Non-viral gene-activated matrices

Next generation constructs for bone repair

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In the context of producing enhanced therapeutics for regenerative medicine, our laboratory develops gene-activated matrices (GAMs) using non-viral gene therapy (GT) in combination with collagen-based scaffolds engineered specifically for tissue repair. Non-viral vectors have been referred to as a minority pursuit in GT but considering the concerns associated with viral vectors and as transient gene expression is such a key consideration, further research is clearly warranted for tissue engineering (TE) applications. Mesenchymal stem cells (MSCs) are well regarded for their capability in bone regeneration but as primary cells, they are difficult to transfect. We have recently optimised the non-viral vector, polyethyleneimine (PEI), to achieve high transfection efficiencies in MSCs. Subsequently, a series of PEI-based GAMs were developed using collagen, collagen-glycosaminoglycan and collagen-nanohydroxyapatite (collagen-nHa) scaffolds whereby transgene expression was detected up to 21 d with the collagen-nHa scaffold providing the most prolonged expression. Moreover, all PEI-based GAMs contained a low plasmid DNA dose of 2 μg which is far below doses often required in previous GAMs. Having successfully developed these GAMs, the ephrinB2 gene has recently been incorporated to produce a novel therapeutic GAM for bone repair. Herein, we discuss our recent investigations in the development and application of non-viral GAMs.

Seeking to revolutionise treatment options, the field of tissue engineering (TE) aspires to 'create new tissue for the therapeutic reconstruction of the human body'.[1] Over the years, many advancements have been made in TE but most success has been in the regeneration of 'soft' tissues such as skin,[2,3] urethra,[4] esophagus[5] and trachea.[6,7] It is quite striking that in 2013, the implantation of bone grafts harvested from a patient or donor, still remains the gold standard treatment strategy for bone repair. Needless to say therefore, a substantial and unmet need for the design of TE therapeutics for intricate and mechanically strong tissue types such as bone and cartilage still exists.

Researchers in the bone TE field often concentrate their efforts on developing a 3D environment, or scaffold, filled with biological, structural and mechanical cues, in the hope of coaxing cells to lay down the matrix of a desired tissue type.[8] A series of collagen-based scaffolds including collagen and composites of collagen-glycosaminoglycan (collagen-GAG),[9-11] collagen-hydroxyapatite[12,13] and collagen-nanohydroxyapatite (collagen-nHa)[14] have been developed for bone repair in our laboratory. The collagen-GAG scaffold was originally created for skin regeneration by Yannas et al.[2] and subsequently engineered in Yannas' laboratory to contain a homogeneous pore structure suitable for bone repair by O'Brien et al. in 2004.[15] Since then, a number of further enhanced, bone-targeted, novel collagen-composite scaffolds have been produced by our group including the collagen-nHa scaffold which will be discussed primarily in this commentary.[14,16,17] Previous work within our laboratory has demonstrated the ability

of a cell free collagen-GAG scaffold and a mesenchymal stem cell (MSC) seeded collagen-GAG scaffold to induce bone formation in a rat cranial defect.[18-20] More recently, a collagen-hydroxyapatite composite scaffold has shown good bone healing after 4 weeks resulting in a completely bridged critical defect.[12] However, for very large bone defects, an extra agent, i.e., in the form of cells or biomolecules, such as therapeutic proteins or genes, is needed where the scaffold alone is insufficient in providing complete healing. With that in mind, although the cell and biomolecule-free bone graft substitutes developed in our lab have produced commendable results to date, we sought to produce next-generation scaffolds by combining them with a gene therapy (GT) approach to further enhance the therapeutic potential of these constructs.

