Mechanisms of Fenofibrate-induced MiR-3189-3p Expression in Glioblastoma

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Introduction

Glioblastoma is the most aggressive type of brain tumor and is characterized by rapid and infiltrative growth. Current therapeutics involving surgical resection, radiation and chemotherapy are largely ineffective, rendering glioblastoma essentially untreatable. MicroRNAs are small, non-coding sequences of single stranded RNA that regulates gene expression by incomplete base paring with messenger RNAs. They bind to the 3' untranslated region of mRNAs and inhibit translation. We previously found that miR-3189-3p inhibits proliferation and migration of glioblastoma cells. Interestingly, expression of this microRNA is low in clinical samples of astrocytomas and glioblastomas, suggesting that a forced expression of miR-3189-3p in these tumors has the potential to slow down or inhibit their growth and migration. Previous studies have shown that miR-3189-3p is co-encoded with Growth Differentiation Factor 15 (GDF15), both of which are up regulated by treatment with the cytotoxic agent, Fenofibrate.

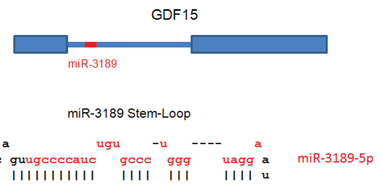

Figure 1. Schematic of the GDF15 gene encoding miR-3189 within the exon and of the stem-loop containing -3p mature microRNA.

GDF15 is a secreted protein belonging to the Transforming Growth Factor-β (TGF-β) superfamily. GDF15 is expressed in response to a variety of stimuli and is known to play roles in various aspects of cellular function including differentiation, proliferation, apoptosis, and regulation of the inflammatory response via Signal Transducer and Activator of Transcription 3 (STAT3) signaling.

Fenofibrate is a peroxisome-proliferator activated receptor alpha (PPAR-α) agonist that is a lipid-lowering drug commonly used for the treatment of cardiovascular disease and various metabolic disorders. Although identified as a PPAR- α agonist, Fenofibrate can also activate PPAR- α independent pathways. In the current study, we have shown that Fenofibrate-mediated upregulation of GDF15 and miR-3189-3p is PPAR- α independent. Furthermore, we have demonstrated that miR-3189-3p expression induces STAT3 phosphorylation, however the specific kinase activity remains to be determined.

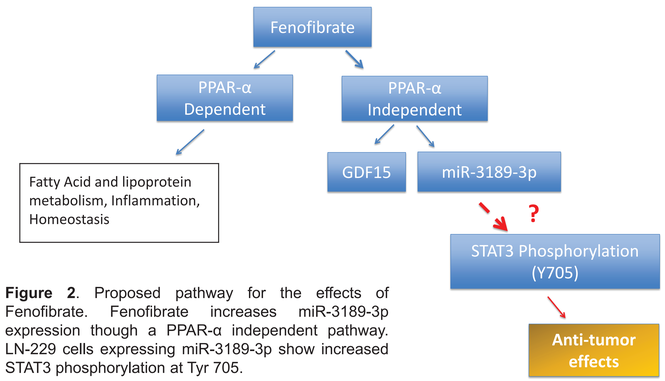

Figure 2. Proposed pathway for the effects of Fenofibrate. Fenofibrate increases miR-3189-3p expression though a PPAR-α independent pathway. LN-229 cells expressing miR-3189-3p show increased STAT3 phosphorylation at Tyr 705.

Methods

LN-229 samples were cultured for 48 hrs under standard growth conditions. For transfection experiments, cells were collected at a density of $4x10^5$/60mm dish and transfected using Lipfectamine 2000 per manufacturer's instruction.

Western blots were performed by scraping the cells in the presence of PBS, followed by centrifugation and disruption of cell pellet in standard lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, pH 8.4, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, Phosphatase Inhibitor and Protease Inhibitor Cocktails (Sigma-Aldrich).

Whole-cell lysates (30 to 40 µg) were separated on a 4–15% SDS-PAGE gel. Phospho-STAT3 antibody were purchased from Cell Signaling Biotechnology. Total STAT3 antibody was from BD Transduction. Anti-14-3-3ζ antibody was purchased from Santa Cruz Biotechnology. AG490 was purchased from EMD Millipore. Tofacitamib Citrate was obtained from Sigma- Aldrich.

For experiment 1, LN-229 cells were treated with 10 mmol PPAR-α antagonist (GW-9662, Enzo Life Sciences) or transfected with 10 µmol siPPAR-α (Santa Cruz) for 24hrs prior to stimulation with 50 µmol Fenofibrate (Sigma-Aldrich). Cells were lysed 48 hrs after incubation with Fenofibrate. For quantitative RT-PCR, total RNA was isolated using the miRVana miRNA extraction kit . RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit for mRNAs or TaqMan assays for microRNAs (Applied Biosystems). Quantitative real-time PCR was performed in duplicate using a Roche LightCycler 480 Real-Time PCR System. Each sample was normalized using GAPDH or RNU6B control (ΔCt) and relative quantification of gene expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. miR-3189-3p mimic and miR-3189-3p inhibitor were purchased from Ambion.

