Novel chemically modified RNA encoding for Nerve Growth Factor shows therapeutic potential to induce innervation for bone regeneration *in vitro*

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INTRODUCTION: Bone is a highly innervated and vascularized tissue. While most research efforts take vascularization into account, focusing on engineering vascularized bone tissue, the role of innervation in bone tissue engineering has often been disregarded. In recent years it has become evident that such innervation activities have an important role in the regulation of bone homeostasis, stimulating bone formation and inhibiting resorption (1). Relevantly, during bone fracture healing, the formation and outgrowth of nerve fibers is a highly important process. These nerve fibers are distributed throughout skeletal tissue, crucial for sensing and responding to stimuli such as mechanical load. Bone morphogenic protein 2 (BMP-2) is commonly used for bone tissue regeneration. However, this growth factor does not induce the formation of nerves. Thus, identifying nerve growth cues, intrinsically expressed during bone healing may be advantageous. Our group has pioneered the use of a new, highly advantageous form of gene therapy, i.e., transcript therapy, for bone tissue healing. Within our research group, this new strategy has previously been utilized to induce the healing of segmental bone defects in rats by delivery of BMP-2 coding, chemically modified messenger RNA (cmRNA) incorporated into a collagen sponge (2). With the view of providing necessary nerve growth cues during bone healing, the aim of the present study is to develop a cmRNA-based strategy to stimulate innervation in the context of bone healing. Specifically, we used a nerve growth factor (NGF) coding sequence and several structural modifications to design a cmRNA capable of inducing NGF expression upon cellular uptake with minimal toxicity.
METHODS: cmRNAs were produced by in vitro transcription (IVT) and carefully characterized before use (2,3). Modifications performed to the mRNA included the use of chemically modified ribonucleotides (i.e., 5-methylcytidine-5'-triphosphate and 2-thiouridine-5'-triphosphate or 5-iodo-uridine and 5-iodo-cytidine) during IVT. cmRNAs coding for eGFP and *Metridia luciferase* were produced and used as reporter systems. As functional cmRNA, NGF coding cmRNA was produced and evaluated. Lipoplexes of all cmRNAs were formed that were assayed for transfection efficiency using bone marrow mesenchymal stem cells (BMSCs) and PC-12 cells (embryonic origin from the neural crest). A co-culture of BMSCs and PC-12 cells was established and transfected with NGFcmRNA. NGF production by BMSCs was evaluated by ELISA. The effect of NGF secretion on PC-12 differentiation was assessed by microscopy. Relevant parameters (e.g., neurite length and area, and number of branchpoints) were calculated. In addition, Phalloidin 488 and β3-Tubulin staining was performed at different times of observation post co-culture of PC-12 and NGFcmRNA-transfected BMSCs. In order to reveal which pathways caused neurite outgrowth in PC-12 cells, bottom-up label free proteomics was performed. For this, cell lysates and supernatants of single as well as co-cultures were used. Cell lysates were used to analyze the proteome of cells while supernatants were used to assess the secretome. The outcome of the liquid chromatography-mass spectrometry data was compared to databases using Proteome Discoverer 2.2 Sequest HT search engine (Thermo Scientific). Statistical analysis was performed using either one-way or two-way analysis of variance (ANOVA) following the GraphPad Prism software recommendations (GraphPad V10.0).
RESULTS SECTION: BMSCs were successfully transfected *in vitro* with lipid particles containing NGFcmRNA. A dose-dependent uptake and protein production was observed. NGF expression peaked at 24 hours post-transfection, and remained significantly high up to 5 days in culture. cmRNA transfection of the cells did not affect their viability and capacity to proliferate *in vitro*. When co-cultured with PC-12, NGF produced by BMSCs was shown to have a beneficial impact on PC-12 differentiation towards the neuronal phenotype. Stimulated PC-12 showed prominent neurite sprouting. This resulted in being highly dose-dependent, with doses of 31.2 and 62.5 pg/cell cmRNA resulting in a dense network of many long neurites with numerous branchpoints. Proteomics analysis revealed most changes in transfected BMSCs occurred in the secretome while in PC-12 this was clearly observed in the proteome. Specific upregulated proteins in transfected BMSCs include numerous translation initiation factors, and ribosomal proteins. The obtained network cluster highlighted that proteins involved in mRNA regulation were present, including several small nuclear ribonucleoprotein polypeptides (SNRPA, SNRPA1, SNRPE). Pathways of downregulated proteins mainly revolved around cellular localization and extracellular matrix regulation (including collagens 1A1, 1A2, 4A1, 5A1, and 6A1). As opposed to changes in transfected BMSCs, changes regarding PC-12 cells following co-culture were mostly observed on a cellular level. This supports cellular changes of PC12 cells shown before by microscopical observations and immunofluorescence. Specific upregulated proteins include neurofilament proteins (NEFH, NEFM, NEFL), β3-Tubulin, and neural cell adhesion molecules (NCAM1). In the obtained network cluster, we recognize several of these proteins (NEFM, NEFH) and also MAP (kinase) proteins, which all play important roles in signal transduction for neuronal differentiation.
DISCUSSION: In recent years it has become increasingly obvious that innervation is deemed highly necessary for the repair and regeneration of many tissues, as it is in organogenesis. An example of a highly innervated tissue is bone. It is thus clear that strategies to heal bone tissue should consider innervation as of vital importance. In this study, we show that transcript therapy can be successfully used to induce in situ expression of NGF protein by target cells aiming to improve innervation in the context of bone healing. As expected, NGFcmRNA transfected mesenchymal stem cells were able to produce NGF protein that at the same time was able to stimulate the differentiation of PC-12 cells toward the neuronal phenotype, demonstrating the functionality of the cell-secreted protein. We speculate that the use of diverse cmRNAs, coding for relevant therapeutic proteins, such as BMPs, NGF, and VEGF may be able to stimulate all crucial processes of osteogenesis, neurogenesis, and angiogenesis to cooccur resulting in a superior, functional bone healing outcome.
SIGNIFICANCE/CLINICAL RELEVANCE: Defective bone healing remains a pressing clinical problem. Approaches to bone healing often consider stimulating angiogenesis but mostly focus on osteogenesis, mineralization, or calcification. Innervation is commonly disregarded. The data summarized in this abstract are the first demonstration that cmRNA can transfect mesenchymal stem cells in a highly efficient and biocompatible manner resulting in functional production of nerve stimulating factors.
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