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Regulation of the Human Fc-Neonatal Receptor alpha-Chain Gene *FCGRT* by MicroRNA-3181

Daniel C. Ferguson and Javier G. Blanco

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, The State University of New York at Buffalo

Abstract

Purpose—*FCGRT* encodes the alpha-chain component of the neonatal Fc receptor (FcRn). FcRn is critical for the trafficking of endogenous and exogenous IgG molecules and albumin in various tissues. Few regulators of FcRn expression have been identified. We investigated the epigenetic regulation of FcRn by two microRNAs (*hsa-miR-3181* and *hsa-miR-3136-3p*) acting on *FCGRT*.

Methods—The binding of candidate microRNAs to the 3'-untranslated region of *FCGRT* was evaluated using luciferase reporter constructs in CHO cells. The effect of microRNAs on *FCGRT* mRNA and FcRn protein expression was evaluated using specific microRNA mimics and inhibitor transfections in A549, HEK293 and HepG2 cells.

Results—*Hsa-miR-3181* mimic reduced luciferase reporter activity by 70.1% (10 nM, $P < 0.0001$). In A549, HEK293 and HepG2 cells, *hsa-miR-3181* decreased *FCGRT* mRNA expression (48.6%, 51.3% and 43.5% respectively, 25 nM, $P < 0.05$). The *hsa-miR-3181* mimic decreased the expression of FcRn protein by 40% after 48 hours (25 nM, $P < 0.001$). The mature form of *hsa-miR-3181* was detected in samples of human liver.

Conclusions—These data suggest that *hsa-miR-3181* is an epigenetic regulator of *FCGRT* expression. The identification of this regulator of *FCGRT* may provide insights into a potential determinant of interindividual variability in FcRn expression.

Keywords

FcRn; *FCGRT*; Epigenetics; MicroRNA; Monoclonal antibodies

Introduction

The human Fc-neonatal receptor gene *FCGRT*, located on chromosome 19, encodes the alpha chain component of the neonatal Fc receptor (FcRn). FcRn is a heterodimer protein resulting from the noncovalent binding between FCGRT and beta-2-microglobulin. Human FcRn functions as a MHC class I-like pH dependent high affinity IgG receptor (1, 2). FcRn aids in immune development and enables the transcytosis of maternal IgG to the fetus across the placental barrier (3–5). FcRn also maintains homeostatic concentrations of IgG and

albumin in blood through a recycling mechanism (6). The interactions between FcRn with the Fc portion of immunoglobulins and specific residues on albumin occur through a series of pH specific binding (pH 6.0) and dissociation (pH 7.4) events that take place within early endosomes and at cell surfaces, respectively (7, 8).

FcRn is responsible for the long serum half-lives of IgG and albumin, which each reach approximately three weeks in humans (9, 10). FcRn knock-out mice display reduced serum half-lives as well as altered tissue distribution of immunoglobulins when compared to wild-type animals (11, 12). FcRn also has a profound impact on the pharmacokinetics and pharmacodynamics of monoclonal antibody drugs. In general, antibody and peptide drugs that contain a human Fc region or are conjugated to albumin exhibit extended plasma half-lives (13–15).

There is limited information in regards to the factors that control the regulation of *FCGRT* and the expression of FcRn in humans. Mostly notably, NF-kappaB signaling influences gene expression through intronic elements in *FCGRT*, and activation of the JAK/STAT-1 signaling pathway by IFN-gamma results in FcRn down regulation (16, 17).

MicroRNAs contribute towards the epigenetic control of gene expression. MicroRNAs are small noncoding RNAs, with a mature form typically 20–22 nucleotides in length, which can bind to the 3-prime untranslated region (3'UTR) of a target mRNA resulting in transcript degradation or inhibition of translation (18). MicroRNAs can contribute to temporal, cell type and disease specific changes in gene expression patterns (19, 20). In certain tissues, the expression of *FCGRT* varies between cell-types. For example, FcRn expression has been detected in mammary gland epithelial cells but is absent from endothelial cells (21). Temporal changes in FcRn expression have been documented in rat, with the expression of intestinal FcRn being higher in suckling pups than in mature animals (22). There is evidence of disease specific changes in FcRn expression (23). For example, FcRn expression was lower in lung cancerous tissue in comparison to non-cancerous tissue (24).

