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Della Simmons
dellasimmons1219@gmail.com

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Investigating the role of miR-190 and miR-200/8 inhibition of the GCS protein in HeLa cells and *Drosophila melanogaster*.

Della Simmons

Mentor- Anya Lyuksyutova

University of Wyoming

Department of Molecular Biology

Project Abstract

Glucosylceramide synthase (GCS or GlcT-1 in *Drosophila*) is a key enzyme in sphingolipid metabolism and obesity. Pharmacological inhibition of GCS in obese mice improves their insulin responsiveness, adipocyte functions, and also reduces inflammation and liver fat content. MicroRNAs are non-coding RNAs that specifically inhibit genes by binding to target sites in the gene's mRNA.

In previous experiments, through the use of transfection, RNA isolation, reverse transcription, and qPCR, we have observed that GCS mRNA levels are decreased in the presence of miR-190 and miR-8/200. This data suggests that our controls have about 2 fold the amount of RNA than our experimentals. If the microRNAs are destroying or inhibiting the RNA, then there should, theoretically, be less RNA in the experimentals as compared to our controls. We have obtained conclusive data regarding human HeLa cells and we are currently in the process of obtaining additional human HeLa cell data and *Drosophila* larvae and fat body samples/data.

Based on preliminary evidence and experimentation that GCS mRNA is regulated by microRNAs and contains well-conserved target sites for microRNAs miR-190 and miR-8/miR-200, we propose to further investigate whether these microRNAs play a direct role in GCS regulation in both HeLa cells and *Drosophila melanogaster*. Further experimentation will include: Further investigation of the effect of miR-8 and miR-190 up regulation on GCS mRNA levels and GCS inhibition in *Drosophila* cells, investigating the effect of microRNAs on overexpressed GCS protein with scrambled miR-190 and miR-200 binding sites in human HeLa cells (to date, the scrambled binding sites that we are planning on using have been determined), investigating the effect of microRNAs on overexpressed GCS protein with scrambled miR-190 and miR-8 binding sites in *Drosophila melanogaster* (to date, the scrambled binding sites have

not yet been determined), and, if the effects of the microRNAs proves to be direct, investigating the effect of these microRNAs in vivo.

PROJECT DESCRIPTION

Background and Significance

Glucosylceramide synthase (GCS) catalyzes the formation of glucosylceramide from ceramide and uridine diphosphate-glucose. GCS is an evolutionary conserved protein, which implies that GCS serves a similar essential function in all organisms. The product of GCS activity, glucosylceramide, is a core precursor molecule for production of most glycosphingolipids, which are major membrane components in lipid micro-domains and have been implicated in various important cellular processes (Watanabe et al., 2010)¹. *Drosophila* fat body resembles the adipose tissue and liver of mammals, and metabolizes and stores nutrients primarily as triacylglycerol and glycogen (Baker and Thummel, 2007)². Levels of *Drosophila* GCS expression in the fat body regulate fat and sugar metabolism (Kohyama-Koganeya et al., 2011)³. While GCS is necessary for normal cell function, deregulation of GCS during obesity is deleterious for the organism. Recent studies have shown that glycosphingolipids are increased in obese rodents and humans. Reducing glycosphingolipid levels by pharmacologically inhibiting GCS improves adipocyte function, insulin resistance and reduces inflammation and other symptoms of morbid obesity (Aerts et al., 2007, Van Eijk et al., 2009)^{4&5}. Furthermore, pharmacological inhibition of GCS results in reduction of triglyceride levels, liver size and general improvement of the fatty liver disease symptoms in rodents (Zhao et al., 2009)⁶. However, currently there is only one GCS inhibitor approved for treatment in humans: Miglustat/Zavesca, which has a series of unpleasant side effects (Aerts et al., 2011, Venier and Igdoura, 2012)^{7&8}. Therefore, it is important to continue investigating new avenues for GCS inhibition. MicroRNAs (miRs) are small non-

