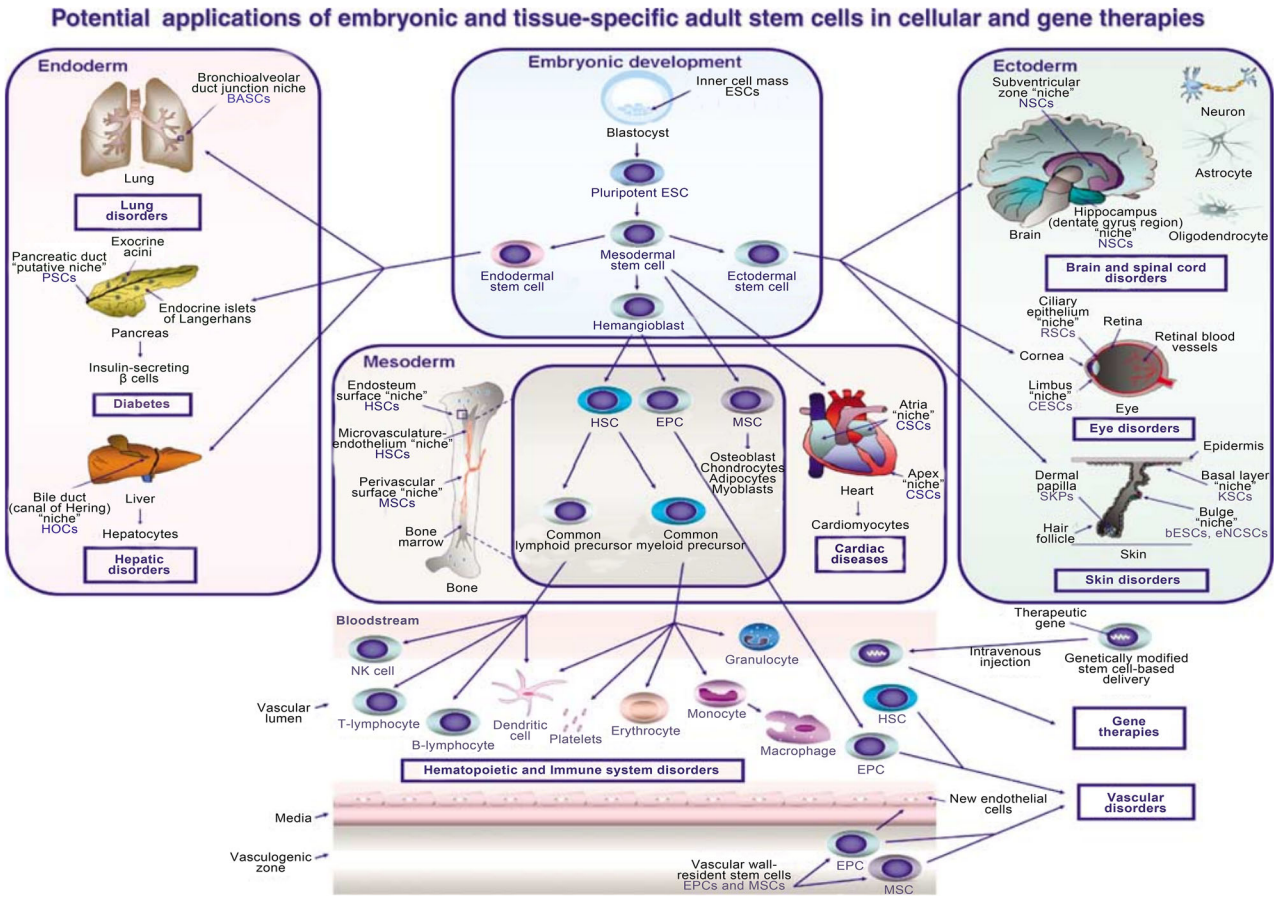

FIGURE 5. A depiction of applicable avenues of gene and cell therapies using stem cells.[48] Reproduced by permission from Macmillan Publishers Ltd: Clinical Pharmacology and Therapeutics, Copyright (2007).[48]

TABLE 1. Literature summary.

