Novel Direct Targets and Functional Roles for MicroRNA-21 in Granulosa Cells and Human Uterine Leiomyomas

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Abstract

MicroRNA-21 (miR-21) is important for maintaining optimal ovulation rates and granulosa cell viability. It is also upregulated in human uterine leiomyomas (ULMs), a disease characterized by the presence of benign tumors on the myometrium. The primary objective of this thesis was to identify miR-21 direct targets in granulosa cells that mediate its important ovarian functions. The secondary objective was to elucidate the role of miR-21 in ULMs. Gene expression and bioinformatic analysis performed on granulosa cells after miR-21 inhibition identified many potential miR-21 direct targets. Luciferase assays revealed that miR-21 regulates the 3'Untranslated Region (3'UTR) of apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (Apobec3), intestinal-specific homeobox (ISX) and ubiquitin-specific protease 30 (USP30). Further research of ISX revealed that miR-21 binds to its 3'UTR and over expression of miR-21 causes inhibition of ISX protein levels. Quantitative RT-PCR and western analysis revealed that ISX regulates scavenger receptor class B type 1 (SRB1) β,β-carotene 15,15′-monooxygenase 1 (BCMO1) and retinoic acid receptor α (RARα) in granulosa cells, genes known to be involved in steriodogenesis, embryogenesis and meiotic resumption in the ovary, respectively. Therefore, miR-21 may be regulating these functions through directly targeting ISX in granulosa cells. Investigation of miR-21 in UtM and UtLM cell lines (cell lines derived from myometrial and leiomyoma tissue, respectively) showed that miR-21 inhibits phosphorylation of elongation factor 2 and prevents expression of cleaved caspase 3 in both cell lines; findings which suggest that miR-21 is important for preventing cell death and maintaining cellular translation in these cell lines. Further research showed that miR-21 inhibits expression of programmed cell death 4 (PDCD-4). Expression analysis of PDCD-4 showed that it is highly expressed in ULM tissue when compared to paired healthy myometrial tissue. Since PDCD-4 is a known tumor suppressor that is suppressed in tumors, this finding indicates that PDCD-4 is playing an alternative role in ULMs compared to other tumorigenic tissue. Since miR-21 is also overexpressed in ULMs, miR-21 is excluded as a means of post-transcriptional gene control that gives rise to PDCD-4 induction in ULMs. Together these studies have identified novel direct targets for miR-21 in granulosa

cells and implicated one of miR-21's most well-studied direct targets, PDCD-4 in a novel functional role in ULMs.

Acknowledgements

I must first thank my advisor, Lane Christenson. A significant portion of this dissertation is made up of studies related to his RO1, while the rest is part of a project that I pursued that is outside the laboratories' focus. Dr. Christenson provided me with the necessary support to make progress on both of these projects. I would also like to thank Drs. Vivian, Petroff, Chennathukuzhi and Wolfe: members of my committee. During my committee meetings and my comprehensive exam, I was challenged to think more holistically and scientifically about the research I was pursuing. Hopefully this document shows some evidence of me rising to meet those challenges

Throughout my graduate experience, I encountered other past and current 'trainee science investigators' who have helped me survive this journey. These people include but are certainly not limited to Xiaoman, Wei-Ting, Jitu, Stephanie, Stephanie, Mena, Brad, Kyle, Darlene, Linda, Brianne, Sine, Riley, Alison, Sara, Fahe, Michelle, Caitlin, Caitlin, Amanda and Bryce. Whether we were laughing, complaining/cursing (A practice that occurred all too often) or just drinking (A practice that should have occurred far more often) our experiences together were what helped me through the difficult times and are, likely, what I will remember from my graduate experience, after I forget all the science I learned.

I must also give a special thanks to two individuals. The first is Vargheese, who essentially functioned as a second mentor to me. Vargheese gave me technical advice when I had problem plaguing one of my experiments; he also helped me better understand the overall significance of my work; and, most importantly, he gave me invaluable life coaching as I struggled to make progress on my PhD and in life, in general. The second is Martha Carletti, a former graduate student under Dr. Christenson. Her previous work laid the foundation for most of what was investigated in this dissertation. Contained in these pages is my effort to build on Martha's work.

While support from people within the discipline of science has been critical to my success, much more of the credit must go to my family. In particular, I would like to single out my parents; Sandy and David. Throughout my life they have always been my lifeline. And as I struggled through graduate school, they never ceased to lend their support even when all they should have been doing is enjoying their retirement. As for the rest of my family there are far too many to mention much less describe how they have contributed to my success in graduate school. Suffice it to say without the support of my very large family including adopted, birth and step, I would have given up long ago on completing my PhD.

And lastly, but certainly not least, I have to give the most thanks and gratitude to my fiancée, Diana. I am not sure how anyone could be more kind and generous and patient and supportive. Instead of belaboring the point of how important Diana has been to my success as a graduate student, I will simply say that, more than anything, Diana in my life has made completion of my doctorate possible.

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V. Chapter 1: Introduction

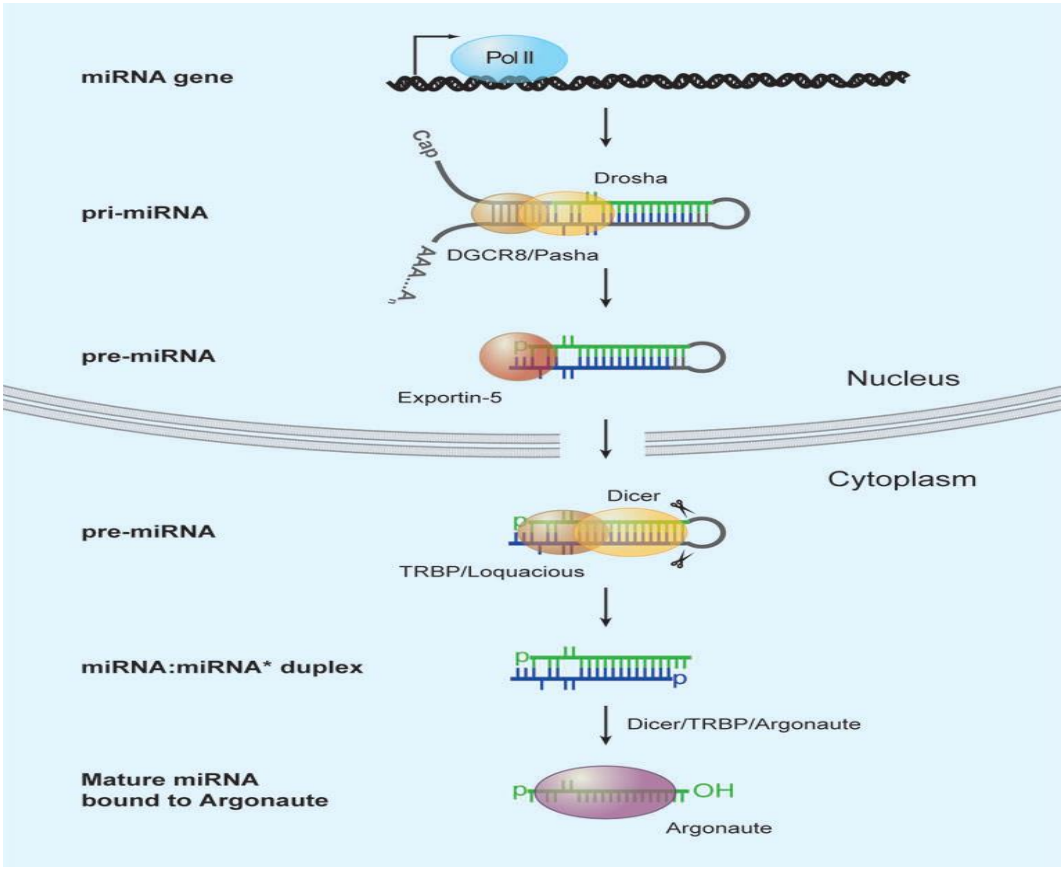
1. Post-Transcriptional Gene Regulation and MicroRNA

The concept of post-transcriptional gene regulation playing a critical role in protein expression was proposed at least as far back as 1969 [1]. Subsequent studies have shown that there is a lack of correlation between steady-state levels of mRNA and the proteins encoded by these mRNA [2, 3] indicating that post-transcriptional gene regulation is an important form of cellular gene control. In 1993, a new class of post-transcriptional gene regulators, microRNA, were discovered [4, 5]. Since their discovery, hundreds of plant and animal microRNA have been identified, which have been shown to be critical mediators of diverse physiological and pathological functions including developmental timing [4-8] organogenesis [9-12], differentiation [11-15], programmed cell death [16-18] embryogenesis [16, 17, 19-21], neurogenesis [15], angiogenesis [11], hematopoiesis [12] , exocytosis [22], tumorigenesis [23, 24], viral defense [25]and insulin secretion [22].

MicroRNA biogenesis occurs through a multi-step process (Figure V-1). Transcription of microRNA typically occurs by polymerase II (pol II) and atypically by polymerase III [26, 27]. Pol II transcribed microRNA contain, similar to mRNA, 5' cap structures, are polyadenylated and may be spliced [26, 28]. After transcription, the microRNA, in its pri-microRNA/immature form, gets processed in the nucleus by a multiprotein complex that contains the RNase III enzyme Drosha and the double stranded RNA binding domain protein DGCR8/Pasha [29]. This complex cleaves the pri-microRNA stem creating a 70 nucleotide precursor microRNA (pre-microRNA) [29]. The pre-microRNA contains a 2 nucleotide 3' overhang that is recognized by exportin 5, which transports the pre-microRNA into the cytoplasm [30]. A RNase III enzyme, Dicer, cleaves the pre-microRNA hairpin loop to produce the 22 nucleotide microRNA:microRNA duplex [31, 32]. The duplex along with Dicer binds to the argonaute protein Ago2 to form the RNA induced silencing complex (RISC) [33]. The miRNA strand with the higher stability at its 5' end is degraded while the strand with the lower stability is incorporated into the RISC where it can post-transcriptionally regulate gene expression [34, 35].

Figure V-1. MicroRNA biogenesis schematic showing compartmental process of the pri-microRNA and the pre-microRNA that takes place in the nucleus and cytoplasm, respectively. Drosha in the nucleus and Dicer in the cytoplasm are two RNase III enzymes important for successful biogenesis of microRNA into its mature form. Exportin five transports the pre-microRNA out of the nucleus. After successful biogenesis the mature microRNA binds to argonaute 2 where it can post-transcriptionally regulate gene expression [36].

Figure V-I


miRNA gene
Pol II
pri-miRNA
Cap
Drosha
DGCR8/Pasha
AAA...A
pre-miRNA
Exportin-5
Nucleus
Cytoplasm
pre-miRNA
Dicer
TRBP/Loquacious
miRNA:miRNA* duplex
Dicer/TRBP/Argonaute
Mature miRNA
bound to Argonaute
OH
Argonaute

MicroRNA regulate gene expression through incorporation into the RISC [37, 38]. Extensive studies have shown that microRNA/RISC target the 3'UnTranslated Region (3'UTR) of an mRNA to which the microRNA has complementarity [39-43]. The first animal miRNA targets were identified in *Caenorhabditis elegans,* which showed that lin-4, the 1st identified microRNA, bound to a complementary sequence in the 3'UTR of lin-14 [4, 5]. Early investigations showed that this binding event regulated gene expression through translational repression having no effect on steady-state mRNA levels [4, 5, 44, 45]. One study showed that Let7a microRNA repressed targets were all skewed to the top of the polysomal gradients where mRNA are not translated while the repressed mRNAs and argonaute proteins accumulated in processing bodies [39]. This indicated that Let7a regulated its targets through translational repression. More recent investigations, however, have revealed that mRNA destabilization and repression are prominent mechanisms by which microRNA regulate gene expression [14, 40-42]. A study investigating miR-122 and its target Cat-1, showed that miR-122 repression of Cat-1 protein caused moderately reduced levels of Cat-1 mRNA levels [14]. Further investigations have revealed that microRNA cause widespread, albeit moderate, changes in mRNA expression levels [41, 46]. One seminal study showed that down regulated mRNA caused by overexpression of a microRNA were overrepresented with sequence motifs in their 3'UTR that were complimentary to the overexpressed microRNA [42], suggesting that, at least, some microRNA direct targets show changes in their steady-state mRNA expression.

To help identify direct targets of microRNA, prediction algorithms have been developed. Studies have shown that free energy of microRNA/mRNA duplex as well as conservation of target sites in related genomes are important in determining specificity and efficiency of binding between microRNA and mRNA 3'UTRs [43, 47, 48]. These algorithms have evolved with the newest iterations starting in 2003, which have now factored in these experimentally derived concepts [21, 49-52]. Two published studies, showed that previously published microRNA/mRNA binding reactions could be identified using the

newest iterations of these algorithms [21, 50]. This finding suggests that it is possible to identify microRNA/mRNA binding events using these algorithms.

These computational methods have suggested that there are two classes of microRNA targets. The first class has perfect sequence complementarity of the mRNA target sequence to that of the 5' end of the microRNA (typically this perfect complementarity consists of seven consecutive nucleotides at the 5' end of the microRNA) and does not require additional base pairings [19, 50, 53, 54]. The second class has imperfect pairing at the 5' end that is compensated with additional pairing at the 3' end of the microRNA [19, 50, 53, 54]. Studies in vertebrates, flies and nematodes showed that detectable sites in the first class were more abundant than that of the second class and that these sites were conserved across species [53]. Algorithms that factor in both classes of microRNA/mRNA target recognition and that utilize cross-species conservation within its algorithm, suggest that there is widespread similarity of genes regulated by microRNA between insects and vertebrates as well as between flies and mammals [53]. Computational algorithms that emphasize cross-species conservation, predict 80-90 percent of the same microRNA binding sites when the same data sets of human 3'UTRs were filtered through their respective algorithms [49, 53]. Together these algorithms estimate that, at least, 30% of all human 3'UTRs are regulated by conserved microRNA [54, 55]. A newer method, which does not factor in cross species conservation, predicts that 74% to 94% of gene transcripts may be microRNA targets [56]. This method, however, has yet to be investigated to determine its efficacy in predicting microRNA/mRNA binding events.

2. Hormonal and Transcriptional Regulation of the Female Reproductive Tract

The female reproductive tract is important for the maturation of fertilizable oocytes, sperm capacitation, fertilization, embryological development, implantation, gestation and parturition. Steroid and peptide hormones as well as appropriately timed changes in gene expression within the ovary, oviduct and uterus are required for proper female reproductive tract function [57-65]. Abnormalities within this system can cause dysfunction and disease within and outside the tract [57, 66-68].

Within the oviduct, ovarian steroids cause morphological and biochemical changes that are important for fertilization and embryonic development [69, 70]. Studies have also shown that several oviductal proteins are induced and secreted within the oviduct that are important for binding of sperm and oocyte, sperm penetration and early embryological development [69, 70]. In the uterus, the interplay of ovarian, pituitary and peptide hormones transform the endometrium of the uterus to a stage in which it is receptive to the blastocyst [71-73]. This hormonal regulation of the uterus causes widespread transcriptional changes in gene expression critical for implantation of the blastocyst [71-73].

In the ovary, both FSH and LH from the pituitary are important for oocyte development and ovulation. FSH induces many transcriptional changes as well as an increase in estradiol production, which eventually leads to the luteinizing surge of LH [74-77]. The surge leads to changes in gene expression, an increase antrum volume and follicular size and further maturation of the oocyte [74-77]. Eventually there is a breakdown of the ovarian cumulus layer surrounding the oocyte within the follicle allowing for ovulation of a fertilizable oocyte [78, 79]. Following ovulation the corpus luteum forms, which secretes progesterone, which is critical hormonal stimulation critical for maintenance of the early stages of pregnancy [80].

The period of time between the initiation of the LH surge and ovulation is the periovulatory period. During this period, LH acts on periovulatory follicles, which are mature follicles within the ovary capable of responding to the LH surge [75]. Within periovulatory follicles mural granulosa cells line the follicular wall and contain LH receptors while cumulus granulosa cells surround the oocyte and do not contain LH receptors [81]. Regulation of mural cells occurs through LH binding to its receptor, which leads to an increase in cyclic AMP and intracellular calcium levels [82, 83]. This causes stimulation of many pathways including PKA and MAPK/ERK [84-87] leading to vast changes in gene expression through transcriptional control [88-92].

3. MicroRNA Regulation of Female Reproductive Tract

Recent research has shown that in addition to hormonal and transcriptional regulation, the female reproductive tract is also, in part, under the regulation of post-transcriptional gene regulatory mechanisms. There have been multiple studies in which Dicer, the cytoplasmic RNAse III enzyme critical for microRNA biogenesis was inactivated or downregulated in the Mullerian duct mesenchyme of the female reproductive tract in the mouse. Each of these studies resulted in a significant reproductive phenotype [93-95]. Gonzalez et al, showed that there was a reduction in the size of the uterine horns and oviducts and that cysts formed in the oviducts at the isthmus near the uterotubular junction (the junction that connects the oviduct to the uterus) [93]. These cysts caused impaired transport of oocytes as evidenced by the identification of unfertilized degenerate oocytes found within the cysts [93]. Beads transferred into the mutant oviduct also failed to transport to the uterus further suggesting that Dicer inactivation was causing impaired oocyte and embryo transport with the oviduct [93]. This study also identified a uterine phenotype, showing a reduction in glandular tissue and that the remaining glandular tissue was localized to the myometrium as opposed to its normal location in the endometrium [93]. This uterine phenotype is similar to a rare medical condition found in women known as adenomyosis in which ectopic endometrium is found in the myometrium [93]. This ectopic endometrial expression identified in these mutant mice is correlated with the ectopic expression of Wnt4 and Wnt5a in 8-week old female mice, which was not observed in wild type mice [93]. It is possible, as the authors point out, that this unique myometrial expression pattern of Wnt genes could be causing adenomyosis-like phenotype found in their mutant mice [93].

A study performed by Hong et al, also inactivated Dicer in the Mullerian duct mesenchyme of the female reproductive tract in the mouse [94]. Morphological analysis also showed that the uterus was hypertrophic and the oviduct was highly disorganized [94]. Fertilization did occur when knockout mice were bred to wild type fertile males [94]. In addition fertilized oocytes recovered on day 1 of pregnancy could be successfully developed to the blastocyst stage in culture [94]. However, fertilized oocytes

recovered on day 3 of pregnancy showed developmental defects when compared to wild type controls [94]. Similar to the Gonzalez et al study, there was impaired oviductal transport as there was no evidence of implantation on day 4 of pregnancy [94]. When wild type, fertile males were bred to the Dicer1 knockout females over a 5 month period normal mating behavior was displayed but no offspring resulted, indicating that Dicer is essential for fertility in female mice [94].

A third study performed by Nagaraja et al also inactivated Dicer in the Mullerian ducts and in the mesenchyme of female mice. Similar to findings described above there was compromised oocyte and embryo quality as well as impaired transport in the oviduct [95]. Knockdown of Dicer also caused prominent oviductal cysts and shorter uterine horns similar to findings in the studies described above [95]. Again similar to the two previously described studies, Dicer inactivation lead to female sterility [95]. Nagaraja et al, also identified genes that were induced as a result of dicer knockdown that are potential direct targets of microRNA [95]. Additionally members of the wingless family of secreted glycoproteins (WNT) as well as homeobox transcription factors (both of which function in Mullerian duct differentiation and maturation) were both induced as a result of Dicer knockdown [95].

