Investigation of microRNA products: mir-675-3p and mir-675-5p, from H19, against the Bone Morphogenetic Family (BMP) proteins BMP 7, BMP 4, and BMP 11 for kidney formation during development

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Introduction

Currently, the Kidney Foundation of Canada reports that kidney disease is the 10th leading cause of death in Canada. As of the moment, there is no cure for kidney disease (Kidney Foundation of Canada, 2016). A step to furthering our treatment options is to better understand the formation of the kidney in development as research has shown that many adult and pediatric kidney diseases result from an early onset of abnormal kidney development (Bates et al. 2014). One way to better understand kidney formation during development is to study the gene H19 .

H19, a gene that regulates embryonic growth, has been implicated in a series of genetic disease which includes a pediatric kidney cancer known as Wilm's tumor. H19 also encodes two conserved micro-RNAs in its first exon, mir-675-3p and mir-675-5p, which has been associated across select organs in re-generation, proliferation, and differentiation (Liu, Li & Zhang, 2017). Currently, there exists a gap in the literature as to how H19 affects kidney formation during development; however, H19 is a player that shows promise in elucidating kidney development for the aforementioned reasons. This project will aim to study how H19 – specifically, mir-675-3p and mir-675-5p - contributes to the formation of kidney during development. In particular, it will look at its association with the bone morphogenetic protein (BMP) family.

It is important to study the interaction between mir-675-3p and mir-675-5p and members of the BMP family. This is because microRNAs are involved in processes that include differentiation, and the BMP family is ubiquitous in all organ systems for its role in development as signaling molecules (Callis et al. 2007; Wang et al. 2014). For kidney development, BMP signaling is one mediator of the interaction between the developing ureteric bud and the metanephric mesenchyme to initiate mature kidney development (Cain et al. 2008). Therefore, this project will focus on BMP 7, BMP 4, and BMP 11. Each of these proteins, when mutated or absent, are associated with kidney diseases that includes, but are not limited to, bilateral renal agenesis[1], and chronic kidney disease (CKD)[2], and renal hypodysplasia (RHD)[3] (Wang et al. 2014; Refer to Appendix A, pg. 12). Therefore, I hope to further investigate H19, and the

interaction of its microRNA products with prominent members of the BMP family that have been linked with kidney disease.

Question

What is the developmental effect on kidney formation of H19's microRNA products, mir-675-3p and mir-675-5p, on the following proteins from the BMP family: BMP 7, BMP 11, and BMP 4?

Relevance, Potential Impact, Importance

The relevance, importance, and impact of this project lies in two folds: The first is that the function of H19 during development is gaining recognition in the scientific community. This is because H19's characteristics, such as it being a genomic imprinted gene that codes for a lncRNA and microRNAs, contributes to its oncogene and regulative properties. The results obtained by studying H19's functional role for kidney development contribute to our understanding of both kidney development and overall H19 function. Furthermore, the results will provide a foundation for studying the function of H19 for organogenesis overall. One example would be the heart as kidney and heart functions are intricately linked via neurohormonal and sympathetic signalling pathways (Lorenzen and Thum, 2016). The second is that the results will contribute to the potential the microRNAs to serve as a target for therapeutic intervention. Currently, the only treatment options available for those affected by kidney disease include kidney transplantation or dialysis (Kidney Foundation of Canada, 2017). Therefore, by obtaining results, even if they are negative, our understanding of kidney development will be furthered in a meaningful way for both science and medicine.

Hypothesis

Typically, microRNAs negatively regulate gene expression by translational repression and target mRNA degradation; however, a study showed that miRNAs can selectively positively mediate gene expression if there is RNA sequence similarity (Vasudevan, Tong, & Steitz, 2007). It could be a possibility that the microRNAs encoded by H19 has sequence similarity against the interested BMP proteins because of H19's unique characteristics as a lncRNA gene (Liu, Li & Zhang, 2017).