coding RNAs that serve as post-transcriptional inhibitors of gene expression in plants and animals. They act by binding to complementary sites of target mRNAs to induce RNA cleavage or translational repression. GCS levels are ~2 fold higher in *Drosophila* cells deficient in microRNA processing machinery, indicating that microRNAs may be involved in regulating GCS (Rehwinkel et al., 2006)⁹. Computational predictions and genome-wide identification of microRNA targets using TargetScan and PicTar estimate many mRNAs are regulated by microRNAs, and GCS is no exception. In particular, there are two very well conserved microRNA binding sites within 3' untranslated region of *Drosophila* GCS for miR-8 and miR-190, which are also found in human GCS (miR-190 and miR-200) (Appendix 1). Through the use of transfection, RNA isolation, reverse transcription, and qPCR, we observed that GCS mRNA levels are decreased in the presence of miR-190 and miR-8/200. This data suggests that our controls have about 2 fold the amount of RNA then our experimentals (Appendix II). If the microRNAs are destroying or inhibiting the RNA, then there will be less cDNA produced from the reverse transcriptase reaction. From a previous semester of work (spring 2014), we were able to obtain two sets of conclusive data for the HeLa cells. For statistical purposes, we ran an additional set of data (Fall 2104) and were able to obtain another set of conclusive data for HeLa cells. A couple semesters ago (spring 2014), we were only able to obtain a few sets of conclusive data for *Drosophila* (one set of data from fat bodies taken from stage 3 larvae and one set of data taken from flies in the embryonic stage). Because of our lack of conclusive data for *Drosophila*, we spent much of the following semesters collecting larvae and fat bodies in order to isolate RNA. To date, we have obtained conclusive data for both human HeLa cell samples and *Drosophila* samples. Again, our proposition is to investigate miR-190 and miR-8/miR-200 regulation of the GCS levels in *Drosophila melanogaster* and human HeLa cells with respect to

investigating their effect on overexpressed GCS protein along with their effect on GCS protein with scrambled miR-190 and miR-200/8 binding sites.

Summary

The above evidence and experiments strongly suggests that microRNAs regulate GCS in human and *Drosophila* cells and that microRNAs miR-8/200 and miR-190 are likely to be responsible. In this thesis we aim to confirm these findings and further investigate whether miR-8/200 and miR-190 have a direct effect on the regulation of GCS not only in cell culture, but also in *Drosophila melanogaster*.

SPECIFIC AIMS:

Aim I: Defining expression pattern of miR-8, miR-190 and GCS in *Drosophila melanogaster*: To effectively use the *Drosophila* model system, we will first define the expression patterns of GCS, miR-8 and miR-190 in the *Drosophila* embryo, larva and adult by using *in situ* hybridization to detect the GCS mRNA and locked nucleotide in situ hybridization to detect the microRNAs. Previously published expression patterns of GCS, miR-8 and miR-190 suggest that these genes are all expressed embryonically but due to quality of some of the images it is hard to evaluate exactly which tissues have high levels of GCS.

Aim II: Investigating the effect of miR-8 and miR-190 deletion on GCS mRNA and protein levels: We will next acquire flies which lack miR-8 (Karres et al., 2007)¹⁰ and miR-190 (Brown et al., 2002)¹¹ and evaluate the levels of GCS expression in the embryos using both *in situ* hybridization technique and RT-PCR on whole staged embryos. We will measure the levels of GCS RNA by using quantitative PCR and measure GCS protein levels using Western blot and anti-GCS antibody (Kohyama-Koganeya et al., 2004)³. If miR-8 or miR-190 regulates GCS *in vivo* we expect an increase in GCS RNA in these animals.

Aim III: Investigating the effect of miR-8 and miR-190 upregulation on GCS mRNA and protein levels: Finally, we will over-express miR-8 and miR-190 using the UAS-Gal4 system and evaluate the effect of microRNA over-expression on GCS RNA and protein levels. We plan to express microRNAs specifically in fat bodies using the R4-Gal4 line as well as in whole animals using the tubulin-Gal4 line. We will measure the levels of GCS by quantitative PCR and measure GCS protein levels using Western blot and anti-GCS antibody (Kohyama-Koganeya et al., 2004)³. If miR-8 or miR-190 regulates GCS *in vivo* we expect to see a decrease in GCS RNA or protein levels.

