ARTÍCULO

Fibroblast growth factor receptor 3 inhibition by small interfering RNAs in achondroplasia

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ABSTRACT

Achondroplasia is a short-limbed dwarfism resulting from mutation and gain-of-function in fibroblast growth factor receptor 3 (FGFR3). Effective therapy for this condition has not as yet been established. We have tested the efficiency of three different small interference RNAs (siRNAs) to abrogate the FGFR3 expression in human immortalized chondrocytes carrying the achondroplasia mutation (G380R). Two siRNA sequences induced markedly decrease of FGFR3 mRNA (up to 75% reduction) and protein levels (up to 61% reduction). Furthemore, siRNA-mediated knockdown of FGFR3 blocked the activation of the downstream signal transduction ERK pathway.

Keywords: Achondroplasia; chondrocytes; fibroblast growth factor receptor 3; RNA interference; ERK1/2.

RESUMEN

Inhibición del receptor FGFR3 por ARNs de interferencia para la acondroplasia

La acondroplasia es un tipo de enanismo caracterizado por extremidades cortas resultante de una mutación en el receptor de crecimiento de fibroblastos de tipo 3 (FGFR3). Aún no se ha establecido una terapia efectiva para esta enfermedad. Nosotros hemos testado la eficiencia de tres diferentes *small interference RNAs* (*siRNAs*) para bloquear la expresión del receptor FGFR3 en condrocitos humanos inmortalizados portadores de la mutación acondroplásica (G380R). Dos secuencias de siRNAs indujeron un marcado descenso de la expresión de ARN mensajero del receptor FGFR3 (hasta un 75%) así como de los niveles de proteína (hasta un 61%). Además, el bloqueo de la expresión del receptor FGFR3 mediado por los *siRNAs* redujo la activación de la cascada de transducción de las ERK.

Palabras clave: Acondroplasia; condrocitos; receptor de crecimiento de fibroblastos de tipo 3; ARN de interferencia; ERK1/2.

1. INTRODUCTION

Achondroplasia is the most common form of dwarfism in humans. This disorder is characterized by a mutation in the gene that encodes for the FGFR3 receptor, being the mutation in 97% of the patients a Glycine to Arginine substitution at position 380.

FGFR3 is a regulator of endochondral bone growth and its mutation causes gain of function disturbing chondrocyte proliferation and differentiation during endochondral ossification process. As a result, the rate of cartilage template formation and turnover required for bone elongation are dramatically reduced (1).

At the molecular level, sustained activation of mutant FGFR3 stimulates several intracellular signalling pathways, including extracellular signal-regulated kinases 1 and 2 (ERK1/2) cascade and signal transducer and activators of transcription (STAT-1) pathway, that account for the critical changes in chondrocyte proliferation (2, 3), differentiation (4) and extracellular matrix homeostasis (5).

Potential therapeutic approaches have been proposed for achondroplasia treatment. The main strategies are based on blocking FGFR3 activation (6, 7) or interfering with the pathways that modulate the downstream propagation of FGFR3 signals (5, 8). However, despite the efforts, achondroplasia remains as an orphan pathology with no pharmacological treatment so far.

RNA interference is a posttranscriptional process triggered by the introduction of double-stranded RNA, which leads to gene silencing in a sequence-specific manner. This is one of the most exciting new findings in functional genomics of the past decade and its potential for experimental and therapeutic purposes is currently under investigation (9).

In this work, we transfected immortalized human chondrocytes carrying the heterozygous achondroplasia mutation (G380R) with three different small interfering RNAs (siRNAs) and analyzed their effect on FGFR3 expression and prolonged signalling to evaluate the therapeutical potential of siRNAs in achondroplasia treatment.

2. MATERIALS AND METHODS

2.1. Cells

Immortalized human chondrocytes carrying the heterozygous achondroplasia mutation (G380R) were generated and characterized by Dr. Laurence Legeai-Mallet (10). Cells were cultured in Dulbecco´s modified Eagle´s medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 1% penicillin/streptomicyn and 500 ng/ml geneticin (all obtained from Invitrogen) and were incubated at 37 ºC with 5% CO_2.

2.2. Small interfering RNA design and Transfection

Using siRNA design software, three siRNA duplexes targeting FGFR3 were obtained from Ambion (Applied Biosystems, Foster City, CA, USA). The three sequences used are shown in Table 1. A scrambled siRNA without sequence homology to any known human gene was also obtained from Ambion and used as a

negative control to prove that silencing of the gene of interest is due to specificity of siRNA and is not due to non-specific effects.