As the name suggests, GT involves the transfer of therapeutic genes, usually in the form of plasmid DNA, for therapeutic purposes. In somatic GT, 'faulty' genes are replaced with healthy alleles to reverse a monogenic disease. In the early days, GT was conceived as a 'last port of call' treatment for lethal single gene disorders and researchers predicted that somatic GT would become commonplace within a decade.[21] However, the emergence of GT as a fully fledged area of medicine has been slow and paved with disappointments and retraction of investment along the way.[22-24] In spite of the relatively few success stories and tragically the occurrence of some fatalities, the number of GT trials underway affirms that there is continued interest and belief in the promise of GT.[25] Interestingly, Glybera, a product that treats the single gene disease lipoprotein lipase deficiency (LPLD), was the first GT product approved by the European Commission in November 2012 making it the first GT product approved by the regulatory bodies in the Western world.[26] This is no doubt a monumental and encouraging outcome for the fields of gene and cell therapy. In addition to treating rare monogenic genetic diseases, GT also holds a lot of promise in the field of TE. Unlike GT for the treatment of single gene diseases where long-term expression and integration of the delivered transgene may be required for effective treatment, there is

no need for constitutive expression in GT for TE applications. A sustained but transient expression of the transgene is more beneficial. In applying GT to TE applications, a vector is necessary to deliver the DNA load to a target cell and transient expression of the genes can be achieved using adenoviral or non-viral vectors.[27-29]

To date, the vast majority of GT research has utilized viral vectors to deliver therapeutic proteins to target cells.[30] Orthopedic GT research has been ongoing for approximately two decades but has not progressed to clinical trials for a number of reasons most of which likely hinge on the unsafe perception of the viral vectors used. Fatal clinical trials involving GT and viral vectors for monogenic disorders have occurred which detracts investment and heightens regulatory attention.[31,32] On the other hand, although they have been referred to as a minority pursuit in GT in general,[22] non-viral vectors might offer a safe and valid alternative and may lead the way to successful clinical translation of orthopedic GT. Considering the concerns associated with using viral vectors and as transient gene expression is such a key consideration, many researchers would agree that non-viral vectors should be the primary choice for GT applications in TE.[33,34] Non-viral vectors are designed to essentially mimic the cell-entry abilities of their viral counterparts.[35] Non-viral gene transfer methodologies offer several advantages, all of which are independent to individual vectors. They can exhibit low immunogenicity, low toxicity, high transfection efficiency, larger plasmid loads, low production cost and they do not insert into the host genome so insertional mutagenesis does not pose a risk.[34] Non-viral vectors also cause a desirable temporary but sustained release of protein from the transfected cell. Simply, therapeutic protein production is temporarily sustained for a limited timeframe after which it subsides. For the aforementioned reasons we opted to develop non-viral gene-activated matrices (GAMs) targeted at bone repair.

Although not new, the idea of a gene delivery vector contained in a biodegradable scaffold is an innovative development in TE.[36] GAMs for bone repair were initially conceived as off-the shelf, non-viral, gene-containing scaffolds in the late

1990s.[36,37] The scaffold essentially acts as a depot for the gene while simultaneously offering structural support and a matrix for new tissue deposition (**Fig. 1**). GAMs can instruct cells to follow a certain lineage in vivo by the single application of the GAM to the defect. Once the construct is in place, there is no need for repeat administration and the in vivo response brought about by the release of the therapeutic protein from cells on the GAM governs effective healing. An important concern in TE is the spatiotemporal delivery of therapeutics from the tissue engineered construct.[38] Sustained delivery mechanisms stand to increase the therapeutic potential as the proteins are present in the defect for a longer period of time to elicit a therapeutic effect. GAMs offer sustained release of the protein as the transfected cells continually secrete protein over time. The scaffold can be treated as a depot whereby the DNA complex stays adhered to the scaffold and infiltrating cells become transfected as they pass through the GAM (**Fig. 1**).

