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Development of human cells with RXFP1 knockdown using retroviral delivery of microRNA against human RXFP1

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Summary

To study the specific actions of relaxin through RXFP1 in human cells, it would be advantageous to develop cell populations with permanent RXFP1 knockdown (KD). We have developed and assessed four microRNA against human RXFP1. One of the four designed microRNA displayed significant RXFP1 KD as assessed by reduced relaxin binding when co-transfected with human RXFP1 into HEK-293T cells. The selected microRNA sequence was subsequently retrovirally delivered into the human dermal fibroblast cell line BJ3 which natively expresses RXFP1. The RXFP1 KD BJ3 cells displayed diminished RXFP1 mRNA expression and complete loss of ability of relaxin treatment to reduce collagen deposition after TGF-β_1 stimulation. The retroviral expression of miRNA to successfully silence RXFP1 expression is an invaluable tool to investigate receptor specificity, signalling and possible off-target effects of newly developed relaxin analogs.

Introduction

The peptide hormone relaxin mediates its actions through the G-protein coupled receptor relaxin family peptide receptor 1 (RXFP1) (1). To further elucidate the specific actions of relaxin and relaxin analogues, we developed microRNA (miRNA) against human RXFP1 and following validation of miRNA targeting utilized retroviruses for cell delivery and RXFP1 KD. Recombinant retroviruses are incapable of self-replication and are designed to integrate a gene of interest or miRNA into the cell's genome with stable expression and marked efficiency in different cell types (2, 3). The relative co-expression of green fluorescence protein (GFP) to miRNA in these viruses was used as marker to sort highly transduced cells using fluorescence activated cell sorting (FACS). To validate this approach, we used human BJ3 cells, a native RXFP1 expressing fibroblast cell line frequently used in the study of RXFP1 mediated collagen remodelling (4).

Methods

Four miRNA were designed against human RXFP1 (accession number NM_021634.2) using Invitrogen BLOCK-iT™ Pol II miR RNAi Designer and Expres-

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sion Vector system and cloned into the pcDNA3.1(+) mammalian expression vector. The miRNA were tested by co-transfection with human RXFP1 in HEK-293T cells and subsequent analysis of Eu-labelled relaxin binding as previously described (5). Data shown are from 3 independent experiments performed in triplicate and are expressed as specific relaxin binding normalised to the specific binding of a RXFP1 only transfection and were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis.

The selected miRNA was cloned into Murine Stem Cell Virus (MSCV) vector and transfected into HEK-293T cells to produce recombinant retrovirus carrying the miRNA transgene. The media containing the recombinant virus was used to transduce BJ3 cells. After transduction, FACS was used to sort high GFP expressing cells, a marker for the levels of miRNA expression, to generate the RXFP1 KD BJ3 cells. The KD of RXFP1 in these cells was assessed using reverse-transcription PCR and collagen remodelling after relaxin treatment. The collagen deposition of wild-type and RXFP1 KD BJ3 cells treated with 4ng/ml TGF-β_1 and 100ng/ml relaxin for 3 days was measured using hydroxyproline assays as performed previously (6). Data shown are from 4 independent experiments performed in duplicate. The collagen content of each treatment was normalised to untreated control cells and analysed using Student's *t*-test.

Results and Discussion

This study set out to develop a viral-based approach to deliver miRNA against RXFP1 to enable KD of RXFP1 in human cells. As there are no high affinity RXFP1 antagonists currently available, this approach will serve as a good tool to study the RXFP1 specific and RXFP1 independent actions of relaxin and relaxin analogues.

Four miRNA targeting the human RXFP1 sequence were designed, cloned and their effectiveness assessed using relaxin binding in HEK-293T cells co-transfected with RXFP1 and each miRNA. One of the four (miRNA 2) was found to significantly KD RXFP1 expression as demonstrated by 60% reduction of specific relaxin binding ($p<0.001$) (Figure 1A). miRNA 2 was selected to develop a recombinant retrovirus to enable RXFP1 KD in human cells.

BJ3 fibroblasts cells were chosen for the retroviral transduction as this line has similar collagen remodelling characteristics to primary fibroblasts with a greater passage capacity. As transduction efficiency varies in different cells, FACS was employed to select the RXFP1 KD BJ3 cells using GFP as a marker for miRNA expression. Assessment of the RXFP1 KD BJ3 cells showed diminished RXFP1 mRNA expression (data not shown). In addition, no reduction in TGF-β_1-stimulated collagen levels were observed in RXFP1 KD BJ3 cells in the presence of high concentrations of relaxin (100ng/ml) (Figure 1B). The complete loss of relaxin stimulated reduction in TGF-β_1-stimulated collagen production in RXFP1 KD BJ3 cells highlights the functional effects of substantial KD of RXFP1 by miRNA 2 transduction (Figure 1B).

This study successfully established a retroviral delivery vector to KD RXFP1 expression in human cells, which can be applied to any cell type. This will allow the identification of RXFP1 independent effects and improve the selection and development of future drugs with greater receptor selectivity and potency.

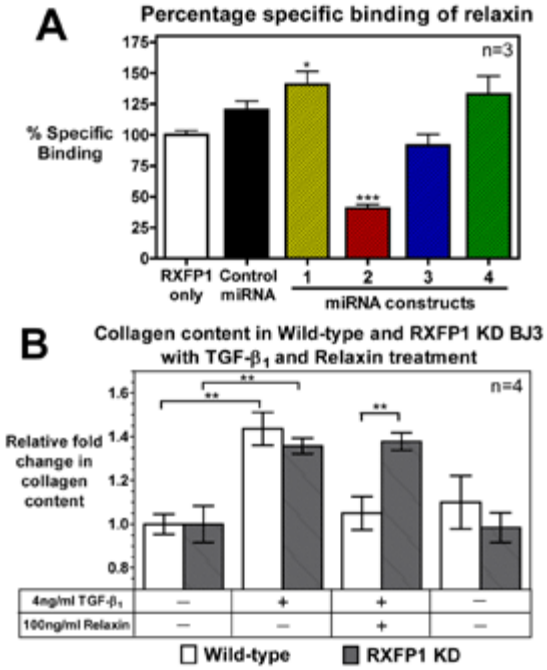

Figure 1. Specific relaxin binding of HEK-293T cells co-transfected with miRNA (A) and collagen content of wild-type and RXFP1 knockdown (KD) BJ3 cells under different treatments (B). (A) Specific binding was determined by the difference in europium labelled relaxin binding with and without unlabelled relaxin. Only miRNA 2 displayed a significant reduction in specific relaxin binding ($p<0.001$). (B). TGF-β1 stimulation resulted in a marked increase in collagen levels in both RXFP1 KD and wild-type BJ3 cells. Relaxin treatment reversed this collagen increase in wild-type cells but not in the RXFP1 KD cells, highlighting the effective RXFP1 knockdown in these cells. Importantly relaxin treatment, or RXFP1 KD, did not influence basal collagen levels in these cells. *$p<0.05$, **$p<0.01$and ***$p<0.001$.

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