Small Interfering RNA Delivery for the Treatment of Hereditary Bone Disease

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Introduction:

Osteogenesis imperfecta (OI) is a heterogeneous bone disease classified into eight different types based on phenotype and genotype [1]. OI type V is caused by a mutation of interferon-induced transmembrane protein 5 (IFITM5) [2]. The mutant protein has an additional five amino acids (Met-Ala-Leu-Glu-Pro) at the N-terminus in individuals with OI type V [2].

IFITM5 is an osteoblast-specific membrane protein previously seen to be a positive regulator for bone formation in *in vitro* studies [3]. Despite this evidence, recent studies show that bone formation of *Ifitm5* knockout mice are comparable to bone formation of the wild type [3,4]. The discrepancy between the *in vitro* and *in vivo* experiments suggests that there exists an *in vivo* mechanism in place to ensure healthy bone formation in the absence of the protein. Given that OI type V is caused by a mutation in IFITM5, suppressing IFITM5 protein translation should restore healthy bone growth for individuals with the disease.

In this report, small interfering RNA (siRNA) was used to suppress the translation of the mutant protein IFITM5 *in vitro*. Four transfection reagents were tested using mouse osteoblast cells: DOTAP, Lipofectamine2000, Lipofectamine RNAiMAX (RNAiMAX), and calcium phosphate (CaP) nanoparticles. Following transfection, cells were lysed to extract and purify RNA. RNA was subsequently converted to DNA using PCR, and q-PCR was used to determine relative gene expression. Among the transfection reagents used, RNAiMAX was the most effective, suppressing gene translation by an average of 60%.

Materials:

Mouse osteoblasts (MC3T3) and MEM-alpha medium (10% FBS) were used throughout the study. Lipofectamine2000 and RNAiMAX were purchased from Invitrogen Corporation by Life Technologies (Carlsbad, CA, USA). DOTAP was purchased from Roche Diagnostics GmbH (Mannheim, Germany). CaP nanoparticles were made using reagents from the CalPhos Mammalian Transfection Kit obtained from Clontech Laboratores, Inc. (Mountain View, CA, USA). Isogen used for RNA extraction was obtained from Nippon Gene Co. (Tokyo, Japan). Real-time PCR (q-PCR) was carried out on a LightCycler System. Glycerol 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

Methods:

All incubations not specified were carried out at 37°C in a 5% CO_2 controlled environment. All siRNA complexes were made following manufacturer protocol.

For liposomal transfection, cells were seeded at 1.5×10^4 cells/cm^2 on six well plates. Cells were immediately exposed to the transfection reagent complexed with either IFITM5 siRNA (50 pmol/well) or control siRNA (SCN01) (50 pmol/well).

TABLE 1:	Condition A	Condition B	Condition C
Total siRNA (ug)	0.34	0.68	1.60
[siRNA] (ug/mL)	20	20	40
[CaP] : [siRNA]	5 : 1	2.7 : 1	2.7 :1

For CaP nanoparticle transfection, cells were seeded overnight at 7.0×10^3 cells/cm^2. The next day, medium was replaced with a mix of serum free medium and CaP nanoparticles complexed with either IFITM5 siRNA or SCN01 siRNA. Table 1 details the three conditions. Three hours after transfection, serum-free medium incubated in a 10% CO_2 environment was added so that the medium volume was doubled. The addition of slightly acidic medium aided the dissolution of the nanoparticles.

After a 24 hr incubation period, all wells were replaced with fresh medium and a mix of mutant IFITM5 plasmid (2 ug/well) and Lipofectamine2000 transfection reagent. A subsequent incubation period of 24 hours ensured complete transfection and replication of the genetic material.

Cells were washed with PBS, and then total RNA was extracted with Isogen according to manufacturer protocol.