Aim IV: Further investigation of the effect of miR-8 and miR-190 up regulation on GCS mRNA levels and GCS inhibition in *Drosophila* larvae and fat bodies. Carrying out additional experiments to verify the effect of miR-8 and miR-190 up regulation on GCS mRNA levels and GCS inhibition in *Drosophila*. The process of collecting *Drosophila* larvae and fat bodies in order to isolate RNA for further testing includes mating, breeding, and the collecting process. Additional experiments would include isolation of RNA from these fat bodies and larvae, RT-PCR of the isolated RNA, and obtaining qPCR results for *Drosophila* that would verify whether miR-8 and mirR-190 were having an effect (specifically an inhibitory effect) on GCS mRNA levels within the fat bodies/larvae of *Drosophila*.

Aim V: Investigating the effect of microRNAs on overexpressed GCS protein with scrambled miR-190 and miR-8 binding sites in *Drosophila melanogaster*. In order to investigate whether or not the effects of miR-190 and miR-8 on GCS in *Drosophila melanogaster* is direct, we are going to produce *Drosophila* larvae that are expressing GlcT-1 (GCS) with a scrambled binding site for either miR-190 or miR-8 (we will be doing the expression of one scrambled binding site at a time). Once the binding site for either miR-190 or

miR-8 have been scrambled, the microRNA will no longer be able to bind to the protein. Therefore, if the effects of the microRNAs are direct, we should see no inhibition of the GCS protein. However, if there is still inhibition of the GCS protein, it suggests that the microRNAs are indirectly inhibiting GCS.

Aim VI: Investigating the effect of miR-200 and miR-190 upregulation on the GCS mRNA and protein levels in human HeLa cells: Run experiments with cells transfected with mir-200, cells transfected with mir-190, and untransfected cells (experiments include transfection, RNA isolation, RT-PCR, and qPCR). These experiments will allow us to determine the effect of microRNA upregulation on GCS *in vitro*.

Aim VII: Investigating the effect of microRNAs on overexpressed GCS protein with scrambled miR-190 and miR-200 binding sites in human HeLa cells. In order to investigate whether or not the effects of miR-190 and miR-200 on GCS in human HeLa cells are direct, we are going to overexpress GCS with a scrambled binding site for either miR-190 or miR-200 (we will be doing one scrambled binding site at a time). Once the binding site for either miR-190 or miR-200 has been scrambled, the microRNA will no longer be able to bind to the protein. Therefore, if the effects of the microRNAs are direct, we should see no inhibition of the GCS protein. However, if there is still inhibition of the GCS protein, it suggests that the microRNAs are indirectly inhibiting GCS.

Aim VIII: If effects of microRNAs prove to be direct, the next step would be to investigate the effect of these microRNAs *in vivo*. If the results of the experiment with the scrambled binding sites prove that the effect of the microRNAs on the inhibition of GCS is direct (there is no inhibition when the scrambled binding sites are present), then our next step would be to test the inhibition, or rather the effect, of the microRNAs *in vivo*. We would do this in one of two

ways. One way in which this could be done is to create transgenic mice in which we would inject into them the DNA encoding for the microRNAs. We could do this in both regular, healthy mice and fat/diabetic mice and record the effect the microRNAs have on the livers of these mice (looking at fatty liver disease). Another way we could pursue investigating this is to overexpress the microRNAs in *Drosophila* (perhaps have the overexpression occur in certain tissues) and look at tissue samples to see what the overexpression of the microRNAs is having on the tissues.

Pitfalls and Limitations:

Our current experimental data does not rule out that miR-190 and miR-200/8 have an indirect effect on GCS. If the proposed experiments confirm that the effect is indeed indirect, although this is still an interesting result, we will have to then switch our investigation to other predicted binding targets of miR-190 and miR-200/8 for having an effect on GCS levels.

For the several semesters, we have been having trouble moving forward with the experiment/research. The processes of mating the flies, waiting for the larvae to come out, and either collecting the larvae or the fat bodies have been long. In order to isolate adequate amounts of RNA to work with, we are trying to collect enough larvae and fat body tissue samples. Along with these limitations, we have had a few minor issues with ordering the correct kits (RNA isolation kit and RT-PCR kit) and having them arrive at reasonable times. Both of these limitations have caused the slow progression of our research, resulting in a lack of results and the lack of change/progress in the current research proposal.