In terms of utilizing non-viral GAMs for the repair of musculoskeletal tissues other than bone, little work has been performed to date in these areas. Although viral vectors have been used to deliver genes to tendons and ligaments,[39] there are no reports on the use of non-viral GAMs. The majority of studies have focused on the repair of articular cartilage possibly due to the ease of access to this tissue. However, due to its relative acellularity, complete avascularity and aneural composition, repairing this complex tissue requires a multistep methodology that necessitates further research to truly reach its full potential. Some studies of note that have described success to date however include a study by Madry et al.[40] where FuGENE 6 was used to successfully deliver insulin-like growth factor 1 (IGF1) gene to lapine articular chondrocytes in an alginate gel suspension delivery system in osteochondral defects demonstrating augmented cartilage repair. Chitosan-gelatin scaffolds have also been utilized to deliver naked transforming growth factor β1 (TGF-β1) plasmid demonstrating enhanced cartilage tissue regeneration within the GAM-treated group compared with controls.[41] Alginate/chitosan polysaccharide capsules have been used to deliver
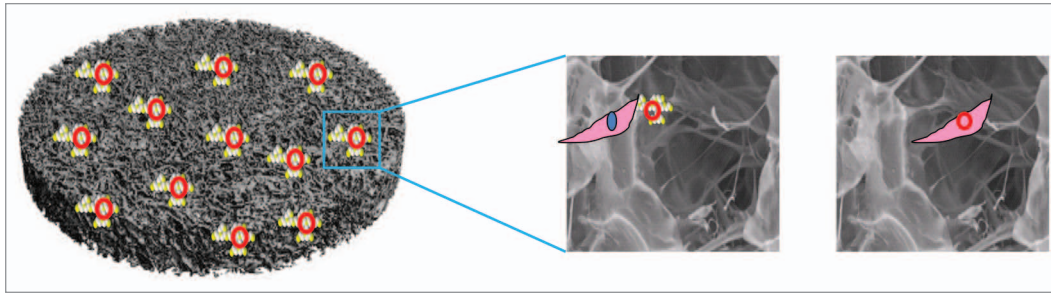
Figure 1. Transfection in gene-activated matrices (GAMs). The scaffold acts as a depot for vector-gene complexes and upon seeding with MSCs, the complexes are taken up and the transgene becomes expressed. The scaffold also retains its key functional role in promoting cell infiltration and as a template for tissue repair.

Sox-9 plasmid DNA to human mesenchymal progenitor cells through the use of the non-viral method nucleofection. In vivo results demonstrated enhanced chondrogenesis within the transfected group compared with untransfected controls.[42] A study by Gelse et al.[43] compared the use of non-viral liposome transfection to that of adeno-associated virus (AAV) and adenovirus (Ad) when delivering BMP2 to chondral lesions on PGA scaffolds but demonstrated inferior results using the non-viral delivery method compared with that of the viral method in terms of cartilage repair. Most recently, Kayabasi et al.[44] have delivered the BMP6 gene to rat MSCs using Lipofectamine on chitosan scaffolds and demonstrated some promising chondrogenic results in vitro. The limited number of non-viral GAM studies performed on the repair of these other musculoskeletal tissues demonstrates that the systems proposed within this review may have the potential to be applied for use in these orthopedic applications and furthermore, they may also have the capacity to be utilized for the regeneration of numerous other tissues.

MSCs are well regarded for their tri-lineage differentiation into bone, cartilage or adipose cells when provided with appropriate cues and their capability in bone regeneration has been extensively documented.[45,46] The lack of an ideal vector for MSC transfection is a significant hurdle in the translation of GT and regenerative medicine. In the recent study from our lab published in the *Journal of Controlled Release*, polyethyleneimine (PEI) was shown to be capable of highly efficient transfection of MSCs.[47] PEI has been previously reported as an efficient transfection

tool in cell lines[48,49] and a small number of stem cells including adipose-derived stem cells and bone marrow-derived MSCs.[50,51] However, as primary cells, MSCs are notoriously difficult to transfect and the highest transfection efficiency previously reported is 19%. By analyzing a number of physical parameters, cell viability and transfection efficiency, across a number of PEI-DNA N/P ratios (ratio of amines in PEI to phosphates in DNA), PEI was optimised for MSC transfection in this study. It was demonstrated that MSCs can be transfected with a transfection efficiency of between 30 and 45% depending on the N/P ratio used.