Several human Fc receptors have previously been identified as targets of specific microRNAs, but the effect of microRNAs on *FCGRT* expression is unknown (25, 26). The goal of this study was to identify microRNAs involved in the regulation of *FCGRT* gene expression. This work identifies a previously unexplored epigenetic component of *FCGRT* expression, and may provide an avenue to continue to define the mechanisms that control FcRn expression in humans.

Materials and Methods

Reagents and MicroRNAs

Human *hsa-miR-3181* (microRNA-3181) and *hsa-miR-3136-3p* (microRNA-3136-3p) mimics, inhibitors, corresponding negative controls, Dharmafect Duo transfection reagent and Dharmafect 4 transfection reagent were obtained from Dharmacon (Lafayette, CO). Phosphate buffered saline (PBS), Tween 20, LB broth, LB agar and kanamycin sulfate were obtained from Sigma Aldrich (St. Louis, MO). PCR primers were synthesized by Sigma

Aldrich, and sequences are listed in Supplemental File A. Restriction enzymes and Antarctic phosphatase were obtained from New England Biolabs (Ipswich, MA). Plasmid blunting and subsequent ligations were performed using the Fast End DNA Repair Kit and Rapid DNA Ligation Kit, respectively (Thermo Fisher Scientific, Waltham, MA)

Cell Culture

Tissue culture treated flasks and plates were obtained from Santa Cruz Biotech (Dallas, TX). Chinese hamster ovary cells (CHO-K1, ATCC CCL-61), human lung epithelial carcinoma cells (A549, ATCC CCL-185), human embryonic kidney epithelial cells (HEK293, ATCC CRL-1573), and human liver hepatocellular carcinoma cells (HepG2, ATCC HB-8065) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). CHO-K1 and A549 cells were maintained in F-12K media and HEK293 and HepG2 cells in DMEM media, each supplemented with 10% fetal bovine serum, and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific). Prior to all transfection experiments CHO-K1, A549, HEK293 and HepG2 cells were allowed to equilibrate in F-12K or DMEM media lacking antibiotics. All cells were maintained at 37°C in a 5% CO₂, 90% humidified environment.

Human Tissue Specimens

The Institutional Review Board of the State University of New York at Buffalo approved this research. Human liver specimens ($N = 5$) were provided by the Liver Tissue Procurement and Distribution System (National Institutes of Health Contract N01-DK-9-2310) and by The Cooperative Human Tissue Network (CHTN, funded by the National Cancer Institute). The purity and integrity of isolated RNA was assessed by measuring the 260nm/280nm absorbance ratio and by gel electrophoresis. Sample demographics are listed in Supplemental File A.

Bioinformatics

A 309 base pair sequence corresponding to the 3'UTR of human *FCGRT* was identified using the National Center for Biotechnology Information gene database reference sequence (Gene ID: 2217). MicroRNAs for experimental analysis were selected using the web-based program miRMap (<http://mirmap.ezlab.org/>) (27).

Reporter Constructs and Site Directed Mutagenesis

A 309 base pair sequence corresponding to the 3'UTR consensus sequence of *FCGRT* was amplified with the Expand High Fidelity PCR system (Roche, Indianapolis, IN) using genomic DNA from a single B-lymphoblastoid cell line (GM10860, Coriell Institute, Camden, NJ). The identity of the PCR product was verified by direct sequencing.

The pMir-Target luciferase reporter vector was obtained from Origene (Rockville, MD). The pMir-Target vector was digested with the restriction enzyme EcoRI, blunted, purified and treated with Antarctic phosphatase. The amplified *FCGRT* 3'UTR PCR product was then ligated into the pMir-Target vector forming the *FCGRT*-3'UTR reporter construct and transformed into XL-1 supercompetent cells (Agilent, Santa Clara, CA). Plasmid DNA was extracted from positive clones and sequenced using pMir-Target vector specific primers and

FCGRT-3'UTR specific primers. *FCGRT*-3'UTR construct for transfections was extracted using the PureYield Plasmid Midiprep system (Promega, Madison, WI).