RESEARCH DESIGN and METHODS:

During this project, we will be using several basic techniques including: designing scrambled binding sites for miR-8 and miR-200, designing mRNA constructs, co-transfection, fly genetics, fusion PCR, RNA isolation (RNeasy Mini kit), and quantitative PCR (Biorad SYBR Green). I

(D. Simmons) have previously and successfully performed qPCR, RNA isolation, RT-PCR, and transfection techniques in this lab, while A. Lyuksyutova (My mentor/ research instructor) has experience with all the other techniques mentioned and will train me (D. Simmons). The instruments we will be using to perform these techniques include: BioRad MyiQ Single Color Real Time PCR Detection System and BioRad precast gels. The fly stocks we will be using will be acquired from Bloomington Drosophila Stock Center. Jay Gatlin has kindly provided the HeLa cells.

Discussion

My research was focused on the effects of microRNAs (specifically miR-8, miR-200, and miR-190) on the protein GCS. To review, glucosylceramide synthase (GCS; also called Glct-1 in *Drosophila* and UGCG in humans, both of which we worked with) is a key enzyme in sphingolipid metabolism. MicroRNAs are non-coding RNAs that are believed to specifically inhibit genes by binding to target sites in the gene's mRNA. Because GCS is an evolutionary conserved protein and it contains well-conserved target sites for the microRNAs miR-8/ miR-200 and miR-190, we wanted to see if these particular microRNAs were having some sort of inhibitory effect on the GCS protein and if this effect was directly caused by the microRNAs. In order to increase our evidence/findings, we decided to work with both *Drosophila Melanogaster* and human HeLa cells.

From previous semesters of research, we were able to obtain conclusive data for human HeLa cells that proved that the miRNAs miR-190 and miR-200 were having some sort of inhibitory effect on the GCS protein. Although we did not obtain conclusive data for *Drosophila Melanogaster* in previous semesters, we were able to collect conclusive data in later experiments. We were able to obtain conclusive data from later samples that the microRNAs

miR-190 and miR-8 were having some sort of inhibitory effect on the GCS protein found in *Drosophila Melanogaster*.

I used the *Drosophila Melanogaster* samples obtained from previous semesters to run further tests to determine the effects the microRNAs were having on the GCS protein. An effect was indicated because the cycle thresholds, when considering a QPCR reaction, for the microRNAs we tested, miR-190 and miR-8, were about one cycle slower than our controls, negmir. This data suggests that our controls have about 2 fold the amount of RNA than our experimentals. If the microRNAs are destroying or inhibiting the RNA, then there will be less cDNA produced from the reverse transcriptase reaction. The smaller amount of cDNA that is ran through the QPCR, the longer it takes for the fluorescence to appear, hence the longer CT cycles. Through this we were able to obtain the conclusive data for the *Drosophila Melanogaster* samples that we were looking for.

The experiments I ran to test the effects of microRNAs in human cells included growing and transfecting human HeLa cells, isolating RNA from these cells, obtaining cDNA from the RNA with the use of RT-PCR, and finally running a qPCR to determine whether the microRNAs were having an effect on the GCS. The idea was to transfect certain HeLa cells with the microRNAs we wanted to look at (miR-200 and miR-190) and also collect untransfected cells. Both the transfected and the untransfected cells would give us data on whether or not the microRNAs were having an effect on the RNA and therefore the protein. In order to properly transfect the HeLa cells, the transfection was done in a hood to avoid contamination of the cell culture. Transfection was done using a transfection method, which included using NeoFX and Opti (both mixtures cause the microRNAs to be incorporated into the cells). These mixtures were then mixed with the microRNAs and this final mixture was then added into wells with Hela cells.

The transfection mixture would infect the cells in the wells with the particular microRNA (miR-200 or miR-190). Along with the transfected cells, we had some cells we did not transfect. These cells would act as our controls so that we would be able to determine whether the microRNAs were having an effect.