Table 1. Small interfering RNAs (siRNAs) sequences.

siRNAs	Sense (5′-3′)	Anti-sense (5′-3′)
SEQ 1	CCGUAGCCGUGAAGAUGCUTT	AGCAUCUUCACGGCUACGGTG
SEQ 2	CCUGCGUCGUGGAGAACAATT	UUGUUCUCCACGACGCAGGTG
SEQ 3	AGGUGUACAGUGACGCACATT	UGUGCGUCACUGUACACCUTG

Transfection complexes were prepared in an Opti-MEM serum free medium (Invitrogen) by mixing 7 μl of siPORT NeoFX transfection reagent (Applied Biosystems) and 300 nM of scramble/FGFR3 siRNAs. Inmortalized human chondrocytes carrying the achondroplasia mutation were platted in a 6 well plate after the addition of transfection complexes. After 72h, RNA and protein were isolated to analyze knockdown efficiency.

2.3. Reverse Transcriptase-PCR

Total RNA was extracted from the achondroplastic chondrocytes using the Nucleospin RNA/Protein Kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer and 1 μg was reverse transcribed using SuperScript III First-Strand synthesis system Kit (Invitrogen).

The FGFR3-specific primers (forward 5′- TGCTGAATGCCTCCCACG -3′ and reverse 5′- CCAGGCTCCACTGCTGATG -3′) that have been previously described (11, 12) were employed for amplification of FGFR3. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as an internal control and GAPDH-specific primers (forward 5′- ACCACAGTCCATGCCATCAC -3′ and reverse 5′- TCCACCACCCTGTTGCTGTA -3′) were purchased from Clontech (Clontech, Mountain View, CA, USA). Reactions were performed using TITANUM Taq DNA polymerase (Clontech). For FGFR3 amplification, reactions conditions were 94 °C for 3 minutes, followed by 35 cycles at 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 10 minutes. The thermal cycling conditions for GAPDH amplification were 94 °C for 5 minutes, followed by 30 cycles at 94 °C for 45 seconds, 60 °C for 45 seconds, 72 °C for 2 minutes and 72 °C for 7 minutes.

PCR products were electrophoresed on 1.5% agarose gel, stained by ethidium bromide and quantified using a Kodak GL 200 Imaging system and Kodak Molecular Imaging software (Kodak, Rochester, NY, USA). The values of the ratio of FGFR3 to GAPDH were calculated and normalized by scramble control when the value of scramble control was defined as 100%.

2.4. Western Blot Analysis

Total protein from achondroplastic chondrocytes transfected with the siRNAs were isolated using the Nucleospin RNA/Protein Kit (Macherey-Nagel) and protein concentration was determined by the Protein Quantification assay (Macherey-Nagel).

15 μg of protein from each sample were subjected to 10% SDS-polyacrilamide gels and were transferred to nitrocellulose membranes. Thereafter membranes were blocked and incubated overnight with anti-FGFR3 (1:100) or pERK1/2 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, blots were incubated with peroxidase-conjugated secondary antibody. Development was performed using ECL system (Amersham, Buckinghamshire, UK). To verify equal loading, membranes were stripped in 62.5 mM Tris-HCl pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol and re-blotted with the antibodies GAPDH (1:2,500) (Abcam, Cambridge, UK) and ERK2 (1:2,000) (Santa Cruz Biotechnology). Films were scanned and a densitometric analysis was performed using Kodak GL 200 Imaging system and Kodak Molecular Imaging software. The intensity of the bands quantified by densitometry was graphed. Data were normalized by FGFR3 protein levels of the scramble control, defined as 100%. In the case of ERK1/2 phosphorylation, data were normalized by ERK1/2 phosphorylation levels of the scramble control, defined as 100%. All the data shown are representative of three independent experiments.

2.5. Statistical Analysis

The differences between the mean values were analyzed with InStat3 software (GraphPad Software, La Jolla, CA, USA) using ANOVA test; statistical significance was considered to be achieved at the $P < 0.05$ level.

3. RESULTS

3.1. Silencing of FGFR3 in human achondroplastic chondrocytes by siRNAs

siRNAs were transfected into human achondroplastic chondrocytes and 72 h later the ability of the selected siRNA sequences to knockdown FGFR3 mRNA and protein levels was analyzed by RT-PCR and Western Blot, respectively (Figure 1). Of the three siRNA sequences tested in achondroplastic chondrocytes, sequences 1 and 2 showed 55% and 75% knockdown of FGFR3 mRNA expression, respectively, as compared to scramble control (Figure 1A). siRNA sequence 3 was less effective at reducing FGFR3 mRNA expression and only 38% reduction was detected.