Results

1) **Fenofibrate-mediated expression of GDF15 and miR-3189-3p is PPAR-α independent.** Quantitative RT-PCR analysis, as expected, revealed increased expression of both GDF15 and miR-3189-3p in all samples treated with Fenofibrate and the addition of the PPAR-α inhibitor (Fig. 3A) or the silencing of this receptor through siRNA (Fig. 3B) did not significantly changed the levels of expression of these two molecules. The effectiveness of siPPAR-α in downregulating PPAR-α mRNA was also evaluated by quantitative RT-PCR in all the samples and results in Figure 3B show this downregulation.

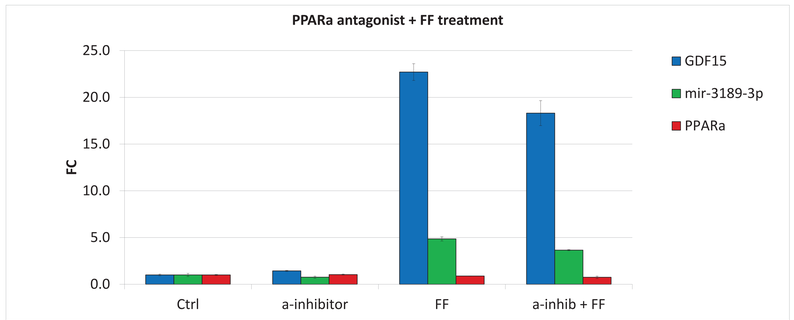

Figure 3A: Real Time PCR. Fenofibrate treatment up regulates miR-3189-3p and GDF15 expression in the presence of PPAR-α antagonist in LN-229 cells.

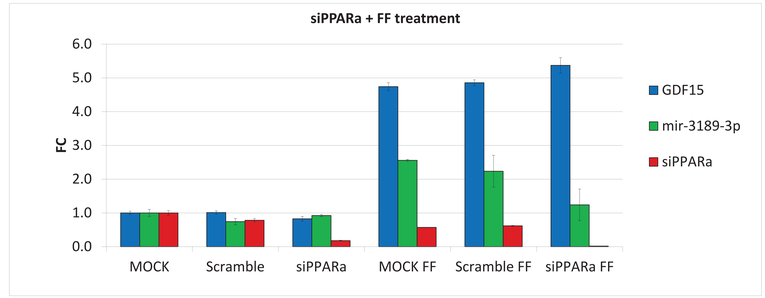

Figure 3B: Real time PCR to detect levels of GDF15, miR-3189-3p and PPAR-α. Fenofibrate treatment up regulates miR-3189-3p expression in in the presence of siPPAR-α. LN-229 cells were incubated for 24h with siPPAR-A before addition of Fenofibrate.

2) **Expression of miR-3189-3p results in increased phosphorylation of STAT3.**

Western blot analysis using an antibody specific to Phospho-STAT3(Y705) revealed increased levels of P-STAT3 in cells expressing miR-3189-3p when compared to controls (Figure 4). The phosphorylation was clearly strong at both 24 and 48 hrs post transfection and it was specific to miR-3189-3p, since the anti-miR-3189-3p (miRNA Inhibitor) was neutralizing this effect. Next, we asked what is the pathway that leads to miR-3189-3p-mediated STAT3-phosphorylation. Since JAK2 is a known activator of STAT3 we utilized a JAK2 inhibitor to determine its effects on STAT3 phosphorylation in our experimental conditions. To this end, we first tested the efficacy of two different JAK2 inhibitors (AG490, JAK2 specific inhibitor, and Tofacitamib, a pan-JAK inhibitor) in the following experimental setting: LN-229 cells were serum starved for 72 hrs prior to stimulation with EGF for 10 minutes. Results indicate that AG490 is inhibiting STAT3 phosphorylation more efficiently than Tofacitamib, and therefore it will be utilized for further experiments.

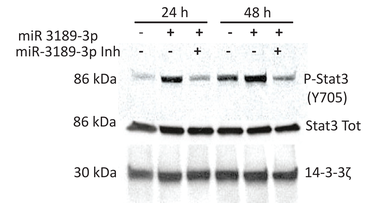

Figure 4; Western blot showing an up regulation of STAT3 at 24 and 48 h over total stat3. Anti-14-3-3ζ antibody was used as a loading control.

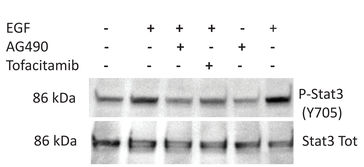

Figure 5; Western blot showing inhibition of phosphorylated STAT3 and total STAT3 at 72h using AG490 and Tofacitamib.

Conclusion

In the current study, we found that increased expression of GDF15 and miR-3189-3p by Fenofibrate treatment is PPAR-α independent. We also found that increased expression of miR-3189-3p results in increased levels of phosphorylated STAT3. Interestingly, in glioblastoma cells, STAT3 functions as a molecular hub, linking extracellular signals to transcriptional control of proliferation, cell cycle progression and immune evasion. Since miR-3189-3p inhibits cell proliferation, it remains to be determined what is the role of phospho-STAT3 in miR-3189-3p-mediated effects.

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