Once the cells had gone through a life cycle (which is about 48 hours for HeLa cells) we collected the cells and isolated the RNA. We isolated the RNA because this is where the effect would be seen (the microRNAs would inhibit or cause damage to the cells' RNA). Because of translation, we know that an inhibited or damaged RNA would cause problems with any proteins that could be translated from that particular RNA. In order to isolate the RNA, we completed a washing of the cells. When you wash cells, you first add a mixture that will break down membranes and get RNA into solution. You then add this solution into a collection column. The collection column is designed to catch RNA while other cell products/materials are being washed away. Once all other cell material is washed away, the RNA is released from the collection column and collected by washing the column with a certain type of water. Once we had collected the RNA, we used a spectrometer to test the concentration and the purity of RNA. This allowed us to determine if, first off, the RNA solution was pure enough to use and second, it gave us a concentration to use. The concentration of RNA was important because we wanted each sample to have the same amount of RNA in order to eliminate any variations different concentrations would cause.

Once we had isolated and calculated our RNA yields, we reverse transcribed the RNA. Although the effect of the microRNAs is seen at the level of RNA, we are currently unable to quantify this affect. In order to quantify the effect of the microRNAs, it is essential to use DNA. This is where the reverse transcription comes into play. By reverse transcribing RNA into DNA,

we would then be able to take the DNA and quantify it. This quantification would allow us to observe any affect from the microRNAs.

In order to reverse transcribe the RNA, we created a mixture that contained the isolated RNA, dNTPs (which are the building blocks of DNA) and a reverse transcriptase (which works in the opposite way RNA polymerase would; it converts RNA into DNA). This process allowed us to obtain cDNA from the isolated RNA. We could then use this cDNA to run a qPCR. A qPCR would allow us to see if the microRNAs were having an effect on the RNA and if they were, what kind of an affect.

So, as stated above, once we had obtained the cDNA, we set up a qPCR reaction. The idea behind a qPCR reaction is to amplify the cDNA in a way that allows the machine to record the amplification (which is how the machine can quantify the effect of the microRNAs on the cDNA and therefore their effect on the RNA). The probe that we used for our qPCR was Taqman. Taqman is a fluorescence probe that allows the machine to test for changes in the cycle (CT/threshold cycle) of the cDNA. Taqman works by releasing its quencher, and therefore becoming fluorescent, at the beginning of its cycle. Through the release of the fluorescence, the machine is able to detect the amount of cDNA in the sample.

From the qPCR, we were able to obtain results for our human HeLa cell samples. The results of our qPCR runs indicated that there was an effect on the RNA by the mircoRNAs. An effect was indicated because the cycle threshold for the microRNAs we tested, miR-190 and miR-8/200, were about one cycle slower than our controls, negmir and untransfected. This data suggests that our controls have about 2 fold the amount of RNA then our experimentals. If the microRNAs are destroying or inhibiting the RNA, then there will be less cDNA produced from the reverse transcriptase reaction. The smaller amount of cDNA that is ran through the qPCR, the

longer it takes for the fluorescence to appear, hence the longer CT cycles.

It is important to note that the only difference between obtaining results from *Drosophila* and obtaining results from human HeLa cells is fat bodies have to be dissected from *Drosophila* larva that have mir-8 and mir-190 overexpressed. This overexpression was done using the UAS Gal4 system. Once these fat bodies are dissected, RNA is isolated from them.

Due to our conclusive findings with human HeLa cells, we wanted to move on to the next stage and find out whether the effects seen were being caused directly or indirectly by the microRNAs. In order to do this, I came up with scrambled binding sites for both miR-190 and miR-200 in UGCG 3'UTR (human). This means that we are planning on making DNA constructs that have scrambled binding sites where miR-190 and miR-200 would bind. If the binding sites are scrambled, then miR-190 and miR-200, in theory, will not be able to bind. If the effect of these microRNAs is direct, then there should be no inhibition/effect on the GCS protein (if they can't bind, they can't destroy or inhibit). Although we have only been able to come up with the scrambled binding sites thus far, it is a future plan to order the constructs and begin this particular step/experiment of the research. I am currently working on determining scrambled binding sites for mir-190 and mir-8 in *Drosophila melanogaster*.