A study performed by Otsuka et al investigated Dicer function in the female reproductive tract using an alternative approach in which a hypomorphic Dicer 1 allele was used to cause Dicer deficiency in the whole animal. This method led to corpus luteum insufficiency that, in part, was due to inadequate vascularization in the ovary [96]. Further analysis showed that adding back the microRNA miR-17-5p and Let-7b after Dicer knockdown, partially restored corpus luteum function [96]. Together these studies implicate dicer, and thereby microRNA, as being critical to proper female reproductive function.

4. MicroRNA-21 (miR-21) in the Ovary and Granulosa Cells

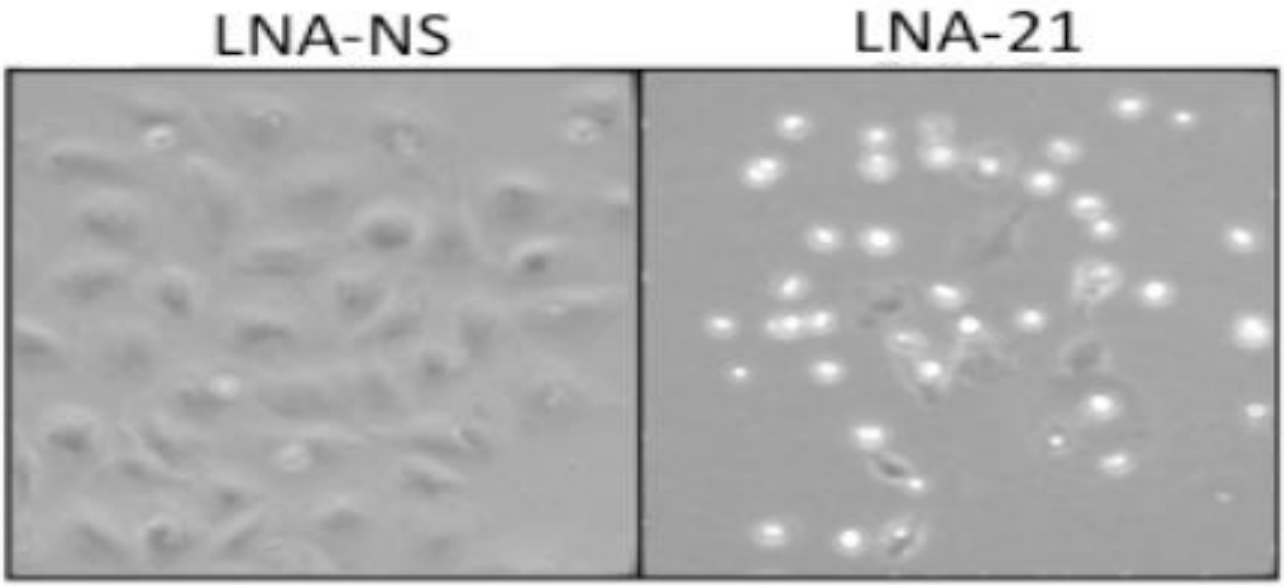
Studies from our lab have further investigated the importance of microRNA in the ovary using mouse models. Specifically our lab identified microRNA that are regulated in granulosa cells by human

chorionic gonadotropin (hCG), a bioequivalent of LH [97]. Of the 212 microRNA that were shown to be expressed in granulosa cells, 13 were dysregulated 4 hours after hCG/LH induction compared to control animals that were not stimulated [97]. Of these 13, 10 were inhibited including miR-483, miR-491, miR-484, miR-329, miR-433-3p, miR-532, miR-431, miR-672, miR-351 and miR-99b [97], while three were induced; miR-132 (16.9-fold), miR-212 (21.7-fold) and miR-21 (4-fold) [97]. QRT-PCR analysis showed that 4 hours after hCG/LH treatment miR-132, miR-212 and miR-21 were 11.4-, 20.5- and 4.0-fold upregulated, respectively [98]. Analysis of the immature forms of each showed that the precursor forms were upregulated prior to or commensurate with that of mature forms, suggesting that hCG/LH is transcriptionally regulating miR-132, miR-212 and miR-21 [97]. Both the precursor and mature forms of each microRNA remain elevated throughout the periovulatory period [97].

Further investigation into miR-21 revealed that it had an important function in the ovary and granulosa cells. Inhibition of miR-21 using a locked nucleic acid, which is an oligo complementary to miR-21 (LNA-21) in cultured granulosa cells caused upregulation of cleaved caspase 3 at 8 hours and 24 hours post-transfection while there was no increase in this apoptotic marker in control treated cells treated with a non-specific LNA (LNA-NS) [99]. Annexin V staining of granulosa cells subsequent to transfection, using the same experimental and control treatments, showed that there was a decrease in cell survival starting at 8 hours post-transfection and that at 24 hours post-transfection only 60% of the cells were alive [99]. Bright field imaging of cells 24 hours after LNA-21 treatment showed the cells rounding up and lifting off the plate (Figure V-2), indicating cell death, while LNA-NS treated cells showed no such phenotype [99]. To confirm that this in-vitro effect occurred in-vivo, 19-day old, immature, female CF-1 mice undergoing a standard 46hr PMSG/16 hr hCG treatment protocol were injected with saline (control) or LNA-21 into the ovarian bursa, 24 hours after PMSG treatment. Sixteen hours after hCG treatment animals were sacrificed and ovaries were isolated and sectioned. TUNEL staining revealed an increase in apoptosis in the LNA-21 treated ovaries [99].

Figure V-II. Bright field imaging of granulosa cells 24 hours after transfection with a non-specific LNA (LNA-NS) (control) and an LNA specific for miR-21 (LNA-21) (a locked nucleic acid that binds and inhibits miR-21 activity). Treatment with LNA-21 caused granulosa cells to round up and begin lifting off plate indicating that cells were dying. Cells treated with LNA-NS showed no such phenotype [99]

Figure V-II



Due to its important anti-apoptotic and pro-ovulatory roles, targets and pathways downstream of miR-21 were investigated. Cultured granulosa cells were treated with anti-NS (control) and anti-21 and labeled Cy3 (anti-NS) and cy5 (anti-21). Protein lysates isolated from cells were subjected to 2-dimensional gel electrophoresis and fluorescent analysis, followed by mass spectrometry. Results showed that phosphorylated forms of elongation factor 2 (EF-2) were upregulated as a result of anti-21 treatment (unpublished data). This finding was confirmed through western analysis, which revealed that phospho-EF-2 is induced in granulosa cells after treatment with anti-21 (unpublished data). EF-2 is an important factor in global translational regulation and when it is phosphorylated global translation is prevented due to EF-2's inability to bind to the ribosome [100, 101]. Induction of phospho-EF-2 after miR-21 inhibition suggests that miR-21 activity is critical for maintaining global translation. Knockdown of miR-21 caused a 3-fold decrease in the amount of S^{35} methionine incorporation in granulosa cells, compared to that of control treatments; providing further confirmation that miR-21 is regulating cellular translation (unpublished data). Further investigation revealed that factors within the AKT pathway (a pathway immediately upstream of EF-2) including AKT, TSC2, mTOR, p70S6K and 4EBP1 had reductions in their phosphorylation states after miR-21 inhibition in granulosa cells (unpublished data). This finding indicates that miR-21 activity is critical for maintaining this pathway in its active form and potentially reveals the mechanism through which miR-21 inhibits phosphorylation of EF-2, thereby maintaining this gene in its active state.

5. MicroRNA-21 (miR-21) in Cancer

Many studies that have implicated miR-21 as being a critical factor in cancer. MiR-21 was originally shown to be overexpressed in human glioblastoma in both high grade glioma samples from humans as well as a number of glioblastoma cell lines [102]. Subsequent studies investigating a vast array of cancers including hepatocellular [103], gastric cancer [104], ovarian cancer[105, 106], cervical carcinoma [107], head and neck cancer cell lines [108] papillary thyroid carcinoma [109], chronic lymphocytic leukemia [110] and diffuse large B cell lymphoma [111] have all shown miR-21 induction. In addition a study

examining aberrant microRNA expression across 540 human samples representing 6 solid tumors, showed that miR-21 was the only microRNA upregulated in all tumor types [112].

There have also been a few studies examining miR-21's mechanism of induction in cancer. Human miR-21 has been mapped to the locus 17q23.2 where it overlaps with TMEM49, a protein encoding gene [26, 113]. Amplification of this chromosomal region has been linked to a number of cancers including breast [114], Hodgkins lymphoma [115], pediatric brain tumors [116] and prostate cancer [117]. Studies have shown that 17q locus induction is, however, not concomitant with upregulation of the miR-21 locus [110, 118]. This suggests that, while miR-21 and the 17q locus tend to be induced in cancer, the two are under separate regulatory control. Studies that have investigated transcriptional regulation of miR-21 and have shown that it is transcribed independently of TMEM49, suggesting that miR-21 is under different transcriptional control than the gene within which it is embedded [113]. This finding is consistent with our results that have shown that while LH does upregulate miR-21, TMEM49 expression levels remain similar between LH treated and non-LH treated ovaries [99].

Previous studies have shown that transcription of miR-21 occurred through the classical biogenesis pathway producing a capped, polyadenylated and unspliced pri-microRNA ~3.5kb in length [26]. Transcriptional regulation of miR-21 has been found to occur through multiple, highly conserved enhancer elements including activator protein 1 (AP-1), C/EBP, nuclear factor 1 (NF-1), Ets/PU.1 and signal transducer and activator of transcription 3 (STAT3) amongst others [113]. Studies have shown that binding to these elements within the miR-21 promoter can be either inhibitory, as in the case of NF-1 and CEPB/β, or stimulatory as in the case of AP-1 and STAT3 [113, 119-121]. It has also been shown that factors can transcriptionally regulate miR-21 in one cell type but not in another. RE-1-silencing transcription factor (REST) blocks miR-21 in mouse embryonic stem cells [122], but does not regulate miR-21 in the Hdh7/7 cell line [123], a cell line derived from embryonic striatum.

Determining transcriptional control of miR-21 is critical because induction of miR-21 in cancer is in part through transcriptional regulation [124]. This is unique in that most microRNAs that are dysregulated in cancer do so without changes in their primary precursors [125], suggesting that the majority of cancer mediated dysregulation of microRNA takes place after transcription. Profiling of microRNA across human cancers has shown that most microRNA dysregulated in cancer are downregulated [124]. One interpretation of these findings is that, in addition to induction of the precursor form of miR-21, that overexpression of miR-21 in cancer may be due to its precursor being a preferential substrate for Drosha and/or Dicer.

Due to its ubiquity in cancer a number investigations have been initiated to determine miR-21's direct targets and functions. This has resulted in identification of numerous miR-21 direct targets. These include phosphatase and tensin homologue (PTEN) [126, 127], reverse-inducing-cysteine-rich protein with kazal motifs (RECK) [128], tissue inhibitor of metalloproteinases (TIMP) [128], leucine rich repeat interacting protein 1 (LRRFIP1) [129], bone morphogenetic protein receptor II (BMPR II) [130], ras homologue gene family member b (RHOB) [131], sprouty2 (SPRY2) [132], mapsin [133], tropomyosin 1 (TPM1) [133, 134] and programmed cell death 4 (PDCD-4) [133, 135, 136]. These targets have been identified across a variety of cell lines including PTEN in cardiac fibroblasts [126] and non-small lung cancer cell lines [127], RECK, TIMP and LRRFIP1 in glioma cells [128, 129], BMPRII in prostate cancer cells [130], RHOB in hepatocellular and breast cancer cell lines [131], SPRY2 in cardiac myocytes [132] and mapsin, TPM1 and PDCD-4 in breast cancer cell lines [133, 134]. These targets are known to regulate tumor progression, invasion, migration, cell growth and proliferation indicating that miR-21 may function to regulate these processes in cancer.

Due to miR-21's functional importance in granulosa cells, some of its previously identified direct targets in cancer cells were investigated in granulosa cells. At 4, 8 and 24 hours after miR-21 inhibition by LNA-21 there was no change in the protein expression levels of PDCD-4, PTEN, TPM1 and SPRY2

when compared to the LNA-NS treated cells [99], suggesting that miR-21 is not directly targeting these genes in granulosa cells.

In addition to identification of its direct targets, studies of miR-21 in cancer have revealed that it has oncogenic functions. Overexpression of miR-21 in hepatocellular cancer cell lines caused increased proliferation, migration and invasion [137]. Induction of miR-21 in MCF-7 breast carcinoma cells and JB6 epidermal cancer cells caused an increase in anchorage independent colony formation, which models neoplastic transformation [138]. Elevation of miR-21 increased the survival rate of myeloma cells [119]. Inhibition studies have shown that when miR-21 expression is reduced there is a decrease in proliferation and growth of MCF-7 cells [135, 139] as well as reduced invasion, intravasation and metastatic capacity of colon cancer cells [140]. There is also a reduction in anchorage-independent colony formation and an induction in apoptosis when miR-21 is reduced [137, 141]. Studies have also shown that miR-21 mediates chemoresistance through the upregulation of pathways that mediate antiapoptotic activity [142, 143]. Together these studies suggest there is a causal and mechanistic link between induction of miR-21 and the maintenance and progression of cancer.

6. Programmed Cell Death 4 (PDCD-4)

PDCD-4 has emerged as one if miR-21's most well-studied direct targets [133, 135, 136, 138, 144-147]. PDCD-4 is a known tumor suppressor that inhibits tumor progression via the inhibition of translation [148]. It contains domains that bind with the scaffolding protein, eukaryotic protein synthesis initiation factor 4G, and the RNA helicase, eukaryotic protein synthesis initiation factor 4A; binding events which inhibit the initiation of translation [149, 150]. Investigations have also shown that PDCD-4 is able to shuttle between the nucleus and cytoplasm and that it contains domains which bind RNA [151], suggesting that PDCD-4 is involved in RNA regulation and/or metabolism. Importantly, studies have linked mouse and human carcinogenesis to inhibition of PDCD-4 expression [152-154]. Exposure of mice to the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate, decreases protein levels of PDCD-4 in skin papillomas and this decrease is attributable to increased proteasomal degradation mediated

through activation of the mTOR pathway [154]. In addition transgenic mice that overexpress PDCD-4 in their epidermis were less susceptible to papilloma formation, carcinoma incidence and papilloma-to-carcinoma conversion than their wild type counterparts exposed to the same carcinogenesis protocol [153]. Global knockdown of PDCD-4 causes the development of spontaneous lymphoma in mice and a significant reduction in life-span [155]. These in-vivo findings have been supported by in-vitro evidence in which exposure of HEK293 cells and keratinocytes to carcinogenesis protocols had decreased levels of PDCD-4 [154]. Furthermore JB6 epidermal cell lines overexpressing PDCD-4 were resistant to neoplastic transformation [152]. Also, AP-1 dependent transcription, an event required for tumorigenesis, is inhibited by PDCD-4 both in-vivo and in-vitro [152, 153].

7. Human Uterine Leiomyomas (ULMs)

ULMs are benign tumors located in the myometrium. They have an overall lifetime incidence rate of between 70% and 80% and are clinically apparent in ~25% of reproductive aged women [156]. ULMs manifest through a variety of symptoms including abdominal and pelvic pain, urinary incontinence, abnormal uterine bleeding and constipation [157, 158]. ULMs are also correlated with reproductive abnormalities and can lead to spontaneous abortions and infertility [159]. ULMs are the cause of 30% of all hysterectomies in the U.S. leading to over 200,000 annually [160]. Most women who are diagnosed with ULMs contain multiple tumors within their uterus, which are classified according to their location. The intramural fibroid is the most common tumor and is located in the smooth muscle of the uterine wall [161]. Less common are tumors located on the uterine surface (subserosal) and the uterine cavity (submucousal) [161].

Through many clinical and epidemiological studies several risk factors have been identified in ULMs. One study showed that young girls who have an early onset of menarche (at or before the age of 10) are at an increased risk to develop ULMs [162]. Multiple clinical studies have shown that parous women are at a lower risk of developing ULMs than nulliparous and that there is a progressive decline in risk with

increasing number of births [162-166]. Increase in age is also positively correlated with higher ULM incidence with a significant increase in diagnosis with women in their forties [165, 167-169]. Menopause has been shown to be a negatively correlated with ULMs as well as a reduced risk of surgery caused by ULMs [163, 165, 170]. Several epidemiological studies have revealed that obesity is correlated with an increased incidence of ULMs [164, 165, 171-175]. Race is also believed to contribute to ULM incidence with African American women having a higher risk of developing the disease than their Caucasian counterparts [167, 176-180].

It is believed that somatic mutations of normal myometrium lead to ULMs [181]. Both hormones and local growth factors have been implicated in driving these mutations. Estrogen is believed to be a major stimulus of ULM growth and it is quite common for women who have fibroids to have their tumors shrink when they undergo menopause (A time period in which women produce less estrogen) [182, 183]. More recent studies have implicated progesterone as playing an important role in fibroid growth [184, 185]. Treatments in which both estrogen and progesterone are reduced by inhibiting gonadotropin release have been effective in reducing tumor size [186]. In addition mifepristone, an anti-progesterone, has also proven effective in tumor reduction [187].

In addition to steroids several mitogenic growth factors have been shown to be elevated in ULMs when compared to healthy myometrial tissue. These include transforming growth factor-β, basic fibroblast growth factor, epidermal growth factor, platelet derived growth factor and insulin like growth factor [188-192]. Steroids often mediate their function through growth factors [59, 60, 193-195] and some studies have shown that sex steroids drive increased growth factor expression in ULMs [196-199].

Genetic predisposition has also been identified as a risk factor in the development of ULMs. Around 40% of fibroids show chromosomal abnormalities and cytogenetic variation in chromosomes 6, 7, 12 and 14 [161]. In addition, a gene mapping study implicated a specific breakage point on chromosome 12 as being critical in the pathobiology of fibroids [200]. Clinical evidence indicates that first degree relatives

in families with two or more verified leiomyoma cases are at a significantly higher risk of obtaining the disease than first degree relatives in families in which there are fewer than two relatives that have the disease [201].

While hormones, growth factors, genetics and other risk factors have been correlated with the disease, the etiology of ULMs remains unknown. Recent profiling experiments have shown that ULMs have a distinct microRNA signature and that miR-21 is induced in ULMs when compared to paired health myometrial tissues [202-204].

8. Statement of Work

Since previous reports from our lab implicated miR-21 as having pro-ovulatory and anti-apoptotic functions in granulosa cells, I wanted to elucidate fundamental molecular events that gave rise to these critical functions through identifying miR-21 direct targets in granulosa cells. To identify these targets, miR-21's effect on steady-state mRNA will be examined as previous reports have shown that microRNA direct targets can be regulated at the mRNA level. The 3'UTR of each differentially expressed gene will be examined, using bioinformatic algorithms, to determine if it has a potential miR-21 binding site. This should reveal a subset of miR-21 regulated genes that will be further tested using 3'UTR luciferase reporters, to identify bona fide miR-21 direct targets. Due to miR-21's critical functional roles in granulosa cells, I believe that these direct targets will be functionally relevant to granulosa cell physiology. Additionally, miR-21 direct targets identified in this study will undergo further experimental analysis to evaluate their functional roles in granulosa cells. Together these studies will begin to reveal the molecular via which miR-21 carries out its functions in granulosa cells.

Lastly, since miR-21 has shown significant induction in ULMs, I will study the role of miR-21 in this uterine pathology. Experiments will initially focus on miR-21's regulation of PDCD-4 (a functionally significant miR-21 direct target in many cell lines) in a cell line derived from ULMs. I will also determine expression patterns of both PDCD-4 and miR-21 in leiomyoma tissue and compare these

patterns to paired healthy myometrial tissue. This study has the potential to yield insights into miR-21 function in a prevalent reproductive disease and inform us as to whether cellular context impacts miR-21 effects.