The literature also shows evidence that associates a mutation or an absence of BMP 7, BMP 4, and BMP 11 results in absent or mal-developed kidneys at birth or post-birth: 1) BMP 7, highly expressed in the distal tubules and collecting ducts of the kidney, has been shown to be protective against CKD. 2) BMP 4 is mainly expressed in the mesenchyme surrounding the branching ureter and its mutations has been shown to be associated with RHD in individuals. 3) BMP 11 knockout mice demonstrate a spectrum of renal abnormalities, with the majority having bilateral renal agenesis (Wetzel et al. 2006). Given that the literature show that the absence or mutation of the interested BMP proteins is associated with maldevelopment of the kidneys, I hypothesize that the H19 microRNA products, mir-675-3p and mir-675-5p, work together to directly positively regulate BMP 7, BMP 4, and BMP 11, which will ultimately promote differentiation of the kidney cells to form proper kidneys.

Predictions and Proposed Experimental Plan

To test that mir-675-3p and mir-675-5p promotes proper kidney development, we will use the immortal kidney cell line HEK293 and the primary human cells, Lonza's Human Renal Cells, to observe cell morphology and the measured BMP RNA and protein levels (Kallen et al. 2014; Lonza, 2017). If the hypothesis is true, then when mir-675-3p and mir-675-5p are not present, we should expect to see in both the HEK293 and the primary human cells, an underdeveloped morphology of the cell and a decrease in both the levels of BMP 7, BMP 4, and BMP 11 RNA and protein. Conversely, if miR-675-3p and mir-675-5p are present in excess, we should expect to see in both the HEK293 and the primary human cells, an overdeveloped morphology of the cell and an increase in both the levels of BMP 7, BMP 4, and BMP 11 RNA and protein.

Experimental Plan:

Part A:

The HEK239 cell line will be obtained and subjected to cell culture in growth medium. The cultured cells will be divided into three groups: The first group will be subjected to transfection of siRNA targeting H19, the second group will be used as a negative control for **Part A**, and the third group will be used for

Part B below (Clemson et al.2009; Matoba et al. 2011). At this point, the growth medium will be switched out for differentiation medium.

Part B: Test for necessity

To test for necessity of mir-675-3p and mir-675-5p, the microRNA products will be removed. In HEK293 cells that have not been transfected with siRNAs targeting H19, they will be transfected with 2'O-methyl antisense oligonucleotides against both the microRNAs (Dey et al 2014). The HEK293 cells will subsequently be cultured differentiation medium.

Part C: Test for sufficiency

To test for sufficiency of mir-675-3p and mir-675-5p, the microRNA products will be added. We will transfect the cultured HEK293 cells, that has been knocked down for H19, with RNA duplexes that contain miR-675-3p and miR-675-5p (Dey et al. 2011). The cells will be cultured in differentiation medium.

For Parts A-C, there will be both a positive and a negative control. The positive control is to validate the efficiency of the siRNA system into the cell. The negative control is to distinguish sequence-specific silencing from non-silencing effects. The positive control that will used for Parts A-C will be the well-characterized positive control, Lamin, The negative controls that will be used for Parts A-C will be a universal non-targeting control, ON-TARGETplus (Dharmacon, 2017). For Parts B-C, the cell morphology, and the levels of RNA and protein of each interested BMP protein will be observed using qRT-PCR and a semi-quantitative Western blot.

Explanation of the proposed experiment:

HEK293 cell line was chosen as the model system instead of knock-out mice because previous studies have characterized the function of H19 with HEK293 so the cell line was chosen to be consistent (Kallen et al. 2013). The cell line also offer practical advantages for a student project: cost-effective, easy to use, and it bypasses ethical concerns associated with the use of animals. To address problems with

contamination of cells e.g mycoplasma, which can affect the results or whether the results can be confidently extrapolated to humans, the experiment will be repeated in primary human cells as they more closely mimic the physiological state of cells in vivo (Kaur and Dufour, 2012). This will help to ensure confidence in our results.

Next, when H19 is expressed, the RNA is found in the nucleus. Usually, siRNA cannot target nuclear RNA; however, studies by many groups have successfully knocked down several nuclear long noncoding RNAs (Clemson et al.2009; Matoba et al. 2011).

Finally, as I am investigating whether the microRNAs upregulates the three BMP proteins, both transcription and translation levels have to be measured. Looking at only the RNA or the protein would give an incomplete picture of what occurs at the molecular level. As I am only looking at three molecules, a qRT-PCR was chosen to detect RNA for its power and sensitivity - an advantage compared to other techniques such as Northern Blots. Similarly, a Western Blot was chosen to detect protein levels for its sensitivity to protein detection by size.

