Delivery of siRNA using cationic star polymers to suppress Runt Related Transcription Factor 2 and Osterix *in vitro*

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Statement of Purpose: Craniosynostosis is a pathosis characterized by the premature fusion of cranial sutures. One in roughly two thousand babies worldwide will be born with craniosynostosis.[1] The putative etiology of craniosynostosis is the overexpression of Bone morphogenetic protein (BMP) and underexpression of a BMP antagonist known as noggin.[2] BMP is a morphogen that induces mesenchymal stem cell differentiation into osteoblasts by activating the runt related transcription factor 2 (Runx2) and Osterix (Osx). These transcription factors control the differentiation of stem cells into osteoblasts and the mineralization of osteoblasts into bone. This study's objective is to target Runx2 and Osx messenger RNA using small interfering ribonucleic acids (siRNA) *in vitro*. We used cationic star nanostructured polymers to encapsulate the siRNAs and deliver it to Human Mesenchymal Stem Cells (HMSC) to prevent BMP induced osteoblast cell differentiation. This approach may be effectively used to prevent genetic diseases such as craniosynostosis.

Methods: Acrylate Cationic (AC)-star nanostructured polymers were prepared from macroinitiators and hydroxyl terminated macromonomers based on poly(ethylene oxide) with DMAEMA-Q and a degradable dimethacrylate crosslinker via arm-first methods using ATRP [3] to deliver siRNAs.

Biocompatibility was tested using live/dead and lactate dehydrogenase (LDH) assays. The mouse calvarial pre-osteoblast subclone 4 cells (MC3T3-E1.4). cells were incubated with the cationic star nanostructured polymers for 24 and 48 hours and stained for viable and dead cells using Live/Dead staining kit (Invitrogen). The control wells did not receive any polymer. LDH assay was performed using Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega G1780).

Transfection efficiency of siRNAs conjugated with fluorescein isothiocyanate (FITC) was determined in HMSC's. siRNA encapsulated AC star nanostructured polymers were added to $1.5x10^6$ cells. Cells were then plated on a U-shaped non-adherent 96 well plate and incubated for 24 and 48 hrs at 37°C and then collected and spun down to remove the media. The cells were then resuspended in DPBS (Dulbeccos Phosphate Buffered Saline) and used for FACS (Fluorescence Activated Cell Sorting) analysis or fixed with 4% paraformaldehyde for confocal imaging. Zetas potential analysis was carried out determine siRNA complexation.

Knockdown efficiency of the Runx2 and Osx siRNAs encapsulated in AC-star nanostructured polymers was determined after 1, 4, 7, 14, and 21 days incubation in the presence or absence of recombinant human BMP 2(rhBMP-2). Runx2 and Osx protein expression in HMSCs was determined using western blot and immunofluorescence staining. Osteoblast differentiation factors, alkaline phosphatase (ALP) and osteocalcin were measured calorimetrically to validate the knock down efficiency.

Results: Live/Dead cell staining showed >95% cell viability at 48 hours suggesting the cationic star nanostructured polymers are biocompatible and non-toxic.

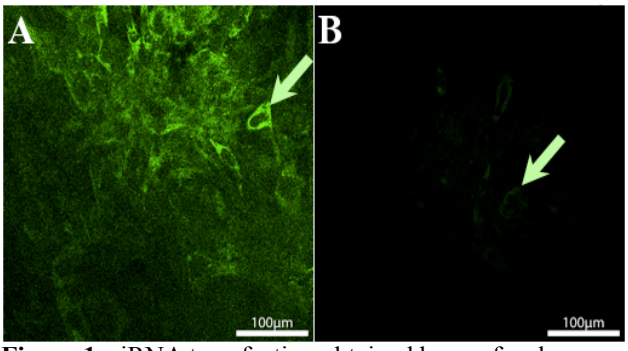

Figure 1. siRNA transfection obtained by confocal microscopy; cationic PEO star polymer with fluorescent siRNA complexation (A) and human mesenchymal stem cells only (B).

The LDH assay also showed < 5% cytotoxicity and were comparable to controls. Zeta potential analysis confirmed the negatively charged Runx2 and Osx siRNA complexation with the AC-star nanostructured polymers. The electrostatic interaction between the cationic polymers and negatively charged siRNA enhanced the delivery and encapsulation of siRNA to the HMSC's. Confocal microscopy and FACS analysis confirmed >100% transfection efficiency after 24 hrs (**Figure 1**). Knock down experiments showed >75% reduction in Runx2 and Osx protein expression when compared to controls that received no siRNAs as early as 4 days.

Conclusion: Acrylated cationic nanostructured star polymers with PEO arms were successfully synthesized. The degradable cationic nanostructured star polymers are biocompatible and non-toxic. FACS and confocal analysis revealed >100% siRNA transfection efficiency into HMSCs. Runx2 and Osx protein knock down efficiency was >75% suggesting this approach can be effectively employed to prevent craniosynostosis.

Reference:

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