VI. Chapter 2: Identification of MicroRNA-21 (miR-21) Direct Targets in Granulosa Cells

1. Abstract

MicroRNA-21 (miR-21) is important for maintaining granulosa cell viability and optimal ovulation rates. To being to elucidate the mechanism by which miR-21 affects these functions, this study sought to identify miR-21's direct targets in granulosa cells. To do so, mouse cultured granulosa cells were treated with an inhibitory nucleotide specific for miR-21 (LNA-21) and a non-specific LNA (LNA-NS), used as a control. At 2, 4 and 8 hours after treatment, arrays were performed to determine differential gene expression analysis between the LNA-NS and the LNA-21 treated cells. Array analysis showed that loss of miR-21 caused dysregulation of 1,950 genes between 1.2- and 3.0-fold across the 3 time points. Of the 1,950 genes, 357 were overexpressed and had at least one potential miR-21 site in their 3'utr according to the algorithms microR, miRanda, PicTar, PITA and TargetScan. Due to their involvement in apoptosis, cell differentation and transformation, 9 of the 357 genes were further analyzed to determine if they were miR-21 direct targets. These included apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (Apobec3), calcium-sensing receptor (CASR), cyclin E1 (CCNE1), tetraspanin CD151 (CD151), intestinal-specific homeobox (ISX), galectin 3 (LGALS),T-lymphoma invasion and metastasis inducing protein (TIAM1), ubiquitin-specific protease 30 (USP30) and WD40-repeat protein (WDR5). The 3' Untranslated Region (3'UTR) of each gene was separately cloned into a luciferase expression vector and cotransfected into granulosa cells along with either pre-miR-21 (a miR-21 overexpression oligo) or pre-miR-NS (control oligo). Overexpression of miR-21 caused a significant reduction in the renilla/firefly ratio of Apobec3, ISX and USP30, suggesting that miR-21 was regulating these genes and implicating them as miR-21 direct targets. There was no difference in the renilla/firefly ratios between cells treated with the miR-21 overexpression oligo and the control oligo in the 6 other genes tested. Apobec3, USP30 and ISX are important factors in mitochondrial morphology, innate immunity and vitamin A regulation, respectively. Direct regulation of these genes may mean that miR-21 is regulating these functions in granulosa cells. This study is the first to identify miR-21 direct targets in granulosa cells; identification of which is critical first step to elucidating miR-21's mechanism of action in granulosa cells.

2. Introduction

MicroRNA are ~22 nucleotide regulatory RNA that function in cell proliferation, cell death, neuronal patterning and modulation of hematopoietic lineage differentiation, amongst other functions [205]. Additionally microRNA play important roles in reproduction including the production of healthy oocytes/embryos, proper transport and implantation of embryos/blastocysts as well as optimal ovulation and fertility rates [93-95].

Investigations from our lab have implicated specific microRNA as important factors in the female reproductive tract [97, 99]. Specifically we have identified 13 microRNA that are regulated by the surge of luteinizing hormone (LH) in mouse granulosa cells; 3 of which (microRNA-132, microRNA-212 and microRNA-21 (miR-21)) are upregulated [97]. Further studies showed that miR-21 functioned in granulosa cell viability and helped maintain optimal ovulation rates [99]. Because of its functional significance, this study sought to identify miR-21 direct targets within granulosa cells in an effort to elucidate the mechanism by which miR-21 carries out its anti-apoptotic and pro-ovulatory functions.

MicroRNA directly target mRNA through binding to complementary sequences on the 3'UTR. This binding event, which is mediated through the RNA Induced Silencing Complex causes repressed protein expression levels [47, 48, 206]. Subsequent to their initial discovery, it was believed that this mRNA/microRNA binding event repressed gene expression through some form of translational control [4, 5, 44, 45]. Studies from different labs pointed to different points at which microRNA mediated translational control took place, including inhibition of initiation of translation, repression after translation initiation and nascent polypeptide degradation [4, 5, 44, 45]. Under these different mechanisms of gene regulation, it was believed that steady-state mRNA levels were not affected by microRNA [4, 5, 44, 45]. It was hypothesized that, unlike plant microRNA which typically have perfect complementarity between the microRNA and mRNA [207], the imperfect base pairing that takes place between mRNA and microRNA in mammalian systems, does not lead to mRNA degradation.

More recent research, however, has disputed this idea. Several studies have shown that microRNA can regulate large number of target mRNAs and that for many highly repressed targets, steady-state mRNA changes is the major component of gene regulation [14, 40, 41]. In addition one study reported that many of the mRNA regulated by microRNA contain binding sites in their 3'UTR that are complementary to the microRNA [42]. This latter finding suggests that mRNA destabilization may occur in mRNA that are directly targeted by microRNA.

Due to its functional importance in the ovary, this study sought to identify miR-21 direct targets in granulosa cells through analyzing its effects on steady-state mRNA levels. To identify these targets, microarrays were used to determine differential gene expression at 2, 4, and 8 hours following knockdown of miR-21 in granulosa cells. Loss of miR-21 caused widespread changes in gene expression and bioinformatics analysis showed that many of the dysregulated genes had putative miR-21 binding sites in their 3'UTR implicating them as potential miR-21 direct targets. Potential miR-21 direct targets in granulosa cells were evaluated using gene specific 3'UTRs linked to a luciferase reporter; three genes intestinal transcription factor (ISX), apilipoprotein B mRNA editing enzyme catalytic polypeptide 3 (apobec3), and ubiquitin specific protease 30 (USP30) were confirmed direct targets. Previous research has implicated ISX, Apobec3 and USP30 in lipid absorption in the intestine, restricting retrovirus mobility and maintenance of mitochondrial morphology, respectively. Through directly targeting these genes, miR-21 may be regulating these processes in granulosa cells.

3. Experimental Procedures

Granulosa cell isolation and Culture

Immature CF-1 female mice were sacrificed at 25 days of age using cervical dislocation in accordance with the protocol for animal sacrifice approved by the Internal Care and Use Committee at the University of Kansas Medical Center. After sacrifice ovaries were removed immediately and placed in M199 collection media (Sigma-Adrich, St. Louis, MO.) supplemented with 10mM HEPES and 0.2% BSA. Media was removed and replaced with M199 media supplemented with 0 .5M sucrose and 1.8mM EGTA. Ovaries were incubated in media in a 37^{o}C water bath for 15 minutes. After incubation, ovaries were washed with M199 media three times. Antral follicles were poked with 28 gauge insulin needles (Becton Dickinson, Franklin Lakes, NJ.) to release granulosa cells. Cells were transferred to a 15ml conical tube and spun for 15 minutes at 800 rcf. Cells were plated in 6 well plates that had been previously coated with fibronectin at a density of 250,000 per well in DMEM F-12 HAM (Sigma-Aldrich, St. Louis, MO.) supplemented with 10% FBS and 1% gentamicin. Cells were incubated at 37^{o}C, 5% CO_2 for 48 hours prior to transfection.

Array Analysis

After 48 hours in culture, granulosa cells were transfected with a locked nucleic acid specific for miR-21 (LNA-21) (5'-+T+C+AGTCTGATAA+G+C+TA-3') (Integrated DNA technologies, Coralville, IA.) or with a non-specific locked nucleic acid (LNA-NS)(5'+C+G+TCAGTATGCG+A+A+TC-3')at a concentration of 5uM per well of a 6-well plate. At 2, 4 and 8 hours post transfection, cells were harvested and quality of RNA was assessed using Agilent Bioanalyzer 2001 (Agilent Incorp., Palo Alto, CA.). RNA was biotin labeled and fragmented according to Affymetrix protocols. Fragmented RNA from each sample was hybridized to the Affymetrix 430E 2.0 arrays (n=3, for each time point) and scanned using the gene array scanner (Affymetrix, Santa Clara, CA.). For each chip analyzed the mean fluorescence was averaged to baseline fluorescence.

Cloning of ISX into siCHECK-2 (Promega) plasmid

The entire 3'UTR (Genome Browser) of Apobec3, CASR, CCNE1, CD151, ISX, LGALS, TIAM1, USP30 and WDR5 was separately amplified from mouse genomic DNA (Table VI-5). Thirty-five cycles of amplification was performed using denaturing, annealing and extension conditions at 94^{o}C, 55^{o}C and 68^{o}C degrees, respectively. Primers were designed such that when the 3'UTRs of Apobec 3, CASR, CCNE1, CD151, LGALS and TIAM1 were amplified each fragment contained a 5' XhoI restriction site and a 3' NotI restriction site (Supplmental Table 3). USP30 and ISX contained 5' SgfI and 3' NotI restriction sites after amplification (Supplmental Table 3). After amplification each DNA fragment was cut with restriction enzymes that matched the sites on the amplified fragments (New England Biolabs, Ipswich, MA.) at 37^{o}C overnight using appropriate buffer. Subsequently cut fragment was cloned into siCHECK-2 (Promega, Madison, WI.) (Vector was cut with same enzymes and under the same conditions as that of the fragment being inserted) using T4 DNA ligase enzyme and appropriate buffer (New England Biolabs, Ipswich, MA.) at 4^{o}C overnight. Plasmid with inserted construct was transformed into DH5α cells (Life Technologies, Carlsbad, CA.) using a standard transformation protocol and grown on LB ampicillin plates. After overnight growth at 37^{o}C, colonies were lifted and grown overnight at 37^{o}C in LB ampicillin media. DNA was isolated and correct clones were confirmed through sequencing.

Luciferase analysis

After 48 hours in culture, granulosa cells were co-transfected with siCHECK-2 plasmid containing the 3'UTR of one of the genes along with pre-microRNA-21 (pre-miR-21) (an immature form of miR-21 that gets processed into the mature form using the cell machinery) (Life Technologies, Carlsbad, CA.) or pre-miR-NS (non-specific pre-miR used as a control). Co-transfections were done using lipofectamine 2000 (Life Technologies, Carslbad, CA.) per the manufacturer's protocol. DNA was transfected at a concentration of 10ng and pre-miRs were transfected at 5uM, per well of a 6-well plate. 24 hours after transfection cells were harvested and lysed using passive lysis buffer (Promega, Madison, WI.). Lysates

were prepared for luciferase using the dual luciferase reporter assay system (Promega, Madison, WI.) per the manufacturers' protocol. Luciferase assays were performed on the Berthold Lumat LB 9501 Luminometer (Wallac, Gaithersburg, MD.). .

Statistical analysis

Student's T-test was used to determine if there were statistically different firefly/renilla expression ratio between the pre-miR-NS and pre-miR-21 treated cells. Statistical significance was set at $p<0.05$. For each luciferase assay performed 3 biological replicates were used (n=3). Each biological replicate contained 3 technical replicates, which were averaged and subsequently used in the statistical analysis.

4. Results and Tables/Figures

Across all three time points there were a total of 1,950 genes that were dysregulated due to LNA-21 treatment compared to LNA-NS treated cells (Table VI-1). At 2 hour there were 148 and 110 upregulated and inhibited, respectively (total, 258); at 4 hour there were 759 and 321 upregulated and inhibited (total, 1080), respectively; at 8 hour there were 372 and 240 upregulated and inhibited, respectively (total, 612) (Table VI-1). There were 40 genes that were up or down regulated at multiple time points at least 1.2 fold due to LNA treatment. Loss of miR-21 in granulosa cells failed to cause any gene to exhibit a more than 3-fold change and only 9 genes had a greater than 2-fold change. Together these data show that miR-21 does modestly affect a vast number of genes at the mRNA level in granulosa cells.

In order to identify potential miR-21 direct targets, genes upregulated more than 1.2 fold were analyzed using the bioinformatics algorithms microR, miRanda, PicTar, PITA and TargetScan to determine if their 3'UTR had potential miR-21 binding sites. Results of this analysis showed that there were 54 genes at 2hr, 201 genes at 4hr and 102 genes at 8hr (357 total) contained putative miR-21 binding sites in their 3'utr (Table VI-2) (Table VI-4).

Due to miR-21's previously defined role in apoptosis in granulosa cells, we selected 9 of the 263 for further investigation These genes had previously reported roles in apoptosis, carcinogenesis and cell differentiation (Table VI-3). Genes chosen included apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (Apobec3), calcium-sensing receptor (CASR), cyclin E1 (CCNE1), tetraspanin CD151 (CD151), intestinal-specific homeobox (ISX), galectin 3 (LGALS), T-lymphoma invasion and metastasis inducing protein (TIAM1), ubiquitin-specific protease 30 (USP30) and WD40-repeat protein (WDR5) (Table VI-3).

To evaluate the ability of miR-21 these gene's 3'UTR in granulosa cells, we carried out 3'UTR luciferase reporter assays. 3'UTR luciferase reporters for CASR, CCNE1, CD151, LGALS, TIAM1 and WDR5 failed to exhibit differential activity following 24 hours of increased miR-21 expression induced by the

co-transfection of pre-miR-21 (Figure VI-1). Three of the 9 gene's 3'UTRs, however, did elicit a significant decrease in the ratio of the renilla/firefly activity following overexpression of miR-21. These genes included ISX, USP30 and Apobec3, which exhibited a 30.1%, 27.8% and 24.3% reduction in the renilla/firefly ratio, respectively, when miR-21 was overexpressed compared to the control treated cells ($n=3$, $p<0.05$) (Figure VI-1).

Table VI-1. Array summary of 1,950 genes that are differentially regulated in granulosa cells ≥1.2 across all three time points following loss of miR-21 compared to control treated cells.

Table VI-1

Time Point	2hr	4hr	8hr
Up-regulated genes after LNA-21	148	759	372
Down-regulated genes after LNA-21	110	321	240
Total	258	1080	612

Table VI-2. Summary of 357 genes across all three time points that were incuded ≥1.2 fold and contained at least one potential miR-21 binding site in their 3'utr per at least one algorithm (microT, miRanda, PicTar, PITA and TargetScan).

Table VI-2

Time Point	2hr	4hr	8hr
Up-regulated after LNA-21 with potential miR-21 binding site	54	201	102

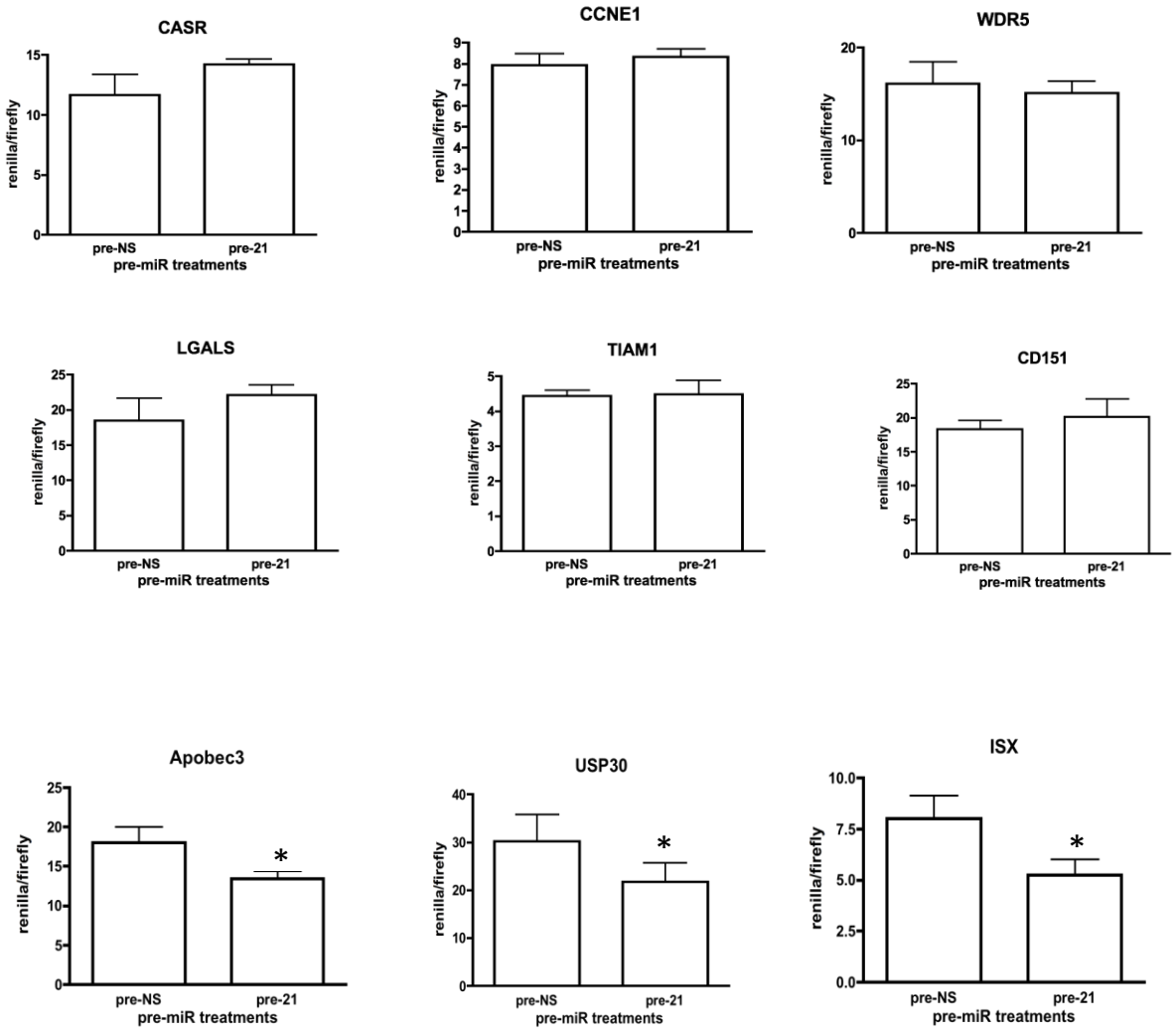
Table VI-3. Characteristics of 9 selected upregulated genes in granulosa cells that are being tested for direct miR-21 binding using 3'UTR luciferase reporters. Genes were chosen due to their roles of apoptosis, carcinogenesis and cell differentiation identified in other tissues.

Table VI-3

gene name	gene symbol	array time point/fold change	function
Apolipoprotein B mRNA editing enzyme catalytic polypeptide 3	**Apobec3**	4hr (1.40 fold increase)	restricts mobility of retroviruses and inhibits mircoRNA mediated decay of mRNA
Calcium-sensing receptor	**CASR**	8hr (1.24 fold increase)	GPCR important in calcium homeostasis
Cyclin E1	**CCNE1**	8hr (1.26 fold increase)	important for cell cycle progression and often overexpressed in cancer cells
Tetraspanin CD151	**CD151**	4hr (1.21 fold increase)	interacts with laminin to promote cell adhesion, important for carcinoma cell motility
Intestinal-specific homeobox	**ISX**	8hr (1.27 fold increase)	plays a role in intestinal differentiation and the gastric carcinogenic pathway
galectin 3	**LGALS**	4hr (1.21 fold increase)	promotes T-cell growth and apoptosis
T-lymphoma invasion and metastasis inducing protein	**TIAM1**	4hr (1.30 fold increase)	regulates cytoskeletal organization, overexpressed in many tumor cell lines
Ubiquitin-specific protease 30	**USP30**	8hr (1.20 fold increase)	functions in mitochondrial morphology
WD40-repeat protein	**WDR5**	4hr (1.33 fold increase)	involved in cell cycle, RNA splicing, transcription and apoptosis

Figure VI-1. Effect of miR-21 overexpression on 3'UTR luciferase reporter activity for select upregulated genes. Granulosa cells were co-transfected with individual gene 3'UTR siCHECK-2 vector and either the pre-miR-NS or pre-miR-21 oligonucleotides. Graphs show mean +/- SEM of the renilla/firefly ratio expression values after each treatment (n=3). *Means +/- SEM are statistically different ($p<.05$).