Upon optimising PEI for MSC transfection, a series of PEI-based GAMs were then developed. It was shown that when merged with collagen, collagen-GAG and collagen-nHa scaffolds, PEI-DNA polyplexes could successfully transfect MSCs. Moreover, all PEI-based GAMs developed contained a low plasmid DNA dose of 2 μg which is far below the higher doses in the order of milligrams which were often required in earlier GAMs.[36,50,52] More specifically, it was also found that scaffold composition can affect transgene expression in the GAMs. While all scaffolds proved capable of successful MSC transfection, the collagen-nHa scaffold demonstrated the highest prolonged levels of gene expression (**Fig. 2**). Temporarily sustained transgene expression was evident in all collagen-based GAMs but the collagen-nHa scaffold resulted in a more prolonged duration of transgene expression over a 14 d period. Similar results were also found in another study from the group by Curtin et al.[17] where sustained transgene expression was more prolonged

on collagen-nHa scaffolds compared with collagen alone scaffolds. Although an investigation of the exact reasons for this was outside the scope of the study, a number of theories are proposed as to why this occurred. First, it may be attributed to the fact that collagen-nHa scaffolds are stiffer substrates than the collagen alone and the collagen-GAG scaffolds. Enhanced gene expression has previously been observed on stiffer biomaterials[53] so it could simply be down to the mechanical strength of the material. It is also possible that it may be attributed to the manner in which the polyplexes are attached to the nHa scaffold. Polyplexes may be bound to the scaffold in one of two possible ways, either by adsorption to the collagen or to the nHa. If the cells preferentially attach to the nHa itself it could explain the higher transfection efficiencies in the nHa-based GAMs. Also, cells respond to their surrounding environment via focal adhesions on the cell surface[11,53,54] and mineralised substrates have been shown to stimulate focal adhesion, MSC motility and migration throughout the matrices.[55] Therefore, it may be that the collagen-nHa scaffold results in the adherence of more cells than the collagen alone and collagen-GAG scaffolds due to increased focal adhesion if the MSCs preferentially attach to nHa. Taken together, these results support the overall hypothesis that the elevated and prolonged levels of gene expression seen in the collagen-nHa scaffolds is likely attributable to increased cellular attachment and mobility in the GAM, meaning that the cells encounter more PEI polyplexes than cells on mineral-free scaffolds contributing to more transfection.

This GAM development study has made a significant contribution to the field of GT and TE. PEI was specifically optimised for MSCs, the target cell in vivo, and combined with a scaffold which was engineered expressly for bone tissue repair to produce a superior construct for TE applications. These GAMs contain a mere 2 µg of polyplex which is a significant reduction on former quantities used thereby decreasing cost and decreasing the quantities of exogenous materials in the injured site. While the GAMs we have developed may have functions in a whole host of applications, the performance of the nHa scaffold is very interesting from a bone repair outlook as nHa has dual benefits in that it prolongs gene expression and has osteoinductive qualities itself.[17] Ultimately, the application of this GAM would involve replacing the reporter genes with therapeutic genes. This goal has since been achieved in the interim by incorporating plasmid DNA encoding the ephrinB2 gene.[56]