Western blot analysis of cell culture lysates from these cells revealed that the amount of FGFR3 protein also decreased (Figure 1B). In particular, densitometry of immunoblots indicated that FGFR3 protein was reduced by 52% and 61% with sequence 1 and 2, respectively. Once more, the sequence 3 induced a lower decrease as compared to the scramble control (20% of reduction).

3.2. Effect of FGFR3 knockdown on ERK1/2 activation

To investigate the impact of FGFR3 silencing on downstream signalling, the phosphorylation status of ERK1/2 was evaluated (Figure 2). siRNA sequence 2 reduced ERK1/2 phosphorylation by 41% as compared to scramble control levels. Although to a lesser extent, siRNA sequence 1 also decreased the levels of phosphorylated ERK1/2 (27% of reduction), whereas siRNA sequence 3 hardly modified the degree of phosphorylation of ERK1/2 when compared to scramble control.

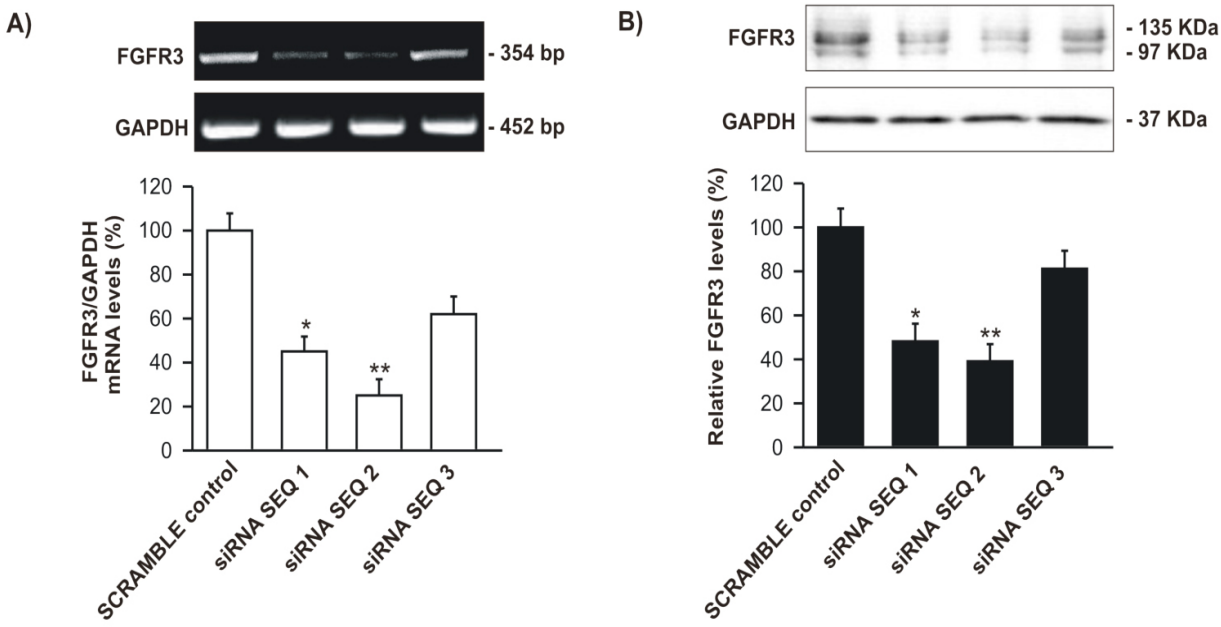

Figure 1. FGFR3 expression after siRNA transfection. siRNAs were introduced into human achondroplastic chondrocytes and FGFR3 mRNA (A) and FGFR3 protein (B) were assessed 72 h later. *P < 0.05, **P < 0.01.