Figure VI-1


CASR
CCNE1
WDR5
LGALS
TIAM1
CD151
Apobec3
USP30
ISX
renilla/firefly
pre-NS
pre-21
pre-miR treatments
*

5. Discussion

This investigation shows that blockage of miR-21 in granulosa cells leads to widespread dysregulation of numerous mRNA. A significant percentage of these genes were shown to be potential miR-21 direct targets according to bioinformatic algorithms. Luciferase reporter analysis of a select number of these miR-21 direct targets implicated 3 as miR-21 direct targets in granulosa cells; USP30, Apobec3 and ISX.

Each of these genes, have previously been shown to have functions that may have significance for granulosa cells and the ovary. Ubiquitin-specific protease 30 (USP30) is a gene that is embedded in the mitochondrial membrane that plays a role in the maintenance of mitochondrial morphology. Depletion of USP30 causes mitochondria to become interconnected and take on an elongated shape [208]. Previous studies have shown that there are elaborate microtubular systems in granulosa cells when compared to other cell types and that morphological changes in the mitochondria have been linked to deviations in steroid hormone output in the ovary [209-211]. It is feasible that USP30 is under miR-21 direct regulation to induce the necessary mitochondrial morphological changes in granulosa cells to properly regulate steroidogenesis. Northern blot analysis has also shown that USP30 has high expression in the gonads when compared to other tissues types further suggesting that this gene may have functional significance in the ovary [208].

Apolipoprotein B mRNA editing enzyme catalytic polypeptide 3's (Apobec3) primary functional role is to confer innate immunity to a wide range of exogenous retroviruses. It carries out this function, in part, through the regulation of immune markers [212]. It is known that the LH surge causes induction of chemokines MCP-1 and IL-8 and that macrophages, neutrophilic granulocytes, and T-lymphocytes are present in the ovarian cells, including granulosa cells, at ovulation. It has been hypothesized that these immune markers may be functioning in tissue remodeling, which occurs during ovulation [213, 214]. MiR-21, which our lab has shown to regulate ovulation, may be directly targeting Apobec3 in an effort to modulate functionally important immunologic markers.

Intestinal-specific homeobox (ISX) is a homeobox transcription factor that regulates vitamin A metabolism and SRB1 expression levels [215]. Vitamin A is known to function in the female germline and female reproduction while SRB1 is critical for cholesterol uptake and steroidogenesis in the ovary. MiR-21 may be targeting ISX to regulate these functions in the ovary. SRB1 is also known to be under LH regulation in the ovary [216, 217]. Our lab has previously shown that miR-21 is also under LH control in the ovary. Together these findings suggest the possibility that the mechanism of SRB1 hormonal control is via miR-21's directly targeting ISX.

Along with identifying miR-21 direct targets, it is also significant that this study failed to implicate Tiam1 as a miR-21 direct target. Tiam1 was induced 1.3 fold four hours after miR-21 inhibition and bioinformatics analysis identified a potential miR-21 binding site in its 3'UTR. Most importantly, Tiam1 has been previously identified as a miR-21 direct target in colon carcinoma cells [218]. While our array and bioinformatics analysis suggested Tiam1 may be a miR-21 direct target, luciferase analysis failed to show that miR-21 could regulate its 3'UTR. This lack of ability to identify the same gene as a microRNA direct target across two different cell types finds significant support in the literature. Specifically for miR-21, while some of its direct targets have been identified in multiple cancer cell lines, the majority have been unique to the cell or tissue type under investigation [126, 129, 130, 132-134, 137, 140, 218]. The lack of ability to identify Tiam1 as a miR-21 direct target in granulosa cells, whether that means it is not an actual target in this cell type or just that this study was unable to identify it as such, is consistent with these previous studies.

Findings from this study are consistent with previous reports that have shown that microRNA do have moderate, yet, statistically effects on steady-state mRNA levels and that microRNA direct targets are regulated at the mRNA level [14, 40, 41]. And while this study identified 3 novel miR-21 direct targets, 6 of the targets examined could not be verified as miR-21 direct targets. Luciferase analysis would need to be performed on the remaining 378 genes that were induced following loss of miR-21 and had potential miR-21 binding sites in their 3'UTR, to determine if they were bona fide miR-21 direct targets.

This study is the first to implicate microRNA direct target in granulosa cells. It is also the first to identify miR-21 direct targets in a physiological system. Further studies will be needed to determine if the 3 currently identified genes are functionally important targets in the context of granulosa cells and/or ovarian biology.

6. Supplemental Data

Table VI-4. List of all genes across all three time points dysregulated ≥ 1.2 fold following inhibition of miR-21 that contain potential miR-21 binding sites in their 3'UTR. Table contains gene symbol, gene name, fold change and p-value.

Table VI-4

Gene Symbol	Gene Name	Fold Change	P-value	Array Time Point
KCNA1	potassium voltage-gated channel; shaker-related subfamily; member 1	**1.24066**	0.0381492	8hr
YAP1	yes-associated protein 1	**1.21551**	0.018231	4hr
MAP2K3	mitogen-activated protein kinase kinase 3	**1.22371**	0.0308353	8hr
2310035C23RIK	RIKEN cDNA 2310035C23 gene	**1.2748**	0.0382897	8hr
RALGPS1	Ral GEF with PH domain and SH3 binding motif 1	**1.25791**	0.0467209	8hr
STX2	syntaxin 2	**1.23431**	0.034218	8hr
CASR	calcium-sensing receptor	**1.23596**	0.0237921	8hr
CNNM4	cyclin M4	**1.2244**	0.0234587	8hr
ISX	intestine specific homeobox	**1.26309**	0.0284002	8hr
ATP8A1	ATPase; aminophospholipid transporter (APLT); class I; type 8A; member 1	**1.24654**	0.0369261	8hr
PSD2	pleckstrin and Sec7 domain containing 2	**1.2342**	0.0287654	8hr
CD244	CD244 natural killer cell receptor 2B4	**1.2094**	0.0378144	8hr
USP47	ubiquitin specific peptidase 47	**1.23215**	0.0353436	2hr
WDR5	WD repeat domain 54	**1.39324**	0.0331789	4hr
MSX1	homeobox; msh-like 1	**1.26411**	0.0262056	4hr
RUNX3	runt related transcription factor 3	**1.19886**	0.0298386	4hr
PDE6G	phosphodiesterase 6G; cGMP-specific; rod; gamma	**1.2723**	0.0302749	4hr
TOM1L2	target of myb1-like 2 (chicken)	**1.22915**	0.00379625	4hr
ATCAY	ataxia; cerebellar; Cayman type homolog (human)	**1.10285**	0.00419113	4hr
DNAJC16	DnaJ (Hsp40) homolog; subfamily C; member 16	**1.28176**	0.0219148	4hr
SPIN1	similar to Spindlin 1 /// spindlin 1	**1.39803**	0.044587	4hr
PCDH12	protocadherin 12	**1.27659**	0.0439822	4hr
CNR1	cannabinoid receptor 1 (brain)	**1.20281**	0.0152991	4hr
TIAM1	T-cell lymphoma invasion and metastasis 1	**1.29708**	0.0395434	4hr
LZTFL1	leucine zipper transcription factor-like 1	**1.24237**	0.0203538	4hr
LIG3	ligase III; DNA; ATP-dependent	**1.25771**	0.046681	4hr
TSSK2	testis-specific serine kinase 2	**1.29503**	0.030274	4hr
MTHFR	5;10-methylenetetrahydrofolate reductase	**1.20036**	0.0308539	2hr
4933439F18RIK	Hypothetical protein (ORF1)	**1.34886**	0.00500596	2hr
NEK6	NIMA (never in mitosis gene a)-related expressed kinase 6	**1.24016**	0.0386376	8hr
RNF144A	ring finger protein 144A	**1.33035**	0.00712759	8hr
DMRTC1A	DMRT-like family C1a	**1.22113**	0.0330858	8hr
SEL1L	deoxyribonuclease 1-like 2 /// similar to Dnase1l2 protein	**1.45314**	0.0470193	8hr
TPCN1	two pore channel 1	**1.38189**	0.00464223	8hr
KCND3	potassium voltage-gated channel; Shal-related family; member 3	**1.24132**	0.0364306	8hr
EPHB2	Eph receptor B2	**1.20284**	0.0442559	8hr

LOC100044065 /// ODZ1	similar to odd Oz/ten-m homolog 1 (Drosophila) /// odd Oz/ten-m homolog 1 (Drosophila)	**1.44758**	0.0263521	8hr
GALNT13	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13	**1.32545**	0.0283911	8hr
MSMB	beta-microseminoprotein	**1.36798**	0.0351251	8hr
BCL7A	B-cell CLL/lymphoma 7A	**1.22852**	0.00949237	8hr
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	**1.24707**	0.011334	8hr
DUSP8	dual specificity phosphatase 8	**1.41019**	0.00814115	8hr
PPP1R10	predicted gene; 100039405 /// similar to protein phosphatase 1; regulatory subunit 10 /// protein phosphatase 1; regulatory subunit 10	**1.33757**	0.0459527	8hr
H1FOO	H1 histone family; member O; oocyte-specific	**1.18956**	0.0397726	4hr
WDR85	WD repeat domain 85	**1.43561**	0.0486218	4hr
2610110G12RIK	RIKEN cDNA 2610110G12 gene	**1.37657**	0.0232366	4hr
CGREF1	cell growth regulator with EF hand domain 1	**1.40623**	0.0349903	4hr
GPR88	G-protein coupled receptor 88	**1.23105**	0.0290101	4hr
SSTR5	somatostatin receptor 5	**1.32819**	0.0387656	4hr
TTC14	tetratricopeptide repeat domain 14	**1.21715**	0.0246102	4hr
BSDC1	BSD domain containing 1	**1.34953**	0.0363251	4hr
IKBKG	inhibitor of kappaB kinase gamma	**1.29765**	0.0407163	4hr
1700052N19RIK	RIKEN cDNA 1700052N19 gene	**1.21604**	0.0283057	4hr
TIMP2	tissue inhibitor of metalloproteinase 2	**1.22514**	0.000312176	4hr
BC053393	cDNA sequence BC053393	**1.30954**	0.00758184	4hr
GAL3ST2	galactose-3-O-sulfotransferase 2	**1.20966**	0.0494251	4hr
STXBP1	syntaxin binding protein 1	**1.3435**	0.0310694	4hr
BMPR1B	bone morphogenetic protein receptor; type 1B	**1.20182**	0.0216408	4hr
SATB1	Special AT-rich sequence binding protein 1; mRNA (cDNA clone MGC:18461 IMAGE:4164993)	**1.20434**	0.0355016	4hr
LCP2	lymphocyte cytosolic protein 2	**1.28997**	0.023994	4hr
CLEC4A2	C-type lectin domain family 4; member a2	**1.40725**	0.0343368	4hr
NFIC	nuclear factor I/C	**1.33857**	0.0247482	4hr
FANCM	Fanconi anemia; complementation group M	**1.30273**	0.0218963	2hr
FGD4	FYVE; RhoGEF and PH domain containing 4	**1.27461**	0.010757	2hr
RAB40B	Rab40b; member RAS oncogene family	**1.22058**	0.0225316	2hr
STAT1	signal transducer and activator of transcription 1	**1.24077**	0.0139625	2hr
TNS4	tensin 4	**1.21525**	0.00546456	2hr
SLC5A7	solute carrier family 5 (choline transporter); member 7	**1.35935**	0.0276498	2hr
MC1R	melanocortin 1 receptor	**1.24041**	0.0135792	8hr
KLRB1C	killer cell lectin-like receptor subfamily B member 1C	**1.30395**	0.0477138	8hr
AK3L1	adenylate kinase 3-like 1 /// similar to adenylate kinase 4	**1.31706**	0.0401146	8hr
CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2	**1.19567**	0.0294399	8hr
LOC100046704 /// NRAS	similar to neuroblastoma ras oncogene /// neuroblastoma ras oncogene	**1.22974**	0.0428806	8hr
FBLN1	fibulin 1	**1.31901**	0.0485345	8hr
NUTF2	nuclear transport factor 2	**1.29939**	0.0176036	8hr

COPG	coatomer protein complex; subunit gamma	**1.27054**	0.0212557	8hr
SYS1	SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	**1.2427**	0.0289906	8hr
SETMAR	SET domain and mariner transposase fusion gene	**1.20264**	0.0325313	8hr
ANAPC11	Anaphase promoting complex subunit 11; mRNA (cDNA clone MGC:35764 IMAGE:5356010)	**1.27847**	0.0318457	8hr
NRP	neural regeneration protein	**1.38904**	0.0263308	8hr
SLC10A7	solute carrier family 10 (sodium/bile acid cotransporter family); member 7	**1.33827**	0.0411596	8hr
NFIX	heat shock transcription factor family member 5	**1.29147**	0.0230708	8hr
2210020M01RIK	RIKEN cDNA 2210020M01 gene	**1.21661**	0.0173907	8hr
UCP2	Uncoupling protein 2 (mitochondrial; proton carrier); mRNA (cDNA clone MGC:13955 IMAGE:4205625)	**1.3288**	0.00108009	8hr
CAP2	CAP; adenylate cyclase-associated protein; 2 (yeast)	**1.31103**	0.0448868	8hr
4833439L19RIK	RIKEN cDNA 4833439L19 gene	**1.22641**	0.0294885	8hr
KCNIP3	Kv channel interacting protein 3; calsenilin	**1.31158**	0.0268974	8hr
SH3GL3	SH3-domain GRB2-like 3	**1.21961**	0.00541832	8hr
EIF4EBP2	eukaryotic translation initiation factor 4E binding protein 2	**1.25029**	0.00143075	8hr
SYS1	SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	**1.2427**	0.0289906	8hr
NPCD /// NPTXR	neuronal pentraxin with chromo domain /// neuronal pentraxin receptor	**1.2506**	0.0418691	8hr
FOXS1	forkhead box S1	**1.34367**	0.0314605	8hr
ETV1	ets variant gene 1	**1.32093**	0.0459477	8hr
RAB36	RAB36; member RAS oncogene family	**1.25816**	0.0348	8hr
MAPK12	mitogen-activated protein kinase 12	**1.2472**	0.00294082	8hr
2810046L04RIK	RIKEN cDNA 2810046L04 gene	**1.23751**	0.00227067	8hr
NEUROG1	neurogenin 1	**1.20242**	0.0116106	8hr
HSF5	heat shock transcription factor family member 5	**1.29147**	0.0230708	8hr
RECQL	RecQ protein-like	**1.19606**	0.0401784	8hr
GNAS	GNAS (guanine nucleotide binding protein; alpha stimulating) complex locus	**1.20489**	0.0259956	8hr
4930579E17RIK	RIKEN cDNA 4930579E17 gene	**1.19629**	0.0332697	8hr
SCN3B	sodium channel; voltage-gated; type III; beta	**1.20291**	0.0124925	8hr
1700065D16RIK	RIKEN cDNA 1700065D16 gene	**1.20812**	0.0120007	8hr
CD151	CD151 antigen	**1.20037**	0.0465499	4hr
LGALS3	lectin; galactose binding; soluble 3	**1.2083**	0.0241608	4hr
RAD23A	RAD23a homolog (S. cerevisiae)	**1.18939**	0.0048281	4hr
DRG2	developmentally regulated GTP binding protein 2	**1.37148**	0.0459712	4hr
TMEM206	transmembrane protein 206	**1.2573**	0.0453215	4hr
APOBEC3	apolipoprotein B mRNA editing enzyme; catalytic polypeptide 3	**1.38165**	0.0372923	4hr
DZIP3	DAZ interacting protein 3; zinc finger	**1.30721**	0.00202659	4hr
TBL1XR1	transducin (beta)-like 1X-linked receptor 1	**1.23375**	0.0409055	4hr
SOCS2	suppressor of cytokine signaling 2	**1.27435**	0.0284393	4hr
UBN2	ubinuclein 2	**1.35502**	0.0335606	4hr
ZFP444	zinc finger protein 444	**1.46436**	0.00163213	4hr
TRAPPC6A	trafficking protein particle complex 6A	**1.63855**	0.00781959	4hr

ANTXR1	anthrax toxin receptor 1	**1.3414**	0.0483811	4hr
ENTPD7	ectonucleoside triphosphate diphosphohydrolase 7	**1.22614**	0.0209234	4hr
ARVCF	armadillo repeat gene deleted in velo-cardio-facial syndrome	**1.30458**	0.0398695	4hr
GPR44	G protein-coupled receptor 44	**1.36093**	0.0276785	4hr
KCNJ6	potassium inwardly-rectifying channel; subfamily J; member 6	**1.25993**	0.044754	4hr
KCNF1	potassium voltage-gated channel; subfamily F; member 1	**1.33999**	0.0152435	4hr
N28178	expressed sequence N28178	**1.31287**	0.029663	4hr
IKBKB	inhibitor of kappaB kinase beta	**1.21493**	0.0130966	4hr
MYO19	myosin XIX	**1.24208**	0.0132445	4hr
CASP9	caspase 9	**1.23768**	0.0322464	4hr
MED20 /// USP49	mediator complex subunit 20 /// ubiquitin specific peptidase 49	**1.28398**	0.00711972	4hr
JAKMIP1	janus kinase and microtubule interacting protein 1	**1.26088**	0.0495906	4hr
SH2D2A	SH2 domain protein 2A	**1.22417**	0.0427331	4hr
CREB3L4	cAMP responsive element binding protein 3-like 4	**1.52371**	0.0250845	4hr
SYS1	SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	**1.20698**	0.0258872	4hr
RNF38	ring finger protein 38	**1.3763**	0.0320749	4hr
EMP2	epithelial membrane protein 2	**1.29212**	0.0188186	4hr
PLDN	pallidin	**1.36334**	0.00265435	4hr
CD93	CD93 antigen	**1.2251**	0.0316378	4hr
KIF5B	Kinesin family member 5B (Kif5b); mRNA	**1.16378**	0.0450234	4hr
4732429D16RIK	RIKEN cDNA 4732429D16 gene	**1.2312**	0.021766	4hr
BC020535	cDNA sequence BC020535	**1.21566**	0.0318979	4hr
ADAM28	a disintegrin and metallopeptidase domain 28	**1.28904**	0.00947245	4hr
S1PR3	sphingosine-1-phosphate receptor 3	**1.38107**	0.00288585	4hr
MARVELD3	MARVEL (membrane-associating) domain containing 3	**1.36351**	0.00394438	4hr
IQGAP1	IQ motif containing GTPase activating protein 1	**1.27375**	0.020949	4hr
GIP	gastric inhibitory polypeptide	**1.28366**	0.0436636	4hr
CSRP3	cysteine and glycine-rich protein 3	**1.24109**	0.0393396	4hr
ATAD4	ATPase family; AAA domain containing 4	**1.34773**	0.0241957	4hr
TNFRSF11A	tumor necrosis factor receptor superfamily; member 11a	**1.23895**	0.00944203	4hr
AIM1L	absent in melanoma 1-like	**1.27806**	0.0471632	4hr
LIPC	lipase; hepatic	**1.29819**	0.0289615	4hr
MGAT3	mannoside acetylglucosaminyltransferase 3	**1.2386**	0.0171148	4hr
NCOR1	nuclear receptor co-repressor 1	**1.32519**	0.0267844	4hr
TBC1D5	TBC1 domain family; member 5	**1.22162**	0.041967	4hr
2810055G20RIK	RIKEN cDNA 2810055G20 gene	**1.21382**	0.00706424	4hr
MPDZ	multiple PDZ domain protein	**1.29698**	0.016222	4hr
CABIN1	calcineurin binding protein 1	**1.2898**	0.0334187	4hr
PDE4B	Phosphodiesterase 4B; cAMP specific; mRNA (cDNA clone IMAGE:5354427)	**1.32027**	0.0340892	4hr
CD93	CD93 antigen	**1.2251**	0.0316378	4hr
DMRT3	Doublesex and mab-3 related transcription factor 3; mRNA (cDNA clone	**1.20375**	0.0434594	4hr