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APPENDIX I: Conservation of mir8/200 and miR-190 binding sites in GCS 3'UTR

miR-190 binding site is strongly conserved in all sequenced vertebrates and *Drosophila* species

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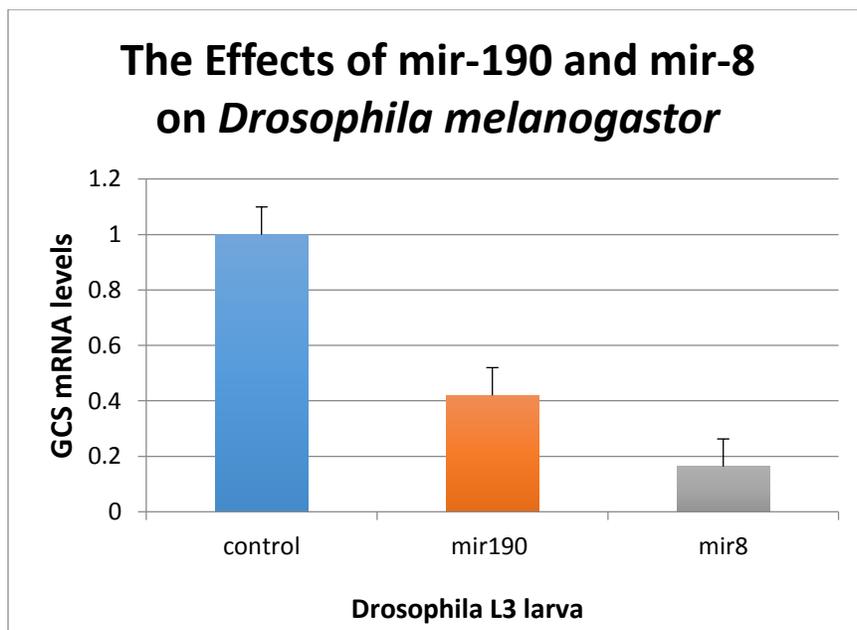
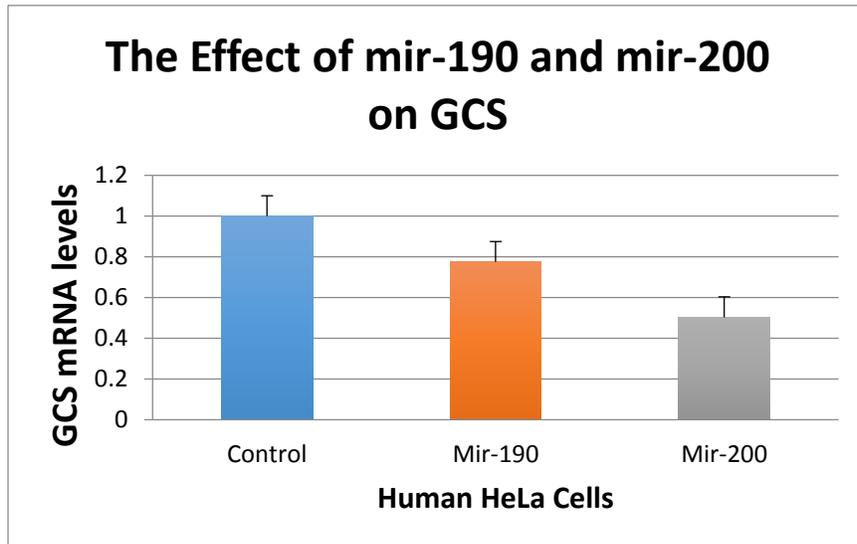
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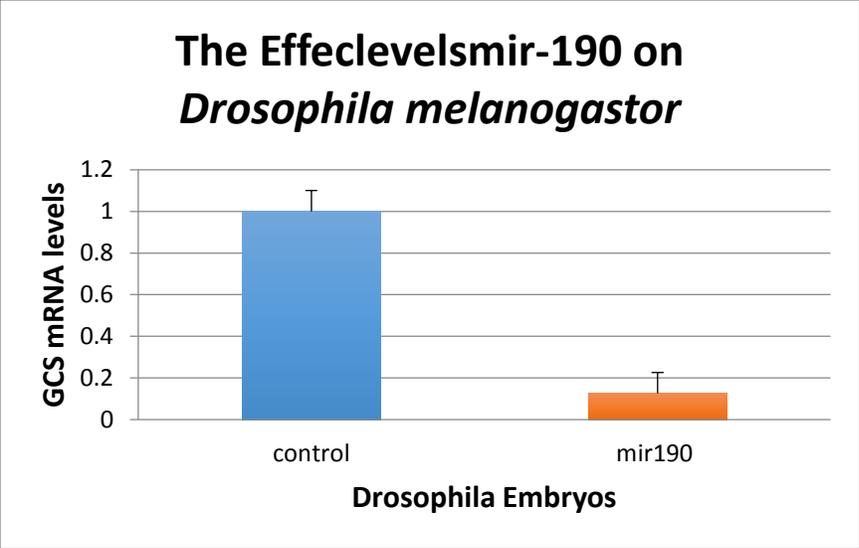
miR-8/200 binding site is strongly conserved in all sequenced vertebrates and *Drosophila* species