Application	Biomaterial/vector	Biomolecule	Cell type	Animal model	References
Biomolecule delivery of soluble signals					
Bone	PLGA microspheres in poly(propylene fumarate) scaffold surrounded by gelatin hydrogel	BMP-2 and VEGF			
Bone	SMO-PCLA-PEG-PCLA-SMO hydrogel	hBMP-2	hMSCs	Ectopic/orthotopic implantation into rat	Kempen *et al.*[32]
Bone				Subcutaneous injection into mouse	Kim *et al.*[35]
Cartilage	Collagen sponge	bFGF	Chondrocytes	Nude mouse	Fujisato *et al.*[21]
Cartilage	Oligo(poly(ethylene glycol) fumarate) hydrogel with gelatin microparticles	TGF-β1	Rabbit marrow mesenchymal stem cells		Park *et al.*[56]
Neovascularization	Alginate hydrogel with PLG microspheres	PDGF and VEGF		Ischemic limb i.m. injection into apoE$^{-/-}$ mouse	Sun *et al.*[76]
Wound healing	Photocrosslinkable chitosan hydrogel	FGF-2		Mutant diabetic mouse	Obara *et al.*[52]
Insoluble biomolecule delivery affecting cell–cell and Cell–ECM interactions					
Bone	PLGA porous scaffold	GRGDS peptide sequence	Human bone marrow cells		Yang *et al.*[88]
Bone	PEG hydrogel	Nanospacing of RGD peptide	Rat MSCs		Wang *et al.*[84]
Neural	Self-assembling nanofiber from peptide amphiphile	IKVAV epitope of laminin	Murine neural progenitor cells		Silva *et al.*[73]
Liver		HA, poly-L-lysine	Primary rat hepatocytes and murine 3T3-J2 fibroblasts		Bhatia *et al.*[6]
Stem cell self-renewal	Polyacrylamide hydrogel	GKKQRFRHRNRKG peptide (vitronectin-derived, GAG-binding)	hESCs and iPSCs		[50]
Viral vectors for *ex vivo* cell engineering					
Bone	Adenovirus	hBMP-2	Rat marrow stromal cells	Orthotopic implantation into rat	Blum *et al.*[7]
Cardiac	Adenovirus	hVEGF	hESC-derived CD133^{+} endothelial progenitors	Rat myocardial infarct model	Rufaihah *et al.*[65]
Cardiac	Adenovirus	hVEGF	Human skeletal myoblasts		
Neural	Herpes simplex virus type-1	rat HGF	Rat marrow stromal cells		
Viral vectors for *in vivo* regeneration					
Bone	Adenovirus coated on allograft	BMP-2	C3H10T1/2 cells for in vitro		
Eye	Recombinant adenovirus 2/2	RPE-specific 65-kDa protein			
Non-viral vectors for *ex vivo* cell engineering					
Bone	Nucleofection with plasmid	Recombinant hBMP-6	Primary porcine adipose tissue-derived stem cells		
Cardiac	PEI nanoparticle	Hypoxia-regulated VEGF	Rabbit skeletal myoblasts		
Non-viral vectors for *in vivo* regeneration					
Bone	Liposomal vector	BMP-2			
Wound healing	Peptide-DNA condensates in fibrin gel	HIF-1α			
Wound healing	Poly(β-amino ester) nanoparticle	Human sonic hedgehog			

TABLE 1. continued.

Application	Animal model	References
Cardiac	Porcine heart model of chronic infarction	Haider *et al.*[24]
Neural	Intracerebal transplantation into rat's ischemic brain	Zhao *et al.*[92]
Viral vectors for *in vivo* regeneration		
Bone	Femoral allograft surgery on female mouse	Yazici *et al.*[89]
Eye	Subretinal injections into young adult patients	Bainbridge *et al.*[2]
Non-viral vectors for *ex vivo* cell engineering		
Bone	Injection into lumbar paravertebral muscle in mouse	Sheyn *et al.*[71]
Cardiac	Female rabbit with acute myocardial infarction model	Yilgor *et al.*[90]
Non-viral vectors for *in vivo* regeneration		
Bone	Transplantation into peri-implant bone defects on pig calvariae	Park *et al.*[55]
Wound healing	Intradermal implantation into mouse	Trentin *et al.*[81]
Wound healing	Intradermal delivery into mouse	Park *et al.*[54]