	MGC:62407 IMAGE:6404988)			
ZHX1	zinc fingers and homeoboxes 1	**1.22034**	0.0292768	4hr
EBF2	early B-cell factor 2	**1.21205**	0.00465592	4hr
B3GALNT2	UDP-GalNAc:betaGlcNAc beta 1;3-galactosaminyltransferase; polypeptide 2	**1.21657**	0.0192898	4hr
5730507A09RIK	RIKEN cDNA 5730507A09 gene	**1.26531**	0.00339387	4hr
666661 /// APOL7C	predicted gene; 666661 /// apolipoprotein L 7c	**1.21095**	0.0310188	4hr
TMEM174	transmembrane protein 174	**1.27495**	0.015729	4hr
CCDC53	coiled-coil domain containing 53	**1.22417**	0.0427331	4hr
ADAMTS20	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif; 20	**1.20064**	0.00446484	4hr
GGTA1	glycoprotein galactosyltransferase alpha 1; 3	**1.47671**	0.00175677	4hr
ARHGEF9	CDC42 guanine nucleotide exchange factor (GEF) 9	**1.20931**	0.0358449	4hr
RNF112	ring finger protein 112	**1.20332**	0.0289161	4hr
C030003D03RIK	RIKEN cDNA C030003D03 gene	**1.28318**	0.043273	4hr
CDC14A /// LOC100047731	CDC14 cell division cycle 14 homolog A (S. cerevisiae) /// hypothetical protein LOC100047731	**1.25287**	0.0104667	4hr
KCNB1	potassium voltage gated channel; Shab-related subfamily; member 1	**1.24783**	0.0264198	4hr
TTC7	tetratricopeptide repeat domain 7	**1.23992**	0.0267143	4hr
RHOH	ras homolog gene family; member H	**1.2172**	0.021773	4hr
SPSB4	splA/ryanodine receptor domain and SOCS box containing 4	**1.3865**	0.0470835	4hr
RBM39	RNA binding motif protein 39	**1.27075**	0.0191579	2hr
SLC7A11	solute carrier family 7 (cationic amino acid transporter; y+ system); member 11	**1.24219**	0.0284351	2hr
PXN	paxillin	**1.32417**	0.0325027	2hr
MAP3K9	mitogen-activated protein kinase kinase kinase 9	**1.20477**	0.0464807	2hr
EDN3	endothelin 3	**1.46281**	0.00197219	2hr
2810403A07RIK	RIKEN cDNA 2810403A07 gene	**1.3593**	0.0251156	2hr
LRCH3	Leucine-rich repeats and calponin homology (CH) domain containing 3; mRNA (cDNA clone MGC:198849 IMAGE:9054814)	**1.23988**	0.00672849	2hr
LIN54	lin-54 homolog (C. elegans)	**1.23177**	0.0364661	2hr
4933403F05RIK	RIKEN cDNA 4933403F05 gene	**1.28247**	0.0229778	2hr
CUGBP2	CUG triplet repeat; RNA binding protein 2	**1.29233**	0.031647	2hr
DGCR8	DiGeorge syndrome critical region gene 8	**1.22353**	0.0126917	2hr
PAFAH1B1	Platelet-activating factor acetylhydrolase; isoform 1b; beta1 subunit; mRNA (cDNA clone MGC:13913 IMAGE:4017963)	**1.30018**	0.0303562	2hr
STEAP2	six transmembrane epithelial antigen of prostate 2	**1.27721**	0.0448114	2hr
STXBP5L	syntaxin binding protein 5-like	**1.24979**	0.0120968	2hr
SEC22A	SEC22 vesicle trafficking protein homologe A (S. cerevisiae)	**1.22315**	0.0179869	2hr
GFOD1	glucose-fructose oxidoreductase domain containing 1	**1.21**	0.0104052	2hr
SCARA5	scavenger receptor class A; member 5 (putative)	**1.47773**	0.0405373	2hr
TMEM29	transmembrane protein 29	**1.40516**	0.00567558	8hr
PHC2	polyhomeotic-like 2 (Drosophila)	**1.27718**	0.0446983	8hr
GHR	growth hormone receptor	**1.22006**	0.003078	8hr
TTC28	tetratricopeptide repeat domain 28	**1.23915**	0.0218533	8hr

CCNY /// LOC100044842	cyclin Y /// similar to cyclin fold protein 1	**1.20531**	0.00794195	8hr
CCNE1	cyclin E1	**1.25368**	0.015062	8hr
NDST1	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	**1.21405**	0.0434991	8hr
FBXO21	F-box protein 21	**1.2031**	0.0340304	8hr
MYLPF	myosin light chain; phosphorylatable; fast skeletal muscle	**1.27022**	0.0271005	8hr
MCM5	minichromosome maintenance deficient 5; cell division cycle 46 (S. cerevisiae)	**1.28009**	0.0419356	8hr
GRHL2	grainyhead-like 2 (Drosophila)	**1.45489**	0.04115	8hr
LEPR	leptin receptor	**1.30297**	0.0468672	8hr
EG232599	Predicted gene; EG232599; mRNA (cDNA clone MGC:156005 IMAGE:40129691)	**1.20106**	0.0220952	8hr
EDA	ectodysplasin-A	**1.23257**	0.0104525	8hr
LDLR	low density lipoprotein receptor	**1.41719**	0.0391926	8hr
ZFP651	zinc finger protein 651	**1.20411**	0.0289022	8hr
CALR3	calreticulin 3	**1.31668**	0.0240547	8hr
CENPA	Centromere protein A; mRNA (cDNA clone MGC:13888 IMAGE:4018429)	**1.3344**	0.0445517	8hr
USP30	ubiquitin specific peptidase 30	**1.19824**	0.0359382	8hr
ZFP473	zinc finger protein 473	**1.21327**	0.0440109	8hr
POU2F1	POU domain; class 2; transcription factor 1	**1.21115**	0.0119038	8hr
ACOT12	acyl-CoA thioesterase 12	**1.273**	0.0254415	8hr
COX4NB	COX4 neighbor	**1.21692**	0.0360243	8hr
GAS7	growth arrest specific 7	**1.22461**	0.049252	8hr
NTSR1	neurotensin receptor 1	**1.27018**	0.020129	8hr
SLC12A3	solute carrier family 12; member 3	**1.32589**	0.0447393	8hr
C530008M17RIK	RIKEN cDNA C530008M17 gene	**1.2**	0.0414148	8hr
VPRBP	Vpr (HIV-1) binding protein	**1.24638**	0.0180449	8hr
PVT1	plasmacytoma variant translocation 1	**1.22902**	0.0187565	8hr
SYT17	synaptotagmin XVII	**1.20427**	0.013803	8hr
GABRB3	gamma-aminobutyric acid (GABA) A receptor; subunit beta 3	**1.22451**	0.0307998	8hr
ASAH2	N-acylsphingosine amidohydrolase 2	**1.21031**	0.0122681	8hr
5730469M10RIK	RIKEN cDNA 5730469M10 gene	**1.2101**	0.00848491	8hr
ELMO1	engulfment and cell motility 1; ced-12 homolog (C. elegans)	**1.27493**	0.0432414	8hr
ENPEP	glutamyl aminopeptidase	**1.29743**	0.0200332	8hr
ACADL	acyl-Coenzyme A dehydrogenase; long-chain	**1.22746**	0.0471311	8hr
ZHX3	zinc fingers and homeoboxes 3	**1.22889**	0.00224961	8hr
ROR1	receptor tyrosine kinase-like orphan receptor 1	**1.20987**	0.0119589	8hr
DMC1	DMC1 dosage suppressor of mck1 homolog; meiosis-specific homologous recombination (yeast)	**1.2226**	0.00382193	8hr
SEC11A	SEC11 homolog A (S. cerevisiae)	**1.29546**	0.0429056	8hr
EOMES	eomesodermin homolog (Xenopus laevis)	**1.27725**	0.0340839	8hr
TRFR2	transferrin receptor 2	**1.3379**	0.00167195	4hr
RANBP3	RAN binding protein 3	**1.25067**	0.049902	4hr
RPS6KA4	ribosomal protein S6 kinase; polypeptide 4	**1.31812**	0.0492866	4hr

MTCH1	mitochondrial carrier homolog 1 (C. elegans)	**1.56096**	0.0210422	4hr
CAPNS1	calpain; small subunit 1	**1.40736**	0.0385973	4hr
ZDHHC3	zinc finger; DHHC domain containing 3	**1.2741**	0.0173486	4hr
SNX12	sorting nexin 12	**1.36964**	0.0391086	4hr
PIGU	phosphatidylinositol glycan anchor biosynthesis; class U	**1.20976**	0.0379166	4hr
SLC25A38	solute carrier family 25; member 38	**1.31786**	0.0276498	4hr
SEC61A2	Sec61; alpha subunit 2 (S. cerevisiae)	**1.23225**	0.0447055	4hr
TBC1D25	TBC1 domain family; member 25	**1.23738**	0.0310281	4hr
WDR41	WD repeat domain 41	**1.44788**	0.0332607	4hr
TOPBP1	topoisomerase (DNA) II binding protein 1	**1.31327**	0.0291106	4hr
WDR41	WD repeat domain 41	**1.25967**	0.0273147	4hr
BBS2	Bardet-Biedl syndrome 2 (human)	**1.31536**	0.016365	4hr
2610021K21RIK	RIKEN cDNA 2610021K21 gene	**1.47377**	0.000387366	4hr
HIF3A /// LOC641092	hypoxia inducible factor 3; alpha subunit /// similar to hypoxia inducible factor 3; alpha subunit	**1.41924**	0.0295638	4hr
CD160	CD160 antigen	**1.29034**	0.028802	4hr
PLSCR4	phospholipid scramblase 4	**1.2333**	0.027773	4hr
DLGAP1	discs; large (Drosophila) homolog-associated protein 1	**1.2339**	0.0283144	4hr
AUH	AU RNA binding protein/enoyl-coenzyme A hydratase	**1.30963**	0.0362311	4hr
PDGFRB	platelet derived growth factor receptor; beta polypeptide	**1.31699**	0.0329807	4hr
PNPLA3	patatin-like phospholipase domain containing 3	**1.47795**	0.0238688	4hr
KCNN3	potassium intermediate/small conductance calcium-activated channel; subfamily N; member 3	**1.34403**	0.0465186	4hr
HES5	hairy and enhancer of split 5 (Drosophila)	**1.26451**	0.04625	4hr
KRT42	keratin 42	**1.45001**	0.0400028	4hr
ZSWIM5	zinc finger; SWIM domain containing 5	**1.44506**	0.0403014	4hr
TMPRSS2	transmembrane protease; serine 2	**1.43772**	0.0362834	4hr
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1	**1.25466**	0.033383	4hr
6430548M08RIK	RIKEN cDNA 6430548M08 gene	**1.24575**	0.0438124	4hr
EXOC6B	exocyst complex component 6B	**1.25007**	0.0223597	4hr
D2BWG1423E	DNA segment; Chr 2; Brigham & Women's Genetics 1423 expressed	**1.23145**	0.00774136	4hr
NCSTN	nicastrin	**1.26415**	0.0152796	4hr
SS18L1	synovial sarcoma translocation gene on chromosome 18-like 1	**1.20556**	0.0109055	4hr
FAM19A5	family with sequence similarity 19; member A5	**1.36901**	0.00950877	4hr
PNOC	prepronociceptin	**1.3553**	0.0202261	4hr
WDR22	WD repeat domain 22	**1.47926**	0.0109485	4hr
HYAL1 /// NAT6	hyaluronoglucosaminidase 1 /// N-acetyltransferase 6	**1.38476**	0.000717346	4hr
MR1	major histocompatibility complex; class I-related	**1.26252**	0.00761025	4hr
USP30	ubiquitin specific peptidase 30	**1.23696**	0.0380664	4hr
CCDC84	coiled-coil domain containing 84	**1.40305**	0.01079	4hr
SLC31A2	solute carrier family 31; member 2	**1.2463**	0.0154858	4hr
SERHL	serine hydrolase-like	**1.50092**	0.0461852	4hr

MYST1	MYST histone acetyltransferase 1	**1.49239**	0.0376414	4hr
GM98	gene model 98; (NCBI)	**1.25344**	0.0379982	4hr
TMEM181 /// TMEM181C-PS	transmembrane protein 181 /// transmembrane protein 181C; pseudogene	**1.36384**	0.0483975	4hr
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	**1.28518**	0.0397991	4hr
MEG3	maternally expressed 3	**1.21647**	0.0486051	4hr
FMOD	fibromodulin	**1.26686**	0.0262299	4hr
PER3	period homolog 3 (Drosophila)	**1.31286**	0.0204295	4hr
OLFR78	olfactory receptor 78	**1.32302**	0.0336662	4hr
DNAJB5	DnaJ (Hsp40) homolog; subfamily B; member 5	**1.32879**	0.0421913	4hr
2900092E17RIK /// PRRT2	RIKEN cDNA 2900092E17 gene /// proline-rich transmembrane protein 2	**1.26888**	0.0323711	4hr
CD80	CD80 antigen	**1.23843**	0.0116944	4hr
ARID3B	AT rich interactive domain 3B (BRIGHT-like)	**1.21779**	0.0023626	4hr
CSF3R	colony stimulating factor 3 receptor (granulocyte)	**1.23806**	0.0419324	4hr
HOXC13	homeo box C13	**1.21624**	0.0135379	4hr
ACTA2	actin; alpha 2; smooth muscle; aorta	**1.24188**	0.000830193	4hr
KBTBD11	Kelch repeat and BTB (POZ) domain containing 11; mRNA (cDNA clone MGC:90804 IMAGE:6852896)	**1.28919**	0.0255294	4hr
P2RX3	purinergic receptor P2X; ligand-gated ion channel; 3	**1.2049**	0.00986529	4hr
ADH6A	alcohol dehydrogenase 6A (class V)	**1.23987**	0.0156428	4hr
PHACTR3	phosphatase and actin regulator 3	**1.42477**	0.0412105	4hr
4930452B06RIK	RIKEN cDNA 4930452B06 gene	**1.26624**	0.000355765	4hr
ERCC8	excision repaiross-complementing rodent repair deficiency; complementation group 8	**1.26181**	0.0030426	4hr
LEPR	leptin receptor	**1.20744**	0.0468575	4hr
DDO	D-aspartate oxidase	**1.24317**	0.0494806	4hr
RAPGEF4	Rap guanine nucleotide exchange factor (GEF) 4	**1.22279**	0.035983	4hr
LIMCH1	LIM and calponin homology domains 1	**1.2013**	0.0141982	4hr
CALCOCO2	calcium binding and coiled-coil domain 2	**1.22109**	0.0309509	4hr
ITGB2L	integrin beta 2-like	**1.21015**	0.0467201	4hr
ELK4	ELK4; member of ETS oncogene family	**1.22889**	0.01276	4hr
APOL11B	apolipoprotein L 11b	**1.20907**	0.0103135	4hr
RBM24	RNA binding motif protein 24	**1.27861**	0.0464648	4hr
ANGPTL3	angiopoietin-like 3	**1.2457**	0.043562	4hr
SHISA2	shisa homolog 2 (Xenopus laevis)	**1.25564**	0.0131316	4hr
1810034E14RIK	RIKEN cDNA 1810034E14 gene	**1.2366**	0.020692	4hr
LGR5	leucine rich repeat containing G protein coupled receptor 5	**1.20803**	0.0226626	4hr
CPLX4	complexin 4	**1.20601**	0.0345655	4hr
DRD1A	dopamine receptor D1A	**1.29043**	0.00856576	4hr
SYPL2	synaptophysin-like 2	**1.21802**	0.0136843	4hr
FRY	PREDICTED: Mus musculus furry homolog (Drosophila) (Fry); mRNA	**1.2278**	0.0406877	4hr
UTY	ubiquitously transcribed tetratricopeptide repeat gene; Y chromosome	**1.2168**	0.0395481	4hr
CACNA2D4	calcium channel; voltage-dependent; alpha 2/delta subunit 4	**1.31956**	0.0211761	4hr

EVI2B	ecotropic viral integration site 2b	**1.24042**	0.00319271	4hr
TLE4	ecotropic viral integration site 2b	**1.24042**	0.00319271	4hr
NRXN3	neurexin III	**1.20262**	0.0491121	4hr
DYM	dymeclin	**1.23266**	0.02255	4hr
B130034C11RIK	RIKEN cDNA B130034C11 gene	**1.20487**	0.00159592	4hr
RPGRIP1	retinitis pigmentosa GTPase regulator interacting protein 1	**1.1985**	0.00545045	4hr
TBC1D14	TBC1 domain family; member 14	**1.21957**	0.0248394	4hr
FAM179B	family with sequence similarity 179; member B	**1.36362**	0.0442118	4hr
GABRA1	gamma-aminobutyric acid (GABA) A receptor; subunit alpha 1	**1.37592**	0.0386429	4hr
PRAMEF12	PRAME family member 12	**1.20598**	0.037564	4hr
AU018091	expressed sequence AU018091	**1.32694**	0.0306449	4hr
PIRA2	paired-Ig-like receptor A2	**1.34356**	0.01633	4hr
LCOR	ligand dependent nuclear receptor corepressor	**1.25776**	0.0273233	4hr
THUMPD3	THUMP domain containing 3	**1.30399**	0.0453354	4hr
NCAPH2	non-SMC condensin II complex; subunit H2	**1.28319**	0.00144291	2hr
ELOVL1	elongation of very long chain fatty acids (FEN1/Elo2; SUR4/Elo3; yeast)-like 1	**1.2169**	0.0283276	2hr
2310079F23RIK	RIKEN cDNA 2310079F23 gene	**1.22044**	0.0254718	2hr
PITPNM2	phosphatidylinositol transfer protein; membrane-associated 2	**1.24444**	0.0193494	2hr
CALD1	caldesmon 1	**1.32562**	0.00509207	2hr
1110005A03RIK	RIKEN cDNA 1110005A03 gene (1110005A03Rik); mRNA	**1.49491**	0.0432868	2hr
ABCC5	ATP-binding cassette; sub-family C (CFTR/MRP); member 5	**1.22803**	0.0441121	2hr
FADS1	fatty acid desaturase 1	**1.26151**	0.0331181	2hr
SEMA4B	sema domain; immunoglobulin domain (Ig); transmembrane domain (TM) and short cytoplasmic domain; (semaphorin) 4B	**1.23599**	0.00276344	2hr
ALPK2	alpha-kinase 2	**1.22457**	0.0279591	2hr
SEC62	SEC62 homolog (S. cerevisiae)	**1.23065**	0.0314432	2hr
IQCH	IQ motif containing H	**1.23077**	0.0132759	2hr
OTOP3	otopetrin 3	**1.21251**	0.0232051	2hr
FAM70A	family with sequence similarity 70; member A	**1.33633**	0.02221	2hr
SKIV2L2	superkiller viralicidic activity 2-like 2 (S. cerevisiae)	**1.27755**	0.0150405	2hr
MS4A2	membrane-spanning 4-domains; subfamily A; member 2	**1.38084**	0.0413534	2hr
SBF2	SET binding factor 2	**1.25571**	0.022509	2hr
PRL7B1	prolactin family 7; subfamily b; member 1	**1.22265**	0.000216473	2hr
DNAHC12 /// DNAHC7L	dynein; axonemal; heavy chain 12 /// dynein; axonemal; heavy chain 7-like	**1.22255**	0.000135741	2hr
E230016M11RIK	RIKEN cDNA E230016M11 gene	**1.28062**	0.0362002	2hr
TRIM24	tripartite motif-containing 24	**1.21951**	0.0182595	2hr
DDX39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	**1.2474**	0.0315811	2hr
NARF	nuclear prelamin A recognition factor	**1.21254**	0.0340665	2hr
1110020G09RIK	RIKEN cDNA 1110020G09 gene	**1.20117**	0.0189711	2hr
CLASP1	CLIP associating protein 1	**1.205**	0.0255391	2hr
TSPYL5	testis-specific protein; Y-encoded-like 5	**1.27133**	0.0447402	2hr

RBMX	RNA binding motif protein; X chromosome	**1.24899**	0.0341292	2hr
RIF1	Rap1 interacting factor 1 homolog (yeast); mRNA (cDNA clone IMAGE:4503215)	**1.40755**	0.0348304	2hr
PTX3	pentraxin related gene	**1.3553**	0.0403225	2hr

Table VI-5. List of 3'UTRs amplified from mouse genomic DNA used for 3'UTR luciferase analysis. Table shows gene abbreviation, length of 3'UTR (base pairs) and sequences of forward and reverse primers.