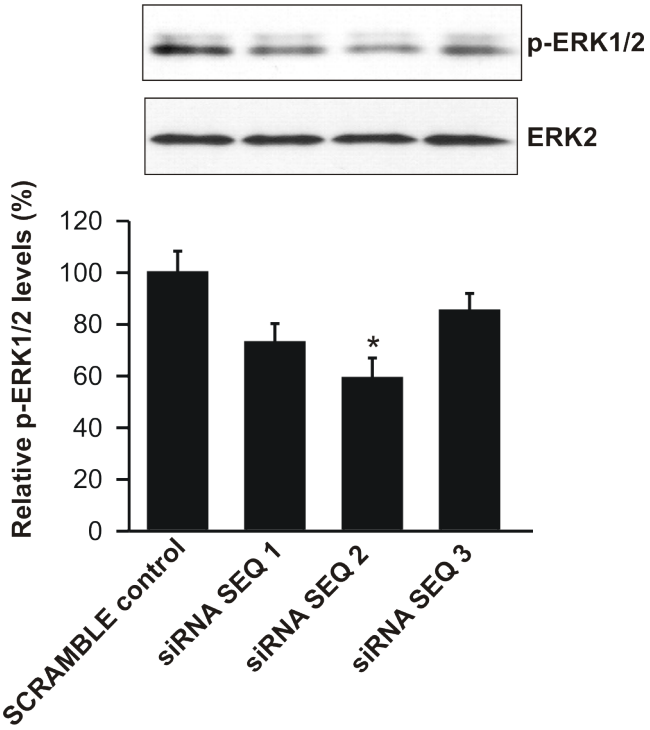

Figure 2. Effect of FGFR3 knockdown on ERK1/2 phosphorylation. Cell lysates of human achondroplastic chondrocytes transfected with siRNAs were successively inmunoblotted with anti-pERK1/2 and anti-ERK2 antibody to verify equal loading. *P < 0.05.

4. DISCUSSION

RNA interference is emerging as a strategy for the highly specific suppression of gene expression, both in vitro and in vivo. The present work shows the use of siRNA duplexes targeting FGFR3 to knockdown the mRNA and protein expression levels of this receptor in immortalized human chondrocytes carrying the achondroplasia mutation (G380R).

Our results are consistent with previous studies demonstrating that siRNAs targeting distinct mRNA sequences results in different silencing efficiency (11, 13). A significant decrease of FGFR3 mRNA and protein levels was observed after transient transfection with siRNA sequences 1 and 2, while the siRNA sequence 3 did not induce FGFR3 down-regulation in the same extent.

Activation of FGFR3 results in trans-autophosphorylation of juxtaposed intracellular kinase domains with subsequent recruitments of signalling molecules leading to phosphorylation and activation of different signalling pathways. Therefore, we determined whether these signalling pathways were also affected by FGFR3 knockdown. In particular, we have analyzed one of the best known and characterized downstream signal transduction pathways coupled to FGFR3, the ERK mitogen activated protein kinase cascade. The sustained activation of this pathway is involved in the inhibitory effect of FGF signalling on chondrocyte proliferation and cartilage matrix production (3, 5). FGFR3 knockdown by siRNAs (siRNAs sequence 1 and 2) attenuated the ERK1/2 phosphorylation suggesting that the negative effects mediated by this pathway could be counteracted by siRNAs targeting FGFR3.

RNA interference strategies have been previously described in different types of cancer cells that have activating mutations in FGFR3. In bladder cancer cells, knockdown of FGFR3 by siRNAs lead to decrease proliferation, reduced clonogenicity and soft agar growth (14). Similarly, siRNA-mediated knockdown of FGFR3 inhibited anchorage-independent growth of adrenal carcinoma cells (12). Inhibition of FGFR3 expression by RNA interference has been also reported in multiple myeloma cells (11). On the other hand, regarding skull and skeletal growth disorders, RNA interference to knock-down the expression of a mutant FGFR2 has been used in Apert syndrome, a classic severe form of craniosynostosis (15). However, to our knowledge we have shown for the first time the use of RNA interference targeting FGFR3 in human achondroplastic chondrocytes.

Although several therapeutic approaches have emerged for achondroplasia treatment, the translation of these therapies into the clinic has not taken place. One of the main troubles for the success of new therapies is the delivery of compounds to growth plate chondrocytes of cartilage. The avascular nature of the cartilage tissue represents a challenge for drug delivery. Conjugation of siRNAs with small molecules that posses affinity for cartilage or chondrocytes could facilitate the target to growth plate chondrocytes of cartilage. In this sense, several strategies have been developed to promote cell/tissue-specific siRNA delivery (16). Additionally, the dense extracellular matrix that surround chondrocytes form an important barrier for drug delivery. However, siRNAs are small enough that diffusion through extracellular matrix should not be an issue.

5. CONCLUSION

Our findings indicate that RNA interference targeting FGFR3 could be a promising therapeutic option for achondroplasia in the future. Hopefully, new advances in delivery strategies to growth plate chondrocytes of cartilage will facilitate the development of siRNA-based therapy.

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