A mutant *FCGRT*-3'UTR reporter construct (mut*FCGRT*-3'UTR) was created using the *FCGRT*-3'UTR as a template with the QuikChange Lightning Multi Site-Directed Mutagenesis Kit, following the manufacturer's instructions (Agilent). Mutagenesis primers were designed to harbor a two-nucleotide substitution within the predicted seed binding sites of microRNA-3181 and microRNA-3136-3p. The mut*FCGRT*-3'UTR was cultured, verified, and purified as described for the *FCGRT*-3'UTR reporter construct.

Dual Luciferase Assays

MicroRNA mimics and inhibitors along with vectors were transfected into CHO-K1 cells using Dharmafect duo transfection reagent following the manufacturer's recommendations. CHO-K1 cells were (70% confluence) were transfected with 100 ng of firefly luciferase vector (*FCGRT*-3'UTR or mut*FCGRT*-3'UTR), 10 ng of pRL-TK renilla luciferase control vector (Promega), and microRNA mimics/inhibitors (final culture media concentrations of 1, 5, and 10 nM) or negative controls (10 nM). Dual luciferase assays were performed using the Dual-Luciferase Reporter Assay System, following the manufacturer's instructions (Promega). Luminescence measurements were recorded with a Synergy HT plate reader using the Gen5 software (BioTek, Winooski, VT).

Quantitative Real-Time Polymerase Chain Reaction and FCGRT mRNA Expression

MicroRNA mimics and inhibitors were transfected into A549, HEK293 and HepG2 cells using Dharmafect 4 transfection reagent following the manufacturer's recommendations. A549, HEK293 and HepG2 cells (70% confluence) were transfected with microRNA mimics or inhibitors (final culture media concentration: 25 nM), or negative controls (25 nM). After 24 hours the culture media was removed and cells were washed with PBS. Total RNA was extracted using Trizol reagent following the manufactures instructions (Thermo Fisher Scientific). Quantitative real-time polymerase chain reactions (qRT-PCR) were performed with a CFX96 C1000 Touch thermocycler, using the iTaq Universal SYBR Green One-Step Kit following the manufacturer's instructions (Bio-Rad, Hercules, CA). For determining microRNA expression, total RNA was isolated from cells and tissue using the miReasy Extraction Kit following the manufacturer's instructions (Qiagen). MicroRNA-3181 expression was determined using the miScript SYBR green PCR system and primer sets, with *U6* and *hsa-miR-93* as normalizer genes (Qiagen). Real time PCR data were analyzed using the Ct method with CFX manager Software.

Immunoblotting and FCGRT Protein Expression

MicroRNA mimics and inhibitors were transfected into A549 cells using Dharmafect 4 transfection reagent following the manufacturer's recommendations. A549 cells (70% confluence) were transfected with 25 nM of microRNA mimic and/or inhibitor, and microRNA mimic and/or inhibitor negative controls. Cell lysates were collected at 24 and 48 hours post transfection using RIPA buffer supplemented with Halt Protease Inhibitor (Thermo Fisher Scientific). Protein concentration of cell lysates was determined with the Pierce BCA protein assay (Thermo Fisher Scientific), with bovine serum albumin as

standard. Cell lysates (35 µg) and recombinant FCGRT peptide (15 ng, Novoprotein, Summit, NJ) were separated on 10% Tris-Glycine gels (Bio-Rad). Proteins were semi-dry transferred to PVDF membranes (Bio-Rad). Membranes were blocked in 5% non-fat dry milk in PBS for one hour at room temperature with slight agitation. Membranes were then probed with anti-FcRn primary antibody (sc-66892, Santa Cruz) at a concentration of 1:500, diluted in 1% non-fat dry milk in PBS overnight. Membranes were washed in 0.05% PBS Tween 20 three times for 5 minutes with agitation. The membranes were then probed with goat anti-rabbit secondary antibody (Santa Cruz) at a concentration of 1:2000 in 5% non-fat dry milk in 0.05% PBS Tween for 1.5 hours at room temperature with slight agitation. The membranes were then washed in 0.05% PBS Tween three times for 5 minutes with agitation. Immunoreactive bands were visualized using Pierce ECL Substrate (Thermo Fisher Scientific) and a ChemiDoc XRS+ Imager and Image Lab software (Bio Rad). Comparisons between FCGRT expression after microRNA mimic/inhibitor or corresponding control transfections were made using band densitometry measurements. Blot images are provided in Supplemental File A.