Table VI-5

Gene Abbreviation	3'UTR Length	Forward Primer Sequence	Reverse Primer Sequence
Apobec3	1060 (BP)	TATACTCGAGCGATGTCTTGAGAGGC	TATAGCGGCCGCGAGACAGACACCC
CASR	771 (BP)	TATACTCGAGCTCCTAATGGAGGGAG	TATAGCGGCCGCCTGCCTTATAGAAT
CCNE1	531 (BP)	TATACTCGAGGACCAAACCTGCCATT	TATAGCGGCCGCGACAGAGTTAAGAA
CD151	784 (BP)	TATACTCGAGCCACGATGATGTCAG	TATAGCGGCCGCTTCGAGTGCTTATA
ISX	555 (BP)	TATAGCGATCGCGCAACTCTCCTCTCTCAGTGATG	TATAGCGGCCGGCAGCCCAACAAAATGACGACAC
LGALS	487 (BP)	TATACTCGAGACCACGCCATGATCTA	TATAGCGGCCGCGGCGCCATTCAGC
TIAM1	2011 (BP)	TATACTCGAGCACGGAGATATGACTT	TATAGCGGCCGCAGGCACAGGGAAG
USP30	1136 (BP)	TATAGCGATCGCGAGTATAGGTCTGA	TATAGCGGCCGCGACCAGAAGAGTC
WDR5	1678 (BP)	TATACTCGAGGTGACTGCTAAGTCCT	TATAGCGGCCGCGGAACAAAATTACA

VII. Chapter 3: MicroRNA-21 (miR-21) Directly Targets Intestinal-Specific Homeobox in Granulosa Cells

1. Abstract

MicroRNA-21 (miR-21) has critical physiological functions in the ovary where it is important for maintaining optimal ovulation rates and in preventing cell death in granulosa cells. The purpose of this project was to elucidate the molecular pathway of miR-21 action in granulosa through identification of functionally relevant miR-21 direct targets. To identify these targets, cultured granulosa cells were treated with an oligonucleotide specific for miR-21 (LNA-21) and a non-specific LNA (LNA-NS) used as a control. Eight hours after treatment, RNA was isolated and array analyses were performed (n=3). Array analysis revealed that loss of miR-21 caused overexpression of 372 and inhibition of 240 genes (612 total) when compared to cells treated with the control oligonucleotide. There were 102 overexpressed genes that contained potential miR-21 binding sites in their 3'UTR per bioinformatics analysis, implicating them as potential miR-21 direct targets. Three genes, including calcium sensing receptor (CASR), cyclin E 1 (CCNE1) and intestinal-specific homeobox (ISX), were chosen for further analysis due to the previously identified functions in carcinogenesis and apoptosis. 3' Untranslated Region (3'UTR) luciferase analysis in granulosa cells showed that miR-21 can bind to the 3'UTR of ISX, while no such binding was observed with the CASR and CCNE1 3'UTRs Overexpression of miR-21 in granulosa cells caused ablation or near ablation of ISX protein. Together these findings confirm that miR-21 is directly targeting and inhibiting ISX in granulosa cells. ISX is a transcription factor that is known to negatively regulate scavenger receptor class B type 1 (SRB1), β,β-carotene 15,15′-monooxygenase 1 (BCMO1) and retinoic acid receptor (RAR) expression in the intestine. Knockdown of ISX with a siRNA specific for ISX in granulosa cells caused increased mRNA levels of each of these genes when compared to cells treated with a non-specific siRNA. Additionally this treatment scheme caused induction of SRB1 protein levels. This study has identified both direct (ISX) and indirect (SRB1, BCMO1 and RAR) targets of miR-21 in granulosa cells. Each of these genes is known to function in a regulatory loop that impacts lipid and vitamin A uptake in the intestine. Also BCMO1 SRB1 and retinoic acid signaling, is known to be important in ovarian development and/or function. Direct and indirect

regulation by miR-21 of these genes suggests the potential of miR-21 regulating these functions in granulosa cells. Identification of these genes is a critical first step towards elucidating miR-21's mechanism of action in granulosa cells.

2. Introduction

MicroRNA are ~22 nucleotide length regulatory RNA that post-transcriptionally regulate gene expression through directly targeting 3'UTRs of mRNA that have complementary binding sites to its sequence [47-50]. MicroRNA have been linked to many functional processes including cell proliferation, cell death, neuronal patterning and modulation of hematopoietic lineage differentiation [36]. In addition to regulating these processes, recent studies from our lab and others have implicated microRNA as critical for optimal female reproductive function. Studies in which Dicer, a critical microRNA biogenic enzyme [31, 32], is knocked down in the female reproductive tract have led to distinct pathologies including morphological changes in the oviduct and uterus, compromised oocyte and embryo quality, impaired embryo transport and implantation rates, decreased ovulation rates and infertility [93-96]. Further studies from our lab have implicated a specific microRNA, microRNA-21 (miR-21) as having important functions in the ovary. MiR-21 is induced by the LH surge and inhibition of miR-21 leads to impaired ovulation rates [98, 219]. Our lab has also shown that viability of mice murine cultured granulosa cells is significantly reduced when miR-21 is knocked down [219].

These findings, which implicate miR-21 in critical physiological functions, parallel a substantial amount of previous research that has implicated miR-21 in pathological processes, specifically in cancer. Profiling experiments have shown that miR-21 is overexpressed in most solid tumors including lung, breast, colon, ovarian and pancreatic cancer [220]. Studies have also revealed that miR-21 has increased expression in carcinogenesis in both mice and humans with causal links between its expression and functions such as hypertrophy, migration and metastasis [221]. These findings have prompted research, which have identified a number of miR-21 direct targets across a variety of cancer cell lines. These, include phosphatase and tensin homologue (PTEN) [126, 127], reverse-inducing-cysteine-rich protein with kazal motifs [128], tissue inhibitor of metalloproteinasis [128], leucine rich repeat interacting protein 1 [129], bone morphogenetic protein receptor I [130], ras homologue gene family member b (RHOB) [131], sprouty2 (SPRY2) [132], mapsin [133], tropomyosin 1 (TPM1) [133, 134] and programmed cell

death 4 (PDCD-4) [127, 132, 134, 135]. Our laboratory examined a select number of these targets in granulosa cells; PDCD-4, PTEN, TPM1 and SPRY2, and failed to identify a difference in protein expression of any of the genes following inhibition of miR-21 [99]. These findings suggests the possibility that miR-21 is directly targeting novel genes in granulosa cells.

Due to its critical role in ovarian/granulosa cell physiology, the purpose of this study was to identify functionally relevant novel miR-21 direct targets in granulosa cells. Since previous reports have shown that direct targets of microRNA have moderate changes in their mRNA levels [41, 42, 222], array expression analysis was used to determine steady state mRNA changes caused by miR-21 inhibition in granulosa cells. Loss of miR-21 caused widespread changes in mRNA levels and bioinformatics analysis implicated many of these genes as potential miR-21 direct targets. This study confirmed that intestinal-specific homeobox (ISX) is a miR-21 direct target in granulosa cells. Further studies showed that ISX regulated the expression of scavenger receptor class B type 1 (SRB1), β,β-carotene 15,15′-monooxygenase 1 (BCMO1) and retinoic acid receptor (RAR). These genes have been identified to be under the regulatory control of ISX and important in lipid uptake and vitamin A metabolism in the small intestine[215]. Also, both RAR and SRB1 have been implicated in critical roles in the ovary including meiotic initiation and steroidogenesis, respectively [223-225]. Through direct and indirect regulation of ISX, SRB1, BCMO1 and RAR, miR-21 may be regulating these functions in granulosa cells.

3. Experimental Procedures

Granulosa cell isolation and cell culture

25 day old CF-1 female mice were sacrificed using cervical dislocation in accordance with the protocol for animal sacrifice approved by the Internal Care and Use Committee at the University of Kansas Medical Center. Ovaries were removed and placed in M199 collection media (Sigma-Aldrich, St. Louis, MO.) supplemented with 10mM HEPES and 0.2% BSA. Media was removed and replaced with M199 media supplemented with 0 .5M sucrose and 1.8mM EGTA. Ovaries were incubated in media in a 37^{o}C water bath for 15 minutes. After incubation ovaries were washed with M199 media (Sigma-Aldrich, St. Louis, MO.) 3X. Antral follicles were poked with 28 gauge insulin needles (Becton Dickinson, Franklin Lakes, NJ.) to release granulosa cells. Cells were transferred to a 15ml conical tube and spun for 15 minutes at 0.8rcf. Cells were plated in 6 well plates that had been previously coated with fibronectin at a density of 250,000 per well in DMEM F-12 HAM (Sigma-Aldrich, St. Louis, MO.) supplemented with 10% FBS and 1% gentamicin. Cells were incubated at 37^{o}C, 5% CO_2 for 48 hours prior to transfection.

Array analysis

After 48 hours in culture, granulosa cells were transfected with a locked nucleic acid specific for miR-21 (LNA-21) (5'-+T+C+A GTCTGATAA+G+C+T A-3')(Integrated DNA technologies, Coralville, IA.) or with a non-specific locked nucleic acid (LNA-NS) (5'+C+G+TCAGTATGCG+A+A+TC-3') at a concentration of 5uM per well of a 6-well plate. At 8 hours post transfection, cells were harvested and quality of RNA was assessed using Agilent Bioanalyzer 2001 (Agilent Incorp. Palo Alto, CA.). RNA was biotin labeled and fragmented according to Affymetrix protocols. Fragmented RNA from each sample was hybridized to the Affymetrix 430E 2.0 arrays (n=3, for each time point) and scanned using the gene array scanner (Affymetrix, Santa Clara, CA.). For each chip analyzed the mean fluorescence was averaged to baseline fluorescence.

Cloning of ISX into siCHECK-2 (Promega) plasmid

The 3'UTR of ISX, CASR and CCNE1 was amplified from wild type mouse genomic DNA. Forward and reverse sequences for ISX 5'- TATAGCTAGCGCGCAACTCTCCTCTCTCAGTGATG-3' /5'-TATAGCGGCCGG CAGCCCAACAAAATGACGACAC-3' , CASR 5'-TATACTCGAGCTCCTAATGGAGGGAG-3'/5'-TATAGCGGCCGCCTGCCTTATAGAAT and CCNE1 5'-TATACTCGAGGACCAAACCTGCCATT-3'/5'-TATAGCGGCCGCGACAGAGTTAAGAA-3'. Thirty-five cycles of amplification were performed using denaturing, annealing and extension conditions at 94^{o}C, 55^{o}C and 68^{o}C degrees, respectively. After amplification, ISX, CCNE1 and CASR was cut with XHO1 and Not1 (restriction sites located on the 5' and 3' end, respectively, of each amplified gene) restriction enzymes (New England Biolabs, Ipswich, MA.) at 37^{o}C overnight using appropriate buffer. After cutting, 3'UTRs were cloned into siCHECK-2 (Promega, Madison, WI.) 3' to the renilla translational stop codon using T4 DNA ligase enzyme and appropriate buffer (New England Biolabs, Ipswich, MA.) at 4 degrees overnight. Plasmid with inserted construct was subsequently transformed into DH5α cells (Life Technologies, Carlsbad, CA.) using a standard transformation protocol and grown on LB ampicillin plates. After overnight growth at 37^{o}C, colonies were lifted and grown overnight at 37^{o}C in LB ampicillin media. DNA was isolated and clones were confirmed through sequencing.

Site Directed Mutagenesis of ISX/siCHECK-2 plasmid

Mutagenesis of the miR-21 site in the ISX 3'utr was performed using the GeneArt Site-Directed Mutagenesis PLUS Kit (Life Technologies, Carlsbad, CA.) per the manufacturers protocol. Primer designed to perform the mutagenesis contained the following sequences; 5'-GGATGAAGTCCAGAGTCATAGGCTCCT-3' and 5'-GGTAAAAGAAGAGCCTATGACTCTGG-3'. The mutagenesis reaction mutated 5 bases on the 3' end of the miR-21 site in the ISX 3'utr as indicated in figure 2. Successful mutagenesis was confirmed through sequence analysis.

Luciferase analysis

After 48 hours in culture, granulosa cells were co-transfected with sicheck2 plasmid containing the 3'utr of one of the genes along with pre-microRNA-21 (pre-miR-21) (an immature form of miR-21 that gets processed into the mature form using the endogenous cellular miRNA biogenic machinery) or pre-miR-NS (non-specific pre-miR used as a control). Co-transfections were done using lipofectamine 2000 (Invitrogen, Carlsbad, CA.) per the manufacturers' protocol. The vectors were transfected at a concentration of 10ng and pre-miRs were transfected at 5uM, per well of a 6-well plate. 24 hours after transfection, cells were harvested and lysed using passive lysis buffer (Promega, Madison, WI.). Lysates were prepared for luciferase assay using the dual luciferase reporter assay system (Promega, Madison, WI.) per the manufacturers' protocol. Luciferase assays were performed on the Berthold Lumat LB 9501 Luminometer (Wallac, Gaithersburg, MD.).

Immunoblotting

After 48 hours in culture, granulosa cells were transfected under two different treatment schemes followed by probing with different antibodies per each treatment scheme (described below). 24 hours after transfection cells were harvested and lysed in hypotonic buffer containing 20mM NaCL, 20mM Tris-HCl and 0.1% Triton X-100. After centrifugation at 10,000 X G for 10 minutes to remove cellular debris, protein concentration of the supernatant was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Berkeley, CA.). Fifteen micrograms of each sample was added to appropriate volume of 4X sample buffer (4 mL of glycerol, 0.8g of sodium dodecyl sulfate [SDS], 2.5 mL of 1 M Tris-HCl, 8mL of H2O, 0.05% w/v bromophenol blue). After heating at 95^{o}C for 5 minutes, proteins were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis in 1X running buffer (4.5 g Tris-base, 21.6 glycine, 2 g SDS, 2 L dH2O). The proteins were subsequently transferred to a polyvinylidene fluoride membrane using 1X transfer buffer (6.06 g ofTris-Base, 28 g glycine, 0.2 g SDS, 1,600 mL dH2O, 400 mL MeOH). Membranes were blocked in 5% milk in 1X Tris-buffered saline 0.1% Tween

(TBS-T) for 2 hours. Before and after secondary antibody incubations membranes were washed 3X with TBS-T. West Pico Chemiluminescent (ThermoScientific, Rockford, Il.)substrate was used to visualize the protein antibody complexes.

The first treatment was with pre-miR-21 and pre-miR-NS (control)(Life Technologies, Carlsbad, CA.) at the concentration of 5uM per well of a 6-well plate using lipofectamine 2000, per the manufacturer's protocol. Primary antibody used was anti-ISX (Santa Cruz Biotech., Santa Cruz, CA.)(SC-86151) at a concentration of 1:250 in TBS-T. Secondary antibody used was anti-rabbit IgG at a concentration of 1:1000 in TBS-T. The second treatment was siRNA-ISX and siRNA-NS (control)(Life Technologies, Carlsbad, CA.) at a concentration of 5uM per well of a 6-well plate using lipofectamine 2000, per the manufacturer's protocol. The primary rabbit polyclonal antibody used was anti-SRB1 (Novus Biologicals, Littleton, CO.)(NB400-104) at a concentration of 1:1,000 in 5% milk in TBS-T. Secondary antibody used was anti-rabbit IgG at a concentration of 1:5,000 in TBS-T. After completing the ISX and SRB1 western each blot was stripped in stripping buffer (10 ml of Sodium Dodecyl Sulfate, 390ul of 2-mercaptoethanol, 3.125ml of 1M Tris-HCl (pH 6.8) and 36.485ml of H_2O) for 25 minutes at 50^oC. After stripping, blots were blocked and western was completed as described above with anti-Actin (Santa Cruz Biotech., Santa Cruz, CA.) used as the primary antibody and anti-goat IgG was as the secondary. Antibody conditions were 1:1,000 in 5% milk in TBS-T and 1:5,000 in 5% milk in TBS-T, for the primary and secondary antibodies, respectively.

Quantitative RT-PCR

After 48 hours in culture, granulosa cells were treated with siRNA-ISX and siRNA-NS (Life Technologies, Carlsbad, CA.) at a concentration of 5uM per well of a 6 well plate using lipofectamine 2000 per the manufacturer's protocol. 24 hours after treatment, RNA was isolated using TriZol per the manufacturer's protocol. 250ng of RNA was used in reverse transcription reactions. cDNA was made using the miScript II RT Kit (Qiagen, Hilden, Germany) per the manufacturers protocol. QRT-PCR

analysis was performed to test for amplification levels of RAR, BCMO1, SRB1, CDX2 and ISX between the siRNA-ISX and siRNA-NS treatment groups. Primers used for each were (RAR) 5'-AGTACTGCCGGCTGCAGAA-3'/ 5'-TCGCACCGACTCCTTGGA-3', (BCMO1)5'-TTTCCAAAGCTTTCTCCTACTTGTCT-3'/5'-CCACATTTCATCATCTTGATCAGA-3', (CDX2)5'-GAGCTGGCTGCCACACTTG-3'/5'-GCTCTGCGGTTCTGAAACCA-3', (ISX)5'-GTGTCTGGTCCTGTGCTGACA-3'/5'-CTGAGAGAGTAGGCAGCATTCTGT-3' and (u6)5-'CTCGCTTCGGCAGCACA-3'/5'-AACGCTTCACGAATTTGCGT-3' (used as a control) . Each reaction was performed on the ABI Prism 7900HT Sequence Detection system (Life Technologies, Carslbad, CA.) under the conditions of stage 1-50.0^{o}/2:00, stage 2- 95.0^{o}/10:00, stage 3-95.0^{o}/:15 and 60^{o}/1:00, stage 3-95.0^{o}/:15, 60.0^{o}/:15 and 95.0^{o}/:15. Each sample was performed in triplicates and the average was used to determine the relative expression levels between it and U6. To calculate relative fold change, the delta-delta CT method [226] was used.

Statistical analysis

For each of the quantitative assays performed in this study (3'utr luciferase assays, western blotting, and qRT-PCR) students t-tests were formed to determine if there was a statistical difference between control and experimental treatment groups. Each assay used an N=3 and N=4, as indicated in the results. Statistical significance was set at $P<0.05$ for each assay analyzed. Statistical analysis was not performed in the ISX blot shown in the results (figure 3) as the ISX signal was ablated or nearly ablated after pre-miR-21 treatment. Instead, all 4 experiments performed are shown in results.