that may overlap, greatly increasing complexity. Although many of the techniques discussed in this article can be used for temporal and spatial control of delivery in a broad sense, they generally do not control *in vivo* delivery with the cellular and subcellular spatial resolution and second-scale temporal resolution that may be in many ways ideal. Coupled with this engineering limitation is the basic science limitation of not having precise knowledge about these precise spatial and temporal requirements. Despite the use of various computer-controlled 3D cell printing techniques, spatiotemporally controlling multiple microenvironments within close regions remains a challenge. Newer techniques involving computational topology design and solid free-form fabrication help alleviate some disadvantages, yet the remaining challenges include but are not limited to vascularization, host integration, resolution and porosity, and the seeding and co-culturing of multiple cell types.[28,68] Furthermore, the mechanisms by which complex tissues are orchestrated to develop and heal are largely unknown.[57] Therefore, there are opportunities in the field for the invention of higher resolution *in vivo* biosensing technologies, extracellular and intracellular drug delivery technologies with increased spatial and temporal control, and cell delivery and scaffold technologies with increased spatial control and organization of diverse cell types.

FUTURE DIRECTIONS

In this manuscript, we have discussed the work of many world leaders involved in delivering biomolecules for regenerative medicine applications. The different types of delivery include extracellular delivery of soluble biomolecules, either as a bolus or through controlled release systems; delivery of insoluble factors, often through biomaterial-based scaffolds; and intracellular nucleic acid delivery to program a target cell on a genetic level. Examples of applications are in diverse areas of regenerative medicine such as tissue engineering of bone,[30,38] cartilage, muscle, blood vessels,[17,29,87] the heart, and the eye, neuroengineering,[67] and wound healing (Fig. 5; Table 1).

Many of the future directions in this field are associated with delivery that is engineered to be more biomimetic. This includes more precise spatial and temporal control of delivery as well as sequential delivery of multiple factors in the manner that best mimics natural healing mechanisms. Future directions include greater investigation and characterization on the microscale and nanoscale of the microenvironment in developing and healing tissues as well as creating synthetic bioengineered microenvironments that successfully reproduce the biological complexity of natural tissues including controlled release of the needed insoluble and soluble biomolecules. Regulatory hurdles for cellular, tissue, and gene therapies are in many ways more complicated than for small molecules due to the added safety concerns associated with cellular materials. For example, characterization and purity of cells is critical, but as cell populations can often contain heterogeneity, ensuring purity and homogeneity of cells and their combination with biomaterials and signaling biomolecules in a precisely controlled way is a future direction of the field. Cell fate *in vivo* and ensuring that any delivered cells do not differentiate, proliferate, or migrate in an unintended manner is key as well for both safety and efficacy. These concerns make the pathway from discovery of a new regenerative medicine therapy on the bench to translation in the clinic more tortuous.

In the past few years research efforts developing highly specific genome editing tools such as zinc nuclease fingers could possibly open the doors for safer, more efficacious methods to control gene expression to promote regeneration.[4,40,47,59] Zinc nuclease fingers alleviate some complications more traditional types of gene vectors face such as insertional mutagenesis, immune reactions, and high long-term expression. Methods to

pattern topologies[3,13] for spatially controlled protein expression and presentation of biomolecules, as well as gene switches[14,66] which are able to turn on and off in the presence or absence of a molecule will be invaluable for engineering spatiotemporally controlled materials for regenerative applications. Regenerative medicine has enormous potential to treat many areas of medicine and as new basic discoveries are made and new bioengineered technologies invented, therapeutic modalities move closer to helping patients.

CONCLUSIONS

For bioengineered delivery, it is key that needed factors are provided to the right cells at the right time and place to properly control cell and tissue function. Engineering a cell and its microenvironment can be accomplished from the outside-in *via* delivery of soluble and insoluble factors to the outside of a cell as well as from the inside-out *via* gene transfer using both viral and non-viral methods. Delivery combinations of biomolecules, scaffolds, and cells are promising approaches as *in vivo* regenerative medicine therapeutics for a variety of applications.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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