For loading control/normalizer protein detection, blots were striped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) for 10 minutes at room temperature. The membrane was then blocked, and probed with beta-actin primary antibody (sc-130656, Santa Cruz) at a concentration of 1:1000, diluted in 1% non-fat dry milk in PBS for 1.5 hours at room temperature. Subsequent washing, secondary antibody probing, and band detection steps were performed identically as previously described.

Data Analysis

Data processing was performed with Excel 2016 (Microsoft Office: Microsoft, Redmond, WA). Statistical analyses were performed with GraphPad Prism version 7 (GraphPad Software Inc., La Jolla, CA). Comparison between the means of a single treatment and control were performed with Student's *t* test. Comparisons between multiple treatment group means to a control were performed with 1-way ANOVA and Dunnett's post-test. Data are expressed as the mean $\pm$ standard deviation (SD). Differences between means were considered to be significant at $P < 0.05$.

Results

Effect of microRNA-3181 and microRNA-3136-3p on FCGRT 3'UTR Luciferase Reporter Activity

Bioinformatic analyses revealed microRNA-3181 (NCBI Gene ID: 100422972) and microRNA-3136-3p (NCBI Gene ID: 100422859) to be the lead candidates for binding to the 3'UTR region of *FCGRT*. Dual luciferase assays were performed in CHO-K1 cells transfected with reporter constructs carrying the intact 3'UTR of *FCGRT* (*FCGRT*-3'UTR) or mutations in the 3'UTR of *FCGRT* (*mutFCGRT*-3'UTR) for the seed sequences of microRNA-3181 and microRNA-3136-3p (Figure 1A). Using the non-mutated *FCGRT*-3'UTR construct, luciferase activity was reduced for the microRNA-3181 mimic at all concentrations tested (e.g., 70.1% reduction relative to control at 10 nM, *** $P < 0.0001$. Figure 1B), but not for the microRNA-3136-3p mimic (Figure 1C). Co-transfection of

microRNA-3181 mimic and inhibitor (10 nM each) restored luciferase activity (~117% relative to control at 10 nM, $*P < 0.05$. Figure 1D). To confirm microRNA-3181 binding to the predicted seed site in the 3'UTR of *FCGRT*, luciferase assays were conducted using the *mutFCGRT*-3'UTR vector. No statistically significant reductions in luciferase activity were observed at any concentration of microRNA-3181 mimic tested (Figure 1E).

Effect of microRNA-3181 on FCGRT mRNA Expression in A549, HEK293 and HepG2 Cells

The effect of microRNA-3181 mimic and inhibitors on *FCGRT* expression was examined in A549, HEK293 and HepG2 cells. The expression of *FCGRT* was significantly reduced in all three cell lines 24 hours following a 25 nM transfection with the microRNA-3181 mimic (A549: 48.6% $*P < 0.05$, HEK293: 51.3% $*P < 0.05$, HepG2: 43.5% $*P < 0.05$; Figure 2A,C,E). The expression of *FCGRT* mRNA was also reduced in a dose dependent manner 24 hours following transfection with the microRNA-3181 mimic in A549 cells (28.5%, 45.6%, 54.6% and 57.0% reduction relative to control at 1, 5, 10 and 25 nM, respectively; Supplemental File A). Corresponding transfections with the microRNA-3181 inhibitor increased *FCGRT* expression, in a dose dependent manner (11.9%, 21.4%, 42.9% and 40.6% increase relative to control at 1, 5, 10 and 25 nM, respectively; Supplemental File A). To confirm the role of the individual microRNA-3181 mimic and inhibitor in the modulation of *FCGRT* expression, co-transfections were performed in each cell line. The co-transfection of microRNA-3181 mimic and inhibitor (25 nM each) did not significantly reduce or increase the expression of *FCGRT* mRNA in cells 24 hours post transfection (Figure 2B,D,F).