4. Results and Tables/Figures

Steady-state mRNA changes in granulosa cells after loss of miR-21

MiR-21's effect on steady state mRNA levels in granulosa cells was assessed to identify miR-21 direct targets. At 8 hours after miR-21 knockdown in granulosa cells arrays were performed. There were a total of 611 genes that were dysregulated with 372 being overexpressed and 240 being inhibited. Of the 372 overexpressed genes following loss of miR-21, 102 had potential miR-21 sites in their 3'UTR according to bioinformatics algorithms (Table VII-2) (microT, miRanda, PicTar, PITA, TargetScan). Three genes, CASR (increased 1.2 fold), CCNE1 (increased 1.3 fold) and ISX (increased 1.3 fold), were chosen for further analysis due to their previously identified role in carcinogenesis and apoptosis (Table 1.).

3'UTR luciferase analysis of CASR, CCNE1 and ISX

To determine if miR-21 binds to the 3'UTRs of CASR, CCEN1 and/or ISX, the 3'UTR of each was separately cloned into the siCHECK-2 (Promega, Madison, WI.) vector. Granulosa cells were co-transfected with siCHECK-2 construct and pre-miR-21. Control cells were treated with a non-specific pre-miR (pre-miR-NS). The renilla/firefly ratio of siCHECK-2 containing CASR and CCNE1 3'UTRs did not change when miR-21 was overexpressed (Figure VII-1). However, overexpression of miR-21 did cause the renilla/firefly ratio of siCHECK-2 containing the ISX 3'UTR to decrease 30.1% when compared to the pre-miR-NS treated cells ($n=3$, $p<.05$) (Figure VII-1). To determine if renilla/firefly ratio change was due to specific binding, 5 bases of the 3'UTR of ISX were mutated at the 3' end of its miR-21 putative binding site (TargetScan) (Figure VII-2). An independent experiment in which granulosa cells were treated with siCHECK-2 containing the mutated ISX 3'UTR resulted in renilla/firefly ratio remaining the same between the pre-miR-NS and pre-miR-21 treated cells ($n=3$), while there was a 34.0% decline in the renilla/firefly ratio when miR-21 was overexpressed in cells treated with the siCHECK-2 vector containing with wild type ISX 3'UTR ($P<0.05$, $n=3$) (Figure VII-2). Together these results show that miR-21 binds directly to the ISX 3'UTR.

ISX protein expression levels after miR-21 overexpression

To confirm that miR-21 is directly targeting ISX, cultured granulosa cells were treated with pre-miR-21 to overexpress miR-21 and pre-miR-NS, which was used as a control. Overexpression of miR-21 caused ablation or near ablation of ISX expression levels when compared to the pre-miR-NS treated cells ($P<0.05$, $n=4$) (Figure VII-3), suggesting that miR-21 is regulating ISX protein levels in granulosa cells. This western result along with the luciferase analysis, reveal that miR-21 is directly targeting ISX in granulosa cells.

ISX regulation of RAR, BCMO1 and SRB1

RAR, BCMO1, SRB1 and CDX2 have been previously identified to be under ISX regulation in the small intestine. To determine if ISX regulates these genes in granulosa cells, cultured granulosa cells were treated with siRNA specific for ISX (siRNA-ISX) and a non-specific siRNA (siRNA-NS). qRT-PCR analysis revealed that siRNA-ISX treatment caused an 81.7, 80.1 and 36.8 fold increase in RAR, BCMO1 and SRB1 mRNA expression levels, respectively (Figure VII-4). ISX inhibition had no effect on CDX2 expression levels (Figure VII-6). Treatment of cells with siRNA-ISX was effective in reducing the expression of ISX mRNA 130.5-fold when compared to siRNA-NS treated cells (Figure VII-6). Western blotting revealed that inhibition of ISX in granulosa cells using siRNA-ISX caused a 3-fold increase in SRB1 protein expression levels when compared to control treated cells ($p<0.05$, $n=4$) (Figure VII-5).

Table VII-1. List of 3 select genes, cyclin E1 (CCNE1), calcium sensing receptor (CASR) and intestinal-specific homeobox (ISX), upregulated in granulosa cells due to LNA-21 treatment whose 3'UTRs were analyzed using the luciferase assay system. Genes were chosen due to their tumorigenic/apoptotic functions identified in other tissues.

Table VII-1

Gene Name	Gene Symbol	Fold Increase	Function
cyclin E1	CCNE1	1.26 fold increase	important for cell cycle progression
calcium sensing receptor	CASR	1.24 fold increase	GPCR important in calcium homeostasis
intestinal-specific homeobox	ISX	1.26 fold increase	transcription factor involved in inflammatory responses and cellular differentiation

Figure VII-1. Identification of ISX as a direct target of miR-21 in granulosa cells. Each 3'UTR was separately cloned into siCHECK-2 (Promega, Madison, WI.) and transfected into cultured granulosa cells. Pre-miR-21 treatment caused a 30.1% percent reduction in the renilla/firefly ratio of cells transfected with siCHECK-2 containing the ISX 3'UTR when compared to pre-miR-NS treatment (n=3, $p<.05$). There was no statistical change in the renilla/firefly ratio between the pre-miR-NS and pre-miR-21 treatments in granulosa cells transfected with the siCHECK-2 vector containing the 3'UTRs of CCNE1 or CASR (n=3). *Means +/- SEM are statistically different ($p<0.05$).

Figure VII-1

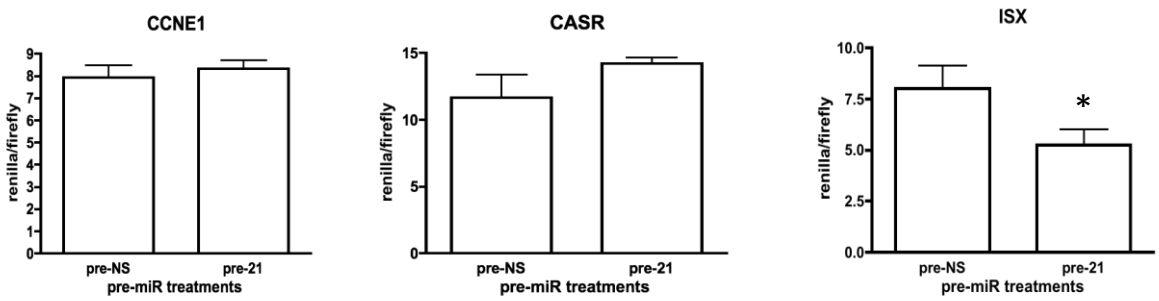
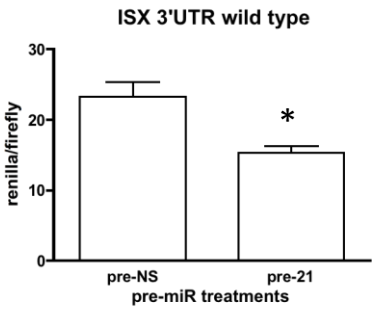
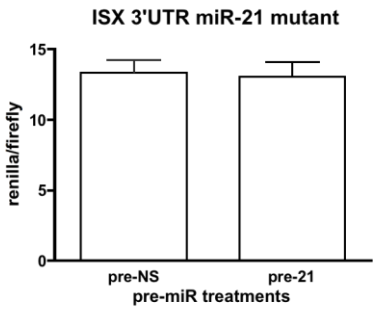

CCNE1
renilla/firefly
0
1
2
3
4
5
6
7
8
9
pre-NS
pre-21
pre-miR treatments
CASR
renilla/firefly
0
5
10
15
pre-NS
pre-21
pre-miR treatments
ISX
renilla/firefly
0.0
2.5
5.0
7.5
10.0
*
pre-NS
pre-21
pre-miR treatments

Figure VII-2. Independent analysis of the ISX 3'UTR miR-21 wild-type and the ISX 3'UTR miR-21 mutant. Pre-miR-21 treatment cased a 34.0% decline in the renilla/firefly ratio in the wild-type ISX 3'utr transfected cells when compared to the pre-miR-NS treatment (n=3, $p<.05$). Conversely there was no statistically significant difference in the renilla/firefly ratio observed between the pre-miR-NS and pre-miR-21 treatments in the ISX 3'UTR mutant (n=3). The boxes show putative miR-21 binding site within the ISX 3'UTR and the disrupted bases (shown in red) in the ISX 3'UTR after mutagenesis reaction. *Means +/- SEM are statistically different ($p<0.05$).

Figure VII-2





3' aguuguagUCA-GAC-UAUUCGAu 5'- miR-21
||| | | |||||||
5' ggggaugaAGUCCAGAAUAAGCUg 3'-ISX 3'utr

3' aguuguagUCA-GAC-UAUUCGAu 5' –miR-21
|||| | |
5' ggggaugaAGUCCAGAGUCATAGg 3' –ISX 3'utr

Figure VII-3. Western analysis of ISX after miR-21 overexpression in granulosa cells. 4 separate experiments are represented in which granulosa cells were treated with a non-specific pre-miR (pre-NS) used as a control or a pre-miR specific for miR-21 (pre-21). Overexpression of miR-21 caused complete or near complete ablation of ISX signal in each of the 4 experiments.

Figure VII-3

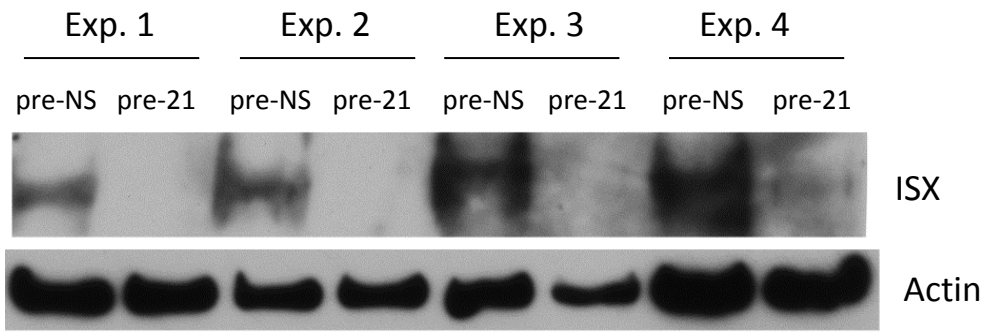

Exp. 1
Exp. 2
Exp. 3
Exp. 4
pre-NS
pre-21
pre-NS
pre-21
pre-NS
pre-21
pre-NS
pre-21
ISX
Actin

Figure VII-4. QRT-PCR analysis of retinoic acid receptor (RAR), β,β-carotene 15,15′-monooxygenase 1 (BCMO1), and scavenger receptor class B type 1 (SRB1) after ISX inhibition in granulosa cells. RAR, BCMO1 and SRB1 were upregulated 81.7 fold, 80.1 fold and 36.8, respectively (n=3, $p<.05$, for each gene tested) after treatment with siRNA-ISX when compared to the siRNA-NS control treatment. *Means +/- SEM are statistically different ($p<0.05$).

Figure VII-4

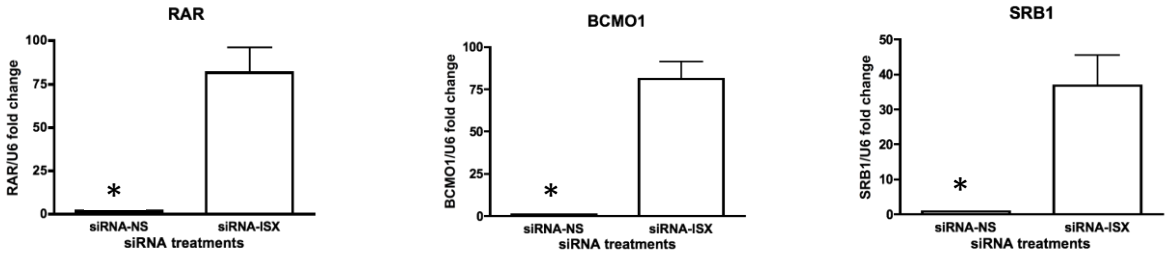
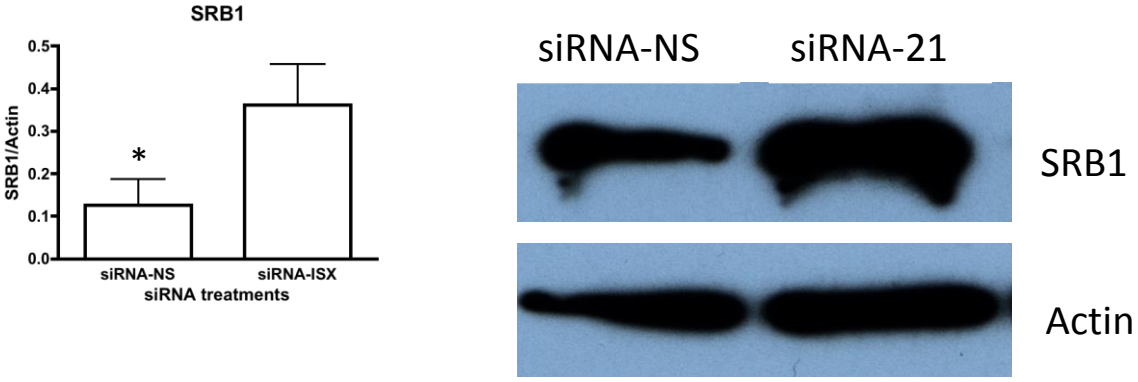

RAR
RAR/U6 fold change
100
75
50
25
0
*
siRNA-NS
siRNA-ISX
siRNA treatments
BCMO1
BCMO1/U6 fold change
100
75
50
25
0
*
siRNA-NS
siRNA-ISX
siRNA treatments
SRB1
SRB1/U6 fold change
50
40
30
20
10
0
*
siRNA-NS
siRNA-ISX
siRNA treatments

Figure VII-5. SRB1 protein expression levels after inhibition of ISX. Treatment of cultured granulosa cells with siRNA-ISX caused a 3-fold increase in SRB1 protein levels when compared to cells treated with si-RNA-NS (n=3, $p<.05$). *Means +/- SEM are statistically different ($p<0.05$).

Figure VII-5


SRB1
SRB1/Actin
0.5
0.4
0.3
0.2
0.1
0.0
*
siRNA-NS
siRNA-ISX
siRNA treatments
siRNA-NS
siRNA-21
SRB1
Actin

5. Discussion

Loss of miR-21 in ovarian granulosa cells can elicit profound effects both in vivo through blockade of ovulation and in vitro through induction of apoptosis [99]. However, analysis of numerous mir-21 targets identified previously in cancer cell lines has failed to identify a single positive target in ovarian granulosa cells [99]. The current study set out to identify direct miR-21 target genes in granulosa cells, as a mechanism to better understand how this post-transcriptional modulator could affect ovarian function. Intestinal-specific homeobox (ISX) mRNA expression was found to be moderately yet statistically induced 1.3-fold 8 hours after blockade of miR-21 activity in granulosa cells. Bioinformatic analysis showed that the 3'UTR of ISX contains a nucleotide sequence that has perfect homology with the seed sequence or the first seven bases of the 5' end of the mature form of miR-21. Luciferase expression analysis of granulosa cells treated with a luciferase vector containing the ISX 3'UTR and a miR-21 overexpression oligonucleotide, revealed that miR-21 does regulate gene expression and that loss of the miR-21 recognition site prevented miR-21 action. Lastly, ISX expression was ablated after miR-21 overexpression in granulosa cells confirming that ISX is a direct target of miR-21 in granulosa cells.

Originally identified in the intestine [227], ISX was shown to be highly expressed in the epithelium from the duodenum to the proximal colon, where it acts as a repressor of multiple genes within the intestine [228]. A recent study by Hsu et al., however, suggests that ISX can also act as a proimmflammatory homeobox gene, as they identified its ectopic expression in hepatocellular carcinoma (HCC) cells [229]. Even more interesting, in the HCC cells, ISX was shown to be a transcriptional activator of the cyclin D1 gene [229]. Thus, it is possible that this transcription factor could be both a positive and negative regulator of gene transcription dependent upon cellular context. The ectopic expression in the HCC is the first to describe ISX in a cell other than intestine, and ours is the first to show expression in a non-transformed ovarian tissue, specifically granulosa cells. As a homeobox gene, ISX, might be expected to play essential roles in cell survival, cell proliferation and inhibition of cell differention, a feature common to other HOX genes [230]. Prior to ovulation the granulosa cells are highly proliferative and require a

constant series of hormonal signals to remain viable and block atresia [89]. Following the ovulatory surge of LH, the granulosa cells terminally differentiate and undergo radical changes in cell morphology associated with the conversion to a highly steroidogenic cell [231]. Consistent with these cellular changes, ISX protein was present in granulosa cells before the LH surge and was reduced by overexpression miR-21, which in vivo is upregulated by the LH-surge [232].

Dietary carotene is typically converted to vitamin A (all-trans-retinol) in the intestine by β,β carotene-15,15'monooxygenase (BCMO1) and vitamin A in turn serves well known roles in vision, reproduction, immune function as well as cell differentiation [233]. Recent studies have implicated scavenger receptor class B type 1 (SCARB1) in the uptake of carotenoids and other lipid soluble vitamins [234], in addition to its well established roles in transfer of cholesterol from high density lipoproteins [223]. Knockout of ISX caused significant changes in intestinal gene expression including a significant increase in the expression of SRB1 and BCMO1, indicating that the ISX transcription factor might be acting as a repressor and in the presence of high vitamin A and downregulate these genes while the absence of the vitamin A derived retinoic acid stimulates ISX expression to limit both metabolism and uptake [228]. Based on these observations and the known expression of these key players in ovarian granulosa cells [216, 217, 235, 236], we examined whether loss of ISX was able to influence downstream target genes.

Knockdown of ISX by siRNA treatment in cultured granulosa cells increased SRB1, BCMO1 and RAR mRNA expression, all three of which have been previously shown to be under negative influence of ISX in the intestine [215, 228]. These findings suggest that ISX may regulate these genes in granulosa cells and that vitamin A uptake/metabolism may be under ISX control in granulosa cells. This hypothesis carries significance because vitamin A has previously been shown to be important for female reproduction and development of the female germ line [237]. Bovine and rodent studies have shown that vitamin A is critical for oocyte meiosis and oocyte maturation and that, under the appropriate conditions, vitamin A supplementation increases fertility rates [238-240]. Indeed, meiotic resumption has been shown to be mediated throughout retinoic acid, an active vitamin A metabolite and that germ cells

deficient in all-trans retinoic acid remained in their undifferentiated state and failed to enter meiosis [239]. Since paracrine signaling between the oocyte and granulosa cells is a known mechanism of oocyte development and function [241], ISX regulation of RAR in granulosa cells may be a control mechanism of meiotic resumption in female germ cells. Retinoic acid signaling, in addition to functioning in meiosis and maintaining viable germ cells during oogenesis, is also essential for cellular growth regulation, differentiation and cell death in a variety of tissues during fetal development [235]. Each of these functions may also be under ISX control through the regulation of RAR in granulosa cells.

ISX regulation of SCARB1 may also function to regulate steroidogenesis in the granulosa cells. Previous studies have shown that SCARB1 is highly expressed in rodent steroidogenic cells where it plays a critical role in cholesterol uptake and steroidogenesis [223]. Studies have also shown that SRB1 expression is hormonally regulated by both FSH and LH [216, 217]. Significantly, previous research has revealed that the luteinizing surge of LH leads to increased signal intensity of SCARB1 in granulosa cells and in luteinized granulosa cells, thereby causing increased cholesterol uptake and steriodogenesis [216]. Studies from our lab have shown that miR-21 is induced by the LH surge [98]. If miR-21 is directly targeting and knocking down ISX after the LH surge, this could be a control mechanism whereby ISX no longer represses SRB1, leading to increased steroidogenesis and luteinization in granulosa cells.