Ephrin ligands and their cognate receptors are involved in governing many cellular processes from cell morphology to vasculogenesis and cell migration.[57,58] Of most relevance to orthopedic applications, it has been shown that some ephrin ligands and receptors, namely ephrinB2 and EphB4, are involved in bone remodelling.[59,60] Bidirectional signaling between an osteoclast expressing ephrinB2 and an osteoblast expressing EphB4 triggers osteoblast differentiation and obstructs osteoclastogenesis.[59-61] The field of biomaterials is currently in the midst of a revolutionary change where progressions in the life sciences are of equal importance to the development of novel biomaterials.[62] The newly discovered role of ephrinB2 and EphB4 interactions in bone prompted the investigation of the overexpression of the ephrinB2 ligand in a GAM in a follow on paper which was also published in the *Journal of Controlled Release* in 2013.[56]
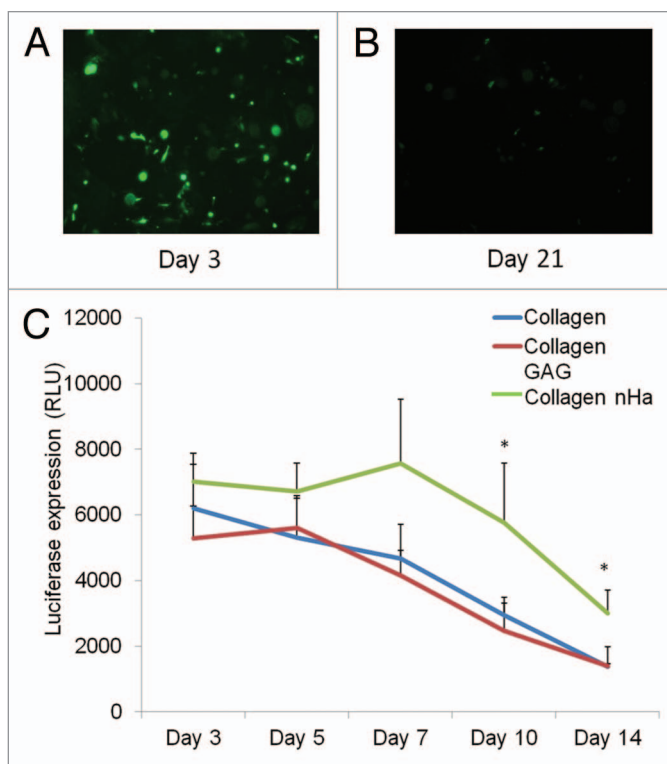
First, in this study, it was shown that ephrinB2 overexpression increases osteogenesis in monolayer human MSCs which was dependent upon an interaction with the EphB4 receptor presented on the surface of adjacent cells. This led to the establishment of a novel PEI-ephrinB2 GAM specifically tailored for bone repair which

Figure 2. Scaffold composition affects gene expression duration in collagen-based GAMs. The images depict MSC transfection within PEI-GFP collagen-GAG GAMs loaded with 2 µg N/P 10 PEI-GFP polyplexes 3 (**A**) and 21 (**B**) days after cell seeding on the matrices. PEI-luciferase collagen-based GAMs were fabricated where luciferase expression was shown to be elevated for a prolonged timeframe in the collagen-nHa GAM. Data plotted shows mean standard deviation (n = 3) and p < 0.05.

enhances osteogenesis, and as we know from the literature, also has the potential to inhibit osteoclastogenesis.[59] The potential pro-anabolic, anti-catabolic scope of PEI-ephrinB2 GAMs in bone regeneration is outlined in **Figure 3**. Furthermore, the original paper detailed successful transient overexpression of the transgene in rat MSCs and in the subsequent paper it was verified that the GAMs had translational capabilities when human MSCs were seeded in place of rat MSCs.

The PEI-ephrinB2 GAM is the first GAM for bone repair which incorporates the ephrinB2 gene and within just 14 d of MSC seeding—a very early timepoint—enhanced osteogenesis was already observed in the GAMs. The finding that ephrinB2-mediated osteogenesis was reliant on EphB4 interaction contributed to the idea that a PEI-ephrinB2 GAM may possess great potential as a select matrix for bone repair. Cytokines such as BMP2 and VEGF can elicit off-site effects and high doses can trigger bone resorption
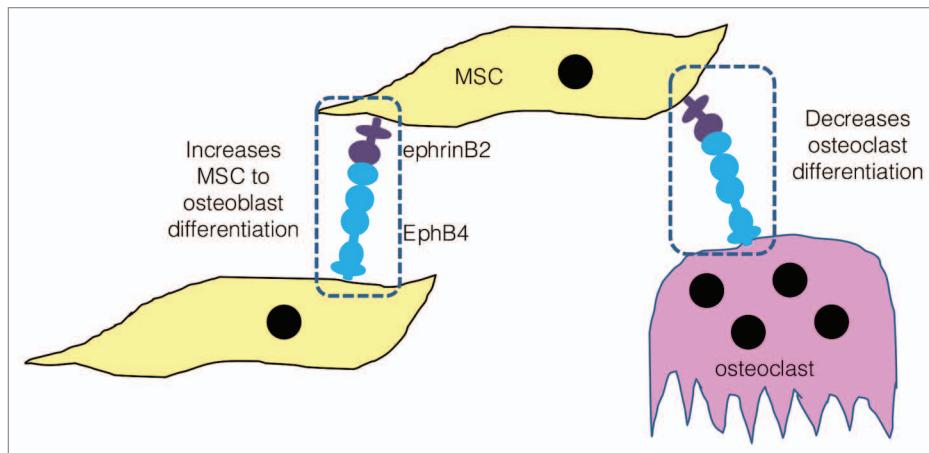
and osteolysis.[63] The secondary requirement for EphB4 interaction means that offsite effects such as these are completely minimised and effects are only exercised in EphB4-expressing cells. The osteogenic enhancements witnessed in monolayer culture and mirrored in the PEI-ephrinB2 GAM provides evidence of the great potential of this therapeutic for in vivo translation.

The discovery that ephrinB2 overexpression enhanced osteogenesis in MSCs was an unusual finding which suggests that ephrin/Eph signaling may be involved within the MSC population itself, independent to previously reported interactions with other cell types. In itself, this implies that ephrinB2-MSCs could be a valuable modification to MSCs utilized in bone repair strategies. Although ephrinB2 has previously been shown to increase angiogenesis in MSCs,[64] this study has shown that very high levels of ephrinB2 overexpression can actually trigger osteogenesis. This result prompted the theory

Figure 3. EphrinB2 GAMs have the potential to exert a pro-anabolic, anti-catabolic effect on bone formation. EphrinB2 overexpression stands to enhance bone formation by two mechanisms, first by increasing osteoblast differentiation and second by blocking osteoclast differentiation.

that ephrinB2 expression is multi-functional with levels of overexpression critical to the therapeutic outcome. Moreover, ephrinB2 can be hypothesized as a 'one size fits all gene' for bone repair in that it has previously been shown to increase angiogenesis,[64] to inhibit osteoclastogenesis[59] and finally, in this successive paper, to increase osteogenesis in human MSCs.

The collagen-nHa scaffold used in the development of these GAMs has also been used in the development of other GAMs in our research group as described earlier. In a study published in 2012 in *Advanced Materials*, Curtin et al.[17] demonstrated that the nHa particles themselves could be used as vectors for successful MSC transfection when the nano-sized Ha particles were co-precipitated with DNA to produce particles of approximately 100nm which could easily cross the cell membrane via endocytosis.[65] A sustained but transient transgene expression profile was noted in these nHa-based GAMs using reporter genes. Furthermore, when the reporter genes were substituted for the osteogenic gene BMP2, significantly enhanced osteogenesis was observed in nHa-BMP2 GAMs upon seeding with MSCs.

Taken together, the work discussed from these publications by our research group provides key support for the use of non-viral vectors in GT-related bone repair indicating that they should not be overlooked in the development of new therapeutics. The combination of gene therapy with scaffolds engineered for tissue regeneration has the capability to provide great potential in orthopedics and other disciplines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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