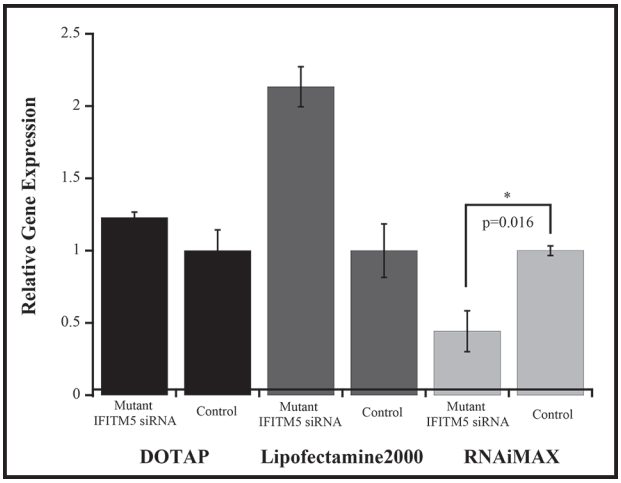

Figure 1: Relative expression of mutant IFITM5 genetic material. Three different commercially available liposomal transfection reagents are compared.

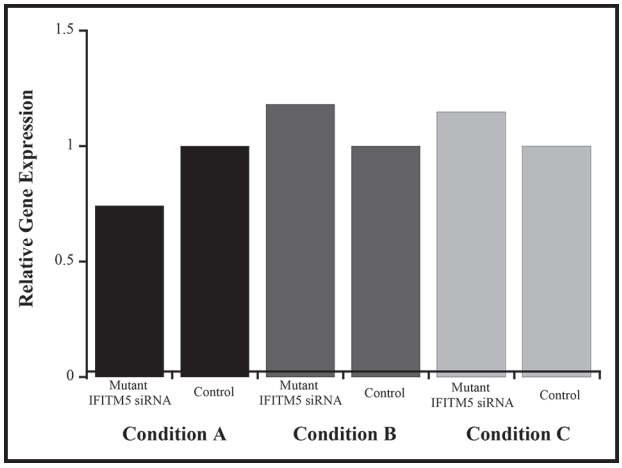

Figure 2: Relative expression of mutant IFITM5 genetic material. Three different compositions of CaP nanoparticles are compared.

Concentration of extracted RNA was determined using UV/Vis spectroscopy. RNA was diluted to 500 ng/uL and then converted to DNA using reverse transcriptase. Resulting DNA was diluted to 5 ng/uL. Expression was quantitatively measured using the cross-point of amplification as determined by real-time polymerase chain reaction (q-PCR).

Results and Discussion:

Figure 1 and 2 show the relative expression of mutant IFITM5 genetic material, comparing osteoblasts transfected with IFITM5 siRNA and control siRNA. Figure 1 highlights the liposomal transfection reagents studied, among which RNAiMAX suppressed IFITM5 gene expression 60% relative to the control. DOTAP and Lipofectamine2000 both failed to suppress gene expression. The success of RNAiMAX is difficult to ascertain because its chemical and structural properties are not distributed by the manufacturer. Additionally, the release mechanism of siRNA into the cell after endocytosis is still unclear. These conceptual barriers prevent a detailed explanation for siRNA suppression using RNAiMAX. Factors that may influence the transfection efficiency include the size of the liposome complex and its chemical composition.

Figure 2 shows the results from individual studies using CaP nanoparticles as the siRNA transfection reagent. The condition with a greater CaP:siRNA ratio and lower concentration of siRNA was seen to suppress gene expression 30% relative to the control.

Because siRNA is foreign genetic material, excess siRNA can induce apoptosis while too little siRNA would decrease the knockdown effect. Therefore, concentration of siRNA is critical to the survival of the cells and transfection efficiency. This experiment suggests that 20 ug/mL of siRNA is sufficient to suppress gene expression, but the CaP study included a single experiment per variation. The reproducibility of these results is yet to be determined.

Conclusions:

The transfection reagent RNAiMAX demonstrates that gene suppression is feasible on the cellular level. Due to the limited information of the compound, RNAiMAX is not a desirable method for *in vivo* studies at this time. CaP nanoparticles as a known biologically degradable compound should be investigated further to determine if it is a viable method to suppress gene expression both on the cellular and physiological level. Furthermore, the method of siRNA delivery subsequent to endocytosis should be investigated for optimization of transfection efficiency.

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