Due to ISX's regulation of BCMO1, SRB1 and RAR, any and, potentially, all of these functions may be under ISX control in granulosa cells/the ovary. And since this study has confirmed that ISX is under miR-21 direct control in granulosa cells, miR-21 may be the master regulator of these functions through direct regulation of ISX and indirect regulation of BCMO1, SRB1 and RAR. Functional studies will need to be performed to determine if miR-21/ISX regulates any/all of the above described functions.

In addition to identifying a novel miR-21 direct target that may have functional relevance in granulosa cells, results from this study are consistent with previous studies that have investigated the role of microRNA on steady-state mRNA levels. Previous investigations have reported that microRNA modestly

regulate large number of target mRNA [14, 40, 41] and that mRNA regulated by microRNA tend to be overrepresented with seed sequences that match the mRNA [42]; a finding which strongly suggests that, at least some, microRNA direct targets show mRNA destabilization. This study identified 612 genes that were moderately, yet, statistically differentially regulated 8 hours after loss of miR-21 when compared to control treated cells. Bioinformatic analysis identified 102 of these genes as being potential miR-21 direct targets based solely on the presence of a recognition site. However, until each of these individual genes are subsequently investigated through use of 3'UTR reporter assays/western blot analyses, we cannot conclude of their relevance to granulosa cell function.

Previous reports from our lab have already shown the physiological importance of miR-21 in the ovarian function and in granulosa cells. Functional studies will need to be performed to determine if miR-21 which is induced by the LH surge is able to then repress the translation or induce degradation of ISX mRNA. In turn, through direct control of ISX levels, genes downstream of this transcriptional repressor are able to increase and many of these have established roles in steroidogenesis. Along with elucidating key molecular events under miR-21 control in granulosa cells, this study has also confirmed previous reports that microRNA affect steady-state mRNA levels and even cause destabilization of the message levels of the genes that microRNA directly target. Further biochemical and molecular assays will be needed to determine if any of the remaining putative miR-21 direct targets identified through bioinformatics are under miR-21 direct control and, if so, whether or not they have functional relevance in granulosa cells/the ovary.

6. Supplementary Data

Figure VII-6. QRT-PCR analysis of intestinal-specific homeobox (ISX) and caudal-related homeobox (CDX2) after ISX knockdown. There was a 130.5 decrease in ISX mRNA expression 24 hours after treatment with a siRNA-ISX when compared to cells treated with siRNA-NS. There was no statistical difference in CDX2 mRNA expression levels between the cells treated with siRNA-NS and siRNA-ISX. *Means +/- SEM are statistically different ($p<0.05$).

Figure VII-6

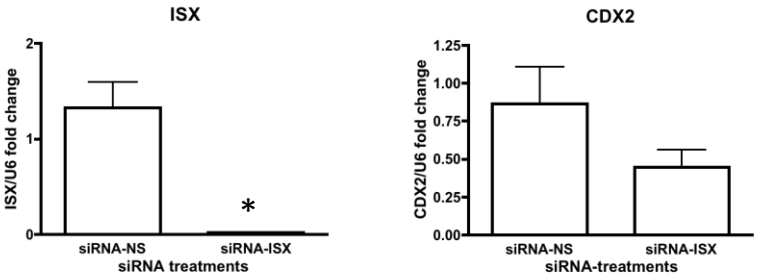

ISX
ISX/U6 fold change
2
1
0
*
siRNA-NS
siRNA-ISX
siRNA treatments
CDX2
CDX2/U6 fold change
1.25
1.00
0.75
0.50
0.25
0.00
siRNA-NS
siRNA-ISX
siRNA-treatments

Table VII-2. List of 102 genes that were upregulated 8 hours after loss of miR-21 and that contained potential miR-21 binding sites. Table lists gene symbol, gene name, fold change and p-value for each gene. Algorithms used were microT, miRanda, TargetScan, PicTar and PITA.

Table VII-2

Gene Symbol	Gene Name	Fold Change	P-value
KCNA1	potassium voltage-gated channel; shaker-related subfamily; member 1	**1.24066**	0.0381492
MAP2K3	mitogen-activated protein kinase kinase 3	**1.22371**	0.0308353
2310035C23RIK	RIKEN cDNA 2310035C23 gene	**1.2748**	0.0382897
RALGPS1	Ral GEF with PH domain and SH3 binding motif 1	**1.25791**	0.0467209
STX2	syntaxin 2	**1.23431**	0.034218
CASR	calcium-sensing receptor	**1.23596**	0.0237921
CNNM4	cyclin M4	**1.2244**	0.0234587
ISX	intestine specific homeobox	**1.26309**	0.0284002
ATP8A1	ATPase; aminophospholipid transporter (APLT); class I; type 8A; member 1	**1.24654**	0.0369261
PSD2	pleckstrin and Sec7 domain containing 2	**1.2342**	0.0287654
CD244	CD244 natural killer cell receptor 2B4	**1.2094**	0.0378144
NEK6	NIMA (never in mitosis gene a)-related expressed kinase 6	**1.24016**	0.0386376
RNF144A	ring finger protein 144A	**1.33035**	0.0071275
DMRTC1A	DMRT-like family C1a	**1.22113**	0.0330858
SEL1L	deoxyribonuclease 1-like 2 /// similar to Dnase1l2 protein	**1.45314**	0.0470193
TPCN1	two pore channel 1	**1.38189**	0.0046422
KCND3	potassium voltage-gated channel; Shal-related family; member 3	**1.24132**	0.0364306
EPHB2	Eph receptor B2	**1.20284**	0.0442559
LOC100044065 /// ODZ1	similar to odd Oz/ten-m homolog 1 (Drosophila) /// odd Oz/ten-m homolog 1 (Drosophila)	**1.44758**	0.0263521
GALNT13	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13	**1.32545**	0.0283911
MSMB	beta-microseminoprotein	**1.36798**	0.0351251
BCL7A	B-cell CLL/lymphoma 7A	**1.22852**	0.0094923
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	**1.24707**	0.011334
DUSP8	dual specificity phosphatase 8	**1.41019**	0.0081411
PPP1R10	predicted gene; 100039405 /// similar to protein phosphatase 1; regulatory subunit 10 /// protein phosphatase 1; regulatory subunit 10	**1.33757**	0.0459527
MC1R	melanocortin 1 receptor	**1.24041**	0.0135792
KLRB1C	killer cell lectin-like receptor subfamily B member 1C	**1.30395**	0.0477138
AK3L1	adenylate kinase 3-like 1 /// similar to adenylate kinase 4	**1.31706**	0.0401146
CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2	**1.19567**	0.0294399
LOC100046704 /// NRAS	similar to neuroblastoma ras oncogene /// neuroblastoma ras oncogene	**1.22974**	0.0428806
FBLN1	fibulin 1	**1.31901**	0.0485345
NUTF2	nuclear transport factor 2	**1.29939**	0.0176036
COPG	coatomer protein complex; subunit gamma	**1.27054**	0.0212557
SYS1	SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	**1.2427**	0.0289906
SETMAR	SET domain and mariner transposase fusion gene	**1.20264**	0.0325313
ANAPC11	Anaphase promoting complex subunit 11; mRNA (cDNA clone MGC:35764 IMAGE:5356010)	**1.27847**	0.0318457
NRP	neural regeneration protein	**1.38904**	0.0263308

SLC10A7	solute carrier family 10 (sodium/bile acid cotransporter family); member 7	**1.33827**	0.0411596
NFIX	heat shock transcription factor family member 5	**1.29147**	0.0230708
2210020M01RIK	RIKEN cDNA 2210020M01 gene	**1.21661**	0.0173907
UCP2	Uncoupling protein 2 (mitochondrial; proton carrier); mRNA (cDNA clone MGC:13955 IMAGE:4205625)	**1.3288**	0.0010800
CAP2	CAP; adenylate cyclase-associated protein; 2 (yeast)	**1.31103**	0.0448868
4833439L19RIK	RIKEN cDNA 4833439L19 gene	**1.22641**	0.0294885
KCNIP3	Kv channel interacting protein 3; calsenilin	**1.31158**	0.0268974
SH3GL3	SH3-domain GRB2-like 3	**1.21961**	0.0054183
EIF4EBP2	eukaryotic translation initiation factor 4E binding protein 2	**1.25029**	0.0014307
SYS1	SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	**1.2427**	0.0289906
NPCD /// NPTXR	neuronal pentraxin with chromo domain /// neuronal pentraxin receptor	**1.2506**	0.0418691
FOXS1	forkhead box S1	**1.34367**	0.0314605
ETV1	ets variant gene 1	**1.32093**	0.0459477
RAB36	RAB36; member RAS oncogene family	**1.25816**	0.0348
MAPK12	mitogen-activated protein kinase 12	**1.2472**	0.0029408
2810046L04RIK	RIKEN cDNA 2810046L04 gene	**1.23751**	0.0022706
NEUROG1	neurogenin 1	**1.20242**	0.0116106
HSF5	heat shock transcription factor family member 5	**1.29147**	0.0230708
RECQL	RecQ protein-like	**1.19606**	0.0401784
GNAS	GNAS (guanine nucleotide binding protein; alpha stimulating) complex locus	**1.20489**	0.0259956
4930579E17RIK	RIKEN cDNA 4930579E17 gene	**1.19629**	0.0332697
SCN3B	sodium channel; voltage-gated; type III; beta	**1.20291**	0.0124925
1700065D16RIK	RIKEN cDNA 1700065D16 gene	**1.20812**	0.0120007
TMEM29	transmembrane protein 29	**1.40516**	0.0056755
PHC2	polyhomeotic-like 2 (Drosophila)	**1.27718**	0.0446983
GHR	growth hormone receptor	**1.22006**	0.003078
TTC28	tetratricopeptide repeat domain 28	**1.23915**	0.0218533
CCNY /// LOC100044842	cyclin Y /// similar to cyclin fold protein 1	**1.20531**	0.0079419
CCNE1	cyclin E1	**1.25368**	0.015062
NDST1	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	**1.21405**	0.0434991
FBXO21	F-box protein 21	**1.2031**	0.0340304
MYLPF	myosin light chain; phosphorylatable; fast skeletal muscle	**1.27022**	0.0271005
MCM5	minichromosome maintenance deficient 5; cell division cycle 46 (S. cerevisiae)	**1.28009**	0.0419356
GRHL2	grainyhead-like 2 (Drosophila)	**1.45489**	0.04115
LEPR	leptin receptor	**1.30297**	0.0468672
EG232599	Predicted gene; EG232599; mRNA (cDNA clone MGC:156005 IMAGE:40129691)	**1.20106**	0.0220952
EDA	ectodysplasin-A	**1.23257**	0.0104525
LDLR	low density lipoprotein receptor	**1.41719**	0.0391926
ZFP651	zinc finger protein 651	**1.20411**	0.0289022
CALR3	calreticulin 3	**1.31668**	0.0240547
CENPA	Centromere protein A; mRNA (cDNA clone MGC:13888 IMAGE:4018429)	**1.3344**	0.0445517

USP30	ubiquitin specific peptidase 30	**1.19824**	0.0359382
FGFR1	fibroblast growth factor receptor 1	**1.1935**	0.0160073
ZFP473	zinc finger protein 473	**1.21327**	0.0440109
POU2F1	POU domain; class 2; transcription factor 1	**1.21115**	0.0119038
ACOT12	acyl-CoA thioesterase 12	**1.273**	0.0254415
COX4NB	COX4 neighbor	**1.21692**	0.0360243
GAS7	growth arrest specific 7	**1.22461**	0.049252
NTSR1	neurotensin receptor 1	**1.27018**	0.020129
SLC12A3	solute carrier family 12; member 3	**1.32589**	0.0447393
C530008M17RIK	RIKEN cDNA C530008M17 gene	**1.2**	0.0414148
VPRBP	Vpr (HIV-1) binding protein	**1.24638**	0.0180449
PVT1	plasmacytoma variant translocation 1	**1.22902**	0.0187565
SYT17	synaptotagmin XVII	**1.20427**	0.013803
GABRB3	gamma-aminobutyric acid (GABA) A receptor; subunit beta 3	**1.22451**	0.0307998
ASAH2	N-acylsphingosine amidohydrolase 2	**1.21031**	0.0122681
5730469M10RIK	RIKEN cDNA 5730469M10 gene	**1.2101**	0.0084849
ELMO1	engulfment and cell motility 1; ced-12 homolog (C. elegans)	**1.27493**	0.0432414
ENPEP	glutamyl aminopeptidase	**1.29743**	0.0200332
ACADL	acyl-Coenzyme A dehydrogenase; long-chain	**1.22746**	0.0471311
ZHX3	zinc fingers and homeoboxes 3	**1.22889**	0.0022496
ROR1	receptor tyrosine kinase-like orphan receptor 1	**1.20987**	0.0119589
DMC1	DMC1 dosage suppressor of mck1 homolog; meiosis-specific homologous recombination (yeast)	**1.2226**	0.0038219
SEC11A	SEC11 homolog A (S. cerevisiae)	**1.29546**	0.0429056
EOMES	eomesodermin homolog (Xenopus laevis)	**1.27725**	0.0340839

VIII. Chapter 4: Roles of MicroRNA-21 (miR-21) and Programmed Cell Death 4 (PDCD-4) in Human Uterine Leiomyomas (ULMs)

1. Abstract

Uterine fibroids (ULM) express increased microRNA-21 (miR-21), yet this oncomir's role in ULM, the leading cause of hysterectomies, is unknown. This study sought to determine if expression of programmed cell death 4 (PDCD-4), a well-known target of miR-21, is altered in autologous leiomyoma and myometrial tissues and whether miR-21 regulates PDCD-4 expression, apoptosis, and global translation in immortalized myometrial (UtM) and leiomyoma (UtLM) cell lines. Leiomyoma tissues robustly expressed the full-length 51kDA isoform of PDCD-4, while normal myometrial tissue had negligible expression, but instead expressed a 29kDA isoform not seen in leiomyoma tissues. Similarly, UtLM cells expressed elevated miR-21 compared to UtM cells and a pattern of PDCD-4 consistent with autologous tissues. Knockdown of miR-21 increased PDCD-4 levels in UtM cells and UtLM cells, indicating that it can regulate PDCD-4 expression, but this regulation was limited. Loss of miR-21 also increased cleavage of caspase-3 (apoptosis marker) and increased phosphorylation of elongation factor -2 (marker of reduced translation) in both cell lines. Both the novel differential PDCD-4 isoform expression seen in these autologous tissues and the increased PDCD-4 in leiomyomas were unexpected. Elevated leiomyoma miR-21 levels would be predicted to decrease PDCD-4 levels, thus leiomyomas differ from other tumors where loss of PDCD-4 has been associated with tumor progression. Our studies indicate regulation of PDCD-4 expression is not a primary miR-21 function in leiomyomas, but instead miR-21 is able to impact cellular apoptosis and translation, through unknown targets, in a manner consistent with its involvement in the pathophysiology of ULM.

2. Introduction

Human uterine leiomyomas (ULMs) are benign tumors located in the smooth muscle of the myometrium. They are clinically apparent in ~25% of reproductive-aged women but the overall incidence is between 70 and 80% [156]. ULMs can cause abnormal uterine bleeding, pelvic pressure, pain, and reproductive dysfunction and they are the most common risk factor for hysterectomy resulting in 200,000 annually in the U.S. [160]. While the etiology that leads to the development of ULMs is unknown, the ovarian steroid hormones, estrogen and progesterone, as well as genetic abnormalities have both been implicated in the development of ULMs [172, 185, 242, 243]. Numerous studies have implicated microRNAs (miRNA) as critical regulators of many disease processes including cancer [220, 244]. A consistent finding observed across ULM miRNA expression studies comparing leiomyoma tissue to autologous myometrial tissue is the induction of miR-21 in ULM tissue [202-204]. Aberrant expression of miR-21 in a number of human cancers including breast, cervical, ovarian, hepatocellular, esophageal, prostate and lung B cell lymphoma has led to miR-21 being referred to as an oncomiR [220, 244]. While miR-21 has received a great deal of attention due to its role in other cancers, little is known regarding the role that miR-21 plays in ULM etiology.

Many functionally important direct targets of miR-21 that regulate apoptosis and/or tumorigenesis have been identified. These include reversion-inducing-cysteine-rich protein with Kazal motifs (RECK), sprouty 2 (SPRY2), phosphatase and tensin homologue (PTEN), T lymphoma and metastasis gene 1 (TIAM1) and tropomyosin 1 amongst others (TPM1) [220]. Another well studied miR-21 target transcript is programmed cell death-4 (PDCD-4) [135, 220]. PDCD-4 has many important functions including cell cycle regulation, neoplastic transformation and apoptotic regulation [148]. In-vitro cell culture models have also implicated PDCD-4 in inhibition of tumorigenesis, through down regulation of carbonic anhydrase II and urokinase receptor [245]. Confirmation of its tumor suppressive role has been demonstrated in mouse models in which overexpression of PDCD-4 in the epidermis led to significant reductions in skin carcinogenesis [153]. Expression analysis of PDCD-4 has shown that it is

down regulated in a variety of tumors providing additional confirmation of PDCD-4's function as a tumor suppressor [135, 220, 246]. In the single study examining miR-21 target genes in ULM, Pan et. al. [247] observed a slight decline in PDCD-4 mRNA levels in cultured primary leiomyoma cells compared to cultured myometrial cells, while paradoxically, showing that PDCD-4 mRNA levels were increased in a transformed leiomyoma cell line and in a leiomyosarcoma cell line, inconsistent with its role in tumor suppression. This study, however, did not address whether PDCD-4 protein expression was regulated by miR-21 in these cell lines [247]. Lastly, no study has examined PDCD-4 expression patterns in isolated leiomyoma or myometrial tissue.

This study investigated miR-21 and PDCD-4 expression patterns in hTert-immortalized leiomyoma (UtLM) and myometrial (UtM) cell lines as well as in leiomyoma and myometrial tissues. Additionally, we determined if miR-21 could post-transcriptionally regulate PDCD-4 and whether miR-21 could elicit effects on apoptosis or translation within the immortalized cell lines. Our results showed a distinct pattern of protein expression for PDCD-4 in leiomyoma tissues when compared to autologous myometrial tissues. Moreover, we observed that miR-21 affected global translation and regulation of apoptosis within myometrial and leiomyoma cells, and PDCD-4 did not reflect the anticipated inverse correlation with miR-21 levels.

3. Experimental Procedures

Tissue Samples

Myometrial and leiomyoma tissue samples were obtained from healthy premenopausal women with symptomatic leiomyomas at the time of elective hysterectomy at Carle Foundation Hospital (Urbana, IL). All tissues were collected under consent for use of discarded human tissue that was approved by the Institutional Review Board at the University of Illinois at Urbana-Champaign and Carle Foundation Hospital. Each tissue sample was assigned an arbitrary identification number on the day it was received and patient information was known only to the physician or physician's nurse. However, physicians provided information about the age of the patient and any medications. All patients were premenopausal (21-50 years old) and had not been on any hormonal medications for six months prior to their hysterectomy. Tissues collected were from both proliferative (53%) and secretory (47%) stages of the menstrual cycle. Upon biopsy, leiomyoma (n=11) and myometrial (n=12) tissues were placed in TRIZOL and processed to RNA in the laboratory (18), most of these tissues were from paired tissues. An additional, 11 autologous pairs of leiomyoma and myometrium tissues were used for protein analysis, seven of the sets of samples for the RNA and proteins samples were derived from the same patients.

Cell Lines and Tissue Culture

The hTert-immortalized leiomyoma cells (hereafter referred to as UtLM) were generated as described previously (38) and were generously provided by Dr. Darlene Dixon (National Institute of Environmental Health Sciences, Research Triangle, NC). The hTert-immortalized myometrial cells (UtM), generously provided by Dr. Rainey (Medical College of Georgia, Augusta, GA), were cultured in DMEM