Effect of microRNA-3181 on FCGRT Protein Expression in A549 Cells

The effect of microRNA-3181 mimic and inhibitors on FcRn protein expression was determined by immunoblotting in A549 cells at 24 and 48 hours post transfection. A human FcRn recombinant peptide was used to determine primary antibody specificity, while a molecular weight marker was used to confirm protein band identity in cell lysates. A band corresponding to the predicted molecular weight of FCGRT was observed at ~ 39 kDa (Figure 3E). Transfections with the microRNA-3181 mimic or inhibitor did not have a significant effect on the expression of FcRn after 24 hours (Figure 3A & B). Expression of FcRn was significantly reduced by 40% in A549 cells 48 hours post transfection with the microRNA-3181 mimic (** $P < 0.001$. Figure 3C). The microRNA-3181 inhibitor did not have an effect on FcRn protein expression 48 hours post transfection (Figure 3D).

Expression of microRNA-3181 in A549 Cells and Human Liver

The expression of microRNA-3181 in A549 cells was evaluated following inhibitor transfections. Consistent with the observed increase in *FCGRT* mRNA expression following microRNA-3181 inhibitor transfections (25 nM), a 58.8% decrease in endogenous microRNA-3181 expression was observed 24 hours post transfection (Figure 2G). Endogenous microRNA-3181 was detected in total RNA extracted from human liver tissue (Figure 2H). Expression of microRNA-3181 showed an approximate 3-fold variation between specimens. An exploratory correlation analysis did not indicate a significant correlation between hepatic *FCGRT* mRNA and microRNA-3181 levels (Supplemental File A).

Discussion

There is a relative paucity of studies focused on epigenetic determinants of variable *FCGRT* gene expression in humans. In this study we focused on two microRNAs, microRNA-3181 (chromosome 16) and microRNA-3136-3p (chromosome 3) as potential regulators of *FCGRT*. We identified human microRNA-3181 as an epigenetic regulator of *FCGRT* expression. FcRn expression in the lung regulates IgG concentrations through recycling and transcytosis (28). In addition, relatively high expression of FcRn in both cancerous and nearby healthy pulmonary tissue was significantly associated with overall survival in a retrospective analysis of patients with non-small cell lung adenocarcinoma (24). The expression of FcRn was also observed to be lower in cancerous tissue compared to nearby healthy lung tissue. Here we used the FcRn expressing cell lines A549, HEK293 and HepG2 as models for exploring the regulation of *FCGRT* by microRNA-3181. In A549 cells, transfection of a microRNA-3181 mimic reduced *FCGRT* mRNA and FcRn protein expression. Interestingly, microRNA-3136-3p, which shares more than 50% of its seed binding sequence with microRNA-3181, had no apparent binding to the 3'-UTR of *FCGRT*. The expression of endogenous microRNA-3181 in A549 cells decreased after transfections with a specific inhibitor of microRNA-3181, which also resulted in increased expression of *FCGRT* mRNA.

Next generation small RNA sequencing endeavors have previously identified microRNA-3181 in human tissues and cell lines (29, 30). No secondary validation of microRNA-3181 or its interaction with gene targets have been reported. In addition, no gene targets of microRNA-3181 have been experimentally identified. Therefore, we confirmed the expression of mature microRNA-3181 transcripts in human liver tissue specimens using qRT-PCR. Hepatic FcRn has been demonstrated to be critical for maintaining proper concentrations of IgG and albumin in blood and preventing their catabolism (31). The presence of microRNA-3181 in human liver tissue warrants studies to explore whether microRNA influences the hepatic expression of *FCGRT*.