Describe and Discuss Possible Result and Discussion

Test for necessity

Result 1

Observation: An under-developed phenotype of the HEK293 kidney cells and primary cells; Decrease in RNA and protein levels of BMP 7, BMP 4, and BMP 11.

Conclusion: mir-675-3p and mir-675-5p is necessary to ensure proper development of kidney cells and the positive regulation of BMP 7, BMP 4, and BMP 11.

Inference: miR-675-3p and miR-675-5p promotes proper kidney cell development and differentiation.

Result 2

Observation: 1) An overdeveloped phenotype of the HEK293 kidney cells and primary cells; Increase in RNA and protein levels of BMP 7, BMP 4, and BMP 11 or 2) Wild-type phenotype of the HEK293 kidney cells and primary cells; No decrease or increase in RNA and protein levels of BMP 7, BMP 4, and BMP 11.

Conclusion: miR-675-3p and miR-675-5p is not necessary for both kidney cell formation and for the positive regulation of BMP 7, BMP 4, and BMP 11.

Inference: miR-675-3p and miR-675-5p does not promote proper kidney development and differentiation.

Test for sufficiency

Result 1

Observation: An overdeveloped phenotype of the HEK293 kidney cells and primary cells; An increase in RNA and protein levels of BMP 7, BMP 4, and BMP 11.

Conclusion: miR-675-3p and miR-675-5p is sufficient for both kidney cell formation and for the positive regulation of BMP 7, BMP 4, and BMP 11.

Inference: miR-675-3p and miR-675-5p promotes proper kidney cell development and differentiation.

Result 2

Observation: 1) An underdeveloped phenotype of the HEK293 kidney cells and primary cells; Decrease in RNA and protein levels of BMP 7, BMP 4, and BMP 11 or 2) Wild-type phenotype of the HEK293 kidney cells and primary cells; No decrease or increase in RNA and protein levels of BMP 7, BMP 4, and BMP 11.

Conclusion: miR-675-3p and miR-675-5p is not sufficient for kidney cell formation and is not sufficient for the positive regulation of BMP 7, BMP 4, and BMP 11.

Inference: miR-675-3p and miR-675-5p does not promote proper kidney development and differentiation.

The hypothesis would be supported if we were to observe that miR-675-3p and miR-675-5p is either sufficient or necessary for both kidney cell formation and for the positive regulation of BMP 7, BMP 4, and BMP 11. It would be strongly supported if we observe that miR-675-3p and miR-675-5p is both sufficient and necessary for both kidney cell formation and for the positive regulation of BMP 7, BMP 4, and BMP 11. If this is the case, we can further investigate if there is a dose-dependent regulation of microRNA to the BMP proteins. For example, BMP 4 dosage is critical for the primordial germ cell formation during embryonic kidney development (Nishinakamaru and Sakaguchi, 2014). This will further elucidate our understanding of the association between the microRNAs and the BMP proteins.

If we were to observe that miR-675-3p and miR-675-5p is not sufficient and not necessary for both kidney cell formation and for the positive regulation of BMP 7, BMP 4, and BMP 11, it would refute the hypothesis. It could be that the microRNA products indirectly positively regulates the BMP proteins. The literature shows that there is a BMP antagonist *Gremlin* that has an inhibitory effect on select BMP proteins at the site of ureteric budding and branching (Cain et al. 2008). In skeletal muscle cells, to promote myoblast differentiation, mir-675-3p and mir-675-5p inhibits Smad transcription factors critical for the BMP pathway (Dev et al. 2014). Future work can explore if the microRNAs negatively regulates *Gremlin* to observe its effect on kidney development.

To sum up, this student project offers an outline to study the effect of mir-675-3p and mit-675-5p against the BMP pathway during kidney development.

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Appendix A

1. Bilateral renal agenesis is a genetic disorder where both kidneys are absent at birth.
2. CKD is a condition characterized by the gradual loss of kidney function over time.
3. RHD is characterized by reduced kidney size or maldevelopment of the renal tissue.