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Oan AAAAAAGGAA--AAAAGAGAAGUAAUUAUUUUUUAUGUUUUAUUAUUAAUUGCUUUUA--AA-AAU
Aca CAAGAAAAG--AAAAGAGAAGUAAUUAUUUUUUAUGUUUUAUUAUUAAUUGCUUUUA--AA-GAU
Gga CAAAAGAG--AAAUUGAAAGUAAUUAUUUUUUAUGUUUUAUUAUUAAUUGCUUUUA--AA-GAG
Xtr ACAAAGGAA--AAAAAAAAGUAAUUAUUUUUUAUGUUUUAUUAUUAAUUGCUUUUA--AA-GAG
miR-200bc/429/548a
Con ..AAAGGAA..AAAAGAGAAGUAAUUAUUUUUUAUGUUUUAUUAUUAAUUGCUUUUA..AA.GAU
  
```

APPENDIX II: The results of our qPCR runs indicated that there was an effect on the GCS levels by the microRNAs in human HeLa cells and *Drosophila melanogaster*.





Appendix III: The following graphs/pictures are the scrambled sites that we have come up with for the binding sites of miR-190 and miR-200 on the UGCG 3' UTR.

UGCG-3' UTR
 Has-mir-200b-3p
 Has-mir-190a-5p

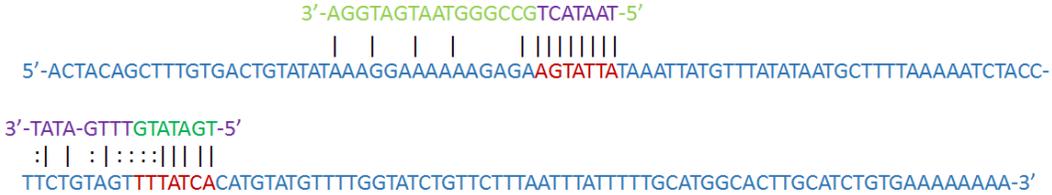


Figure I: This figure (above) shows the binding between UGCG 3' UTR and miR-190 and miR-200 before the binding sites are scrambled.

UGCG-3' UTR
 Has-mir-200b-3p
 Has-mir-190a-5p
 Scrambled regions

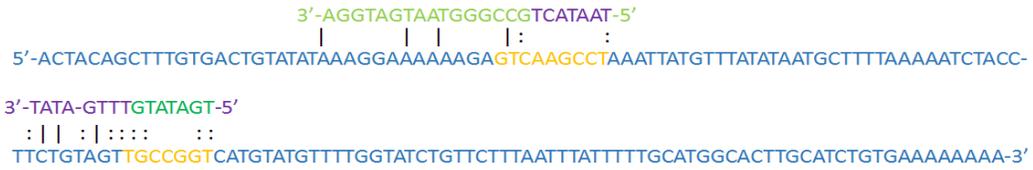


Figure II: This figure (above) shows the binding between UGCG 3' UTR and miR-190 and miR-200 after the binding sites have been scrambled.