The identification of novel inducers and inhibitors of FcRn expression, such as microRNA-3181 mimics or inhibitors, could have pharmaceutical applications. Further improving the pharmacokinetic properties of therapeutic antibodies through manipulation of the drugs interaction with FcRn is an active area of research. Traditional therapeutic antibody engineering efforts have focused largely on tailoring the interaction between FcRn and Fc region of IgG to alter association and dissociation rate constants at acidic and neutral pH (32–34). Alternatively, nullifying the effect of FcRn on serum immunoglobulins through direct FcRn inhibition has been explored, with examples being anti-FcRn antibodies, small molecules, and peptides (35–38). FcRn inhibitors and inducers may prove useful for influencing concentrations of endogenous immunoglobulins as well as the required doses for therapeutic antibodies. One theorized application for an FcRn inhibitor would be to improve intravenous immunoglobulin (IVIG) therapy in chronic immune conditions (39, 40). The work shown here identifies a potential alternative approach to direct inhibition of FcRn function through manipulation of the expression of FcRn using engineered versions of endogenous microRNAs like microRNA-3181.

Our findings provide a foundation to study the effect of microRNA-3181 on FcRn mediated transcytosis and recycling of antibody therapeutics in relevant *in vitro* systems. Additionally, microRNA-3181 may contribute to interindividual variability in FcRn expression. Thus, future studies should investigate whether microRNA-3181 contributes to variable *FCGRT* and FcRn expression in different tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FcRn	Fc-neonatal Receptor
microRNA-3181	hsa-miR-3181
microRNA-3136-3p	hsa-miR-3136-3p
<i>FCGRT</i>	Fc-neonatal receptor gene
3'UTR	3-prime untranslated region
VNTR	Variable number tandem repeat
IVIG	Intravenous immunoglobulin

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Figure 1.

Schematic representation of the *FCGRT-3'UTR* reporter construct showing potential microRNA-3181 and microRNA-3136-3p seed region binding sites (A, top). Representation of *mutFCGRT-3'UTR* reporter construct showing nucleotides altered within the microRNA seed binding regions (A, bottom). Luciferase activities of *FCGRT-3'UTR* construct following microRNA-3181 mimic (B), microRNA-3136-3p mimic (C), and microRNA-3181 mimic plus inhibitor (D) co-transfections in CHO-K1 cells. Effect of microRNA-3181 mimic and *mutFCGRT-3'UTR* report construct co-transfections in CHO-K1 cells (E). In all experiments, luciferase signal was first normalized to co-transfected pRL-TK mediated renilla luciferase signal, and it is expressed relative to the control transfection (microRNA mimic negative control, microRNA inhibitor negative control, or combination of microRNA mimic and inhibitor negative control). Data represent the mean $\pm$ SD of eight separate transfections. *** $P < 0.0001$, (1-way ANOVA, Dunnett's test).

Figure 2.

Expression of *FCGRT* and microRNA-3181 in A549, HEK293 and HepG2 cells. Cells were transfected with 25nM microRNA-3181 mimic (A, C, E) or a combination of mimic and inhibitor (B, D, F), with *FCGRT* expression determined after 24 hours. Data represent the mean $\pm$ SD of three independent experiments (* P <0.05, Student's t test). In all plots, data are shown relative to negative control mimic or a combination of negative control mimic plus inhibitor transfections with expression normalized to *ACTB* expression. Expression of endogenous microRNA-3181 in A549 cells 24 hours following microRNA-3181 inhibitor transfection (G). Data represent the mean $\pm$ SD if two independent experiments. Data are shown relative the inhibitor negative control transfections with expression normalized to *hsa-miR-93* expression. Expression of endogenous microRNA-3181 in hepatic tissue specimens (H). Hepatic expression is shown relative to the lowest expressing sample, and normalized to *U6* and *hsa-miR-93* expression.

Figure 3.

Expression of FcRn in A549 cells following microRNA-3181 mimic (left) and inhibitor (right) transfections. A549 cells were transfected with microRNA-3181 mimic and FcRn expression determined 24 hours (A) and 48 hours (C) post-transfection. Expression of FcRn in A549 cells 24 hours (B) and 48 hours (D) following microRNA-3181 inhibitor transfections. FcGRT band densitometry values are normalized to beta-actin band density, and expressed relative to negative control transfections. Plots represent the mean $\pm$ SD of three independent transfections. ** $P < 0.001$, (Student's *t* test).