Engineered cell-free scaffold with two-stage delivery of miRNA-26a for bone repair

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Abstract: The treatment of non-unions and bone defects is a major challenge. In these situations, autologous bone is the preferred treatment but has several serious limitations. Treatment alternatives including the use of calcium-based scaffolds alone or associated with either growth factors or stem cells have therefore been developed, or are under development, to overcome these shortcomings. Each of these are, however, associated with their own drawbacks, such as the lack of sustained/controlled delivery system for growth factors and poor cell survival and engraftment for stem cells. MicroRNAs (miRNAs), a class of small noncoding RNAs fine-tune the expression of as much as 30% of all mammalian protein-encoding genes. For instance, miRNA26a is able to promote the repair of critical-size calvarial bone defects. Yet, the clinical application of these fascinating molecules has been hampered by a lack of appropriate delivery systems. In an elegant report entitled *cell-free 3D scaffold with two-stage delivery of miRNA-26a to regenerate critical-sized bone defects*, Zhang *et al.* 2016, developped a non-viral vector with high affinity to miR-26a that ensured its efficient delivery in bone defects. Engineered scaffolds were able to induce the regeneration of calvarial bone defects in healthy and osteoporotic mice. Taken together, these data pave the way for the development of advanced bone substitutes that at least will match, and preferably supersede, the clinical efficiency of autologous bone grafts. However, the transfer from the bench to the bedside of such scaffolds requires further investigations including (I) a better understanding of the underlying biological mechanisms involved in bone formation via miRNA26a; (II) evidences of polymer scaffold biocompatibility upon its complete degradation; and (III) demonstration of the engineered scaffold functionality in defects of clinically relevant volume.

Keywords: Bone; microRNAs (miRNAs); scaffold; bone grafts

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The treatment of non-unions and bone defects are a major challenge in orthopaedic, maxillo-facial and neurosurgeries. Segmental bone defects after trauma or tumor surgery do not heal spontaneously, and need special reconstruction techniques which are very expensive, take a long time to heal and have unpredictable results. In these challenging situations, autologous bone is the preferred treatment. It consists in taking bone from a donor site (iliac crest or fibula for the most common sites), and grafting it into the bone defect (1). Grafts of this kind are osteoconductive (they provide a scaffold on which bone cells can proliferate), osteoinductive (they induce undifferentiated cells proliferation and their differentiation into osteoblasts), and osteogenic (they provide a reservoir of skeletal stem and progenitor cells that can form new bone). Since autologous bone grafting has several serious limitations [such as limited volume of bone in the donor site, longer surgery, donor site morbidity, possible infections and residual pain, which affect up to 30% of the patients (2)], it has become necessary to develop alternative techniques (3). For these reasons, surgeons use banked bone and natural or synthetic calcium-based ceramics. These bone substitutes are available in almost unlimited quantities and are osteoconductives. They act as a scaffold for the ingrowth of neovasculature and

directional migration of osteogenic precursor cells from the surrounding tissues into the defect site. In these scaffolds, bone formation depends on the existence of osteocompetent cells in the scaffold vicinity and is limited to scaffold periphery when addressing large bone defects.

To overcome these limitations, *in vitro* expanded mesenchymal stem cells [also referred as multipotent stromal cells (MSCs)] have been combined with porous scaffolds with the hope that these cells could either form new bone or enhance functions pertinent to new bone formation (4). The proof of concept of such strategy has been performed in clinically-relevant animal models and demonstrated that MSCs significantly enhanced bone formation (5-8). However, the osteogenic capability of these tissue constructs did not match the one of autologous bone grafts. Alternatively, bone morphogenetic proteins (BMPs), a group of growth factors, have been used to favor bone repair. These molecules, which were originally discovered for their ability to induce bone formation, have been used in clinical settings for bone regeneration and repair since the last decade (9). However, the clinical experience using such compounds has not met expectations. In fact, despite their excellent osteoinductive potential, their use is currently strongly controversial because it has been encumbered by numerous and severe clinical complications (10). In conclusion, the results obtained with bone substitutes alone or supplemented with MSCs or growth factors are encouraging but further investigations are needed to provide clinicians effective novel therapeutic alternative modalities that at least match, and preferably supersede, the clinical efficiency of autologous bone grafts.

MicroRNAs (miRNAs) are a class of small, highly conserved, noncoding RNAs of 19–25 nucleotides, which exist widely in eukaryotes (11). After binding to 3'-untranslated regions (3'-UTR) within a target mRNA, miRNAs play a negative role in gene expression by regulating transcript localization, polyadenylation, and translation (11-13). A single miRNA is often involved in several gene regulatory networks. For instance, miR-20a, miR-29b, miR-2861, miR-138, miR-26a, and miR-21 are important regulators of osteoblastic differentiation [for review, introduction of (14)]. Most importantly, the repair of critical-size calvarial bone defects is promoted via the positive regulation of angiogenic-osteogenic coupling using miRNA26a (14). In short, miRNA therapies, similarly to BMP therapies, have two main advantages (I) an "off the shelf" availability and (II) circumvention of a secondary surgery that make them appear as promising treatment strategies for bone repair. Yet, their clinical application has been hampered by a lack of appropriate delivery systems.

In an elegant report entitled *cell-free 3D scaffold with two-stage delivery of miRNA-26a to regenerate critical-sized bone defects*, Zhang *et al.* 2016, developed a non-viral vector with high affinity to miR-26a that ensures its efficient delivery in bone defects (15). To this aim, a vector with short polyethylene glycol (PEG) chains and a low molecular weight cationic polyethylenimine attached to the outer shell of a hyperbranched hydrophobic polyester core was designed. In the presence of miRNA, this hyperbranched polymer vector self-assembled into a nano-sized spherical shell sandwiched between the inner and outer hydrophilic PEG layers. These structures (referred thereafter as polyplexes) exhibit an average diameter of 224 nm. Their release was further controlled by encapsulating them via the double emulsion method in 3 µm biodegradable PLGA microspheres. Scanning electron microscopy studies revealed that the delivery of these polyplexes from microspheres (referred also as the first stage delivery) occured as nanoparticules with almost no morphological discernible changes when compared to genuine polyplexes. Release profiles of miRNA from PLGA microspheres containing polyplexes showed that, in the best case scenario, a burst release of polyplexes followed by a sustained release of polyplexes for longer than a month was achieved. The delivery of miRNA into cells by polyplexes (referred also as the second stage delivery), was assessed using osteoblasts. Data showed that optimized miRNA polyplexes exhibited (I) higher transfection efficiencies and (II) higher miRNA expression inside cells than state of the art polymeric vectors for therapeutic miRNAs. To further spatially control miRNA release, microspheres containing miR-26a polyplexes were immobilized on a nanofibrous cell-free three-dimensional scaffolds. These novel scaffolds were able to induce the regeneration of calvarial bone defects in healthy and osteoporotic mice.

Taken in the broad context of the field, this report is important for several reasons. Firstly, the report by Zhang *et al.* 2016 (15) confirmed previous works by Li *et al.* 2013 that appropriate delivery of miR-26a promoted bone repair in critical-size calvarial defects in rodents (14). Secondly, this report proposed a non-viral vector with very high miRNA binding affinity and negligible cytotoxicity suitable for critical-size bone defect regeneration. Such technology may deliver other therapeutic nucleic acids including DNAs, RNAs, siRNAS, miRNAs and so on. Thirdly, and most interestingly, engineered scaffolds for

miR-26a release exhibited no osteoinductive capacities when implanted in ectopic bone site. These observations are critical as they suggest that such ectopic bone formation is unlikely to occur upon implantation of these engineered scaffolds. If verified in clinically relevant animal model, these observations would confer a major advantage to miR-26a when compared to BMP which have induced at time undesirable ectopic bone formation in clinical studies.

As the field of bone engineering with miRNA moves forward, a detailed elucidation of the cellular and molecular processes that govern the formation of new bone via miR-26a, will be pivotal for further enhancing outcomes in bone repair and ensuring safety. In their report, Zhang X 2016 proposed that the osteogenic action of miR-26a rely on the up-regulation of osteoblast activity through functional targeting of glycogen synthase kinase-3beta (GSK-3beta). Consistent with this hypothesis, GSK-3beta has a miR-26a-binding site in its 3' untranslated region (UTR), and its inhibition improves bone mass and significantly increases mineral apposition rate and bone mineral density in ovariectomized mice (16,17). Nevertheless, upon implantation, engineered scaffolds tend to be subject not only to bone cells but also to inflammatory and blood-forming cells infiltration (18). How the inflammatory and blood-forming cells are regulated by miR-26a and how they contribute to the osteogenesis process remains to be determined.

Optimal matrices for bone regeneration must degrade at a rate commensurate to bone formation and their degradation products should not be toxic. In the report, PLGA microspheres encapsulating miR-26a polyplexes were attached to nanofibrous PLLA scaffold. It is known that PLLA and PLGA are hydrolytically degradated through de-esterification and their monomeric components are removed by highly regulated natural pathway (19). However, PLLA and PLGA degradation products may reduce the local pH value, which in turn, may accelerate the polyesters' degradation rates (20) and induce an inflammatory reaction. The authors claimed that the acid release is not of a significant concern for polymer scaffold with high porosity as used in their study. A downside of the Zhang *et al*. 2016 study is that no attempt were made to measure the extent of scaffold resorption and that scaffold remnants were still observed on histological sections at 2 months (15). Long-term experiments are necessary to elucidate the fate of the newly formed bone upon scaffold resorption and demonstrate the neutrality of scaffold degradation products towards bone formation. Along the same lines, experiments with scaffolds of clinical relevant volumes will be necessary for two reasons: (I) adverse effects due to scaffold degradation might be maximized when addressing the repair of defects of larger volumes; (II) repairing large bone defects may require the use of supraphysiological dose of miR-26a that may trigger undesirable outcomes.

Although these data provide an exciting proof of concept for an attractive alternative to the use of BMPs, application of such system to humans may not be straightforward and require at least a demonstration of its therapeutical potential in large animal models in defects of clinically relevant volume. Last but not least, in order for these engineered scaffolds to find their place in the armentarium of the surgeon, cost/benefit analysis of this new approach are needed if it is to be funded and fully exploited within the current, tough constraints of healthcare budgets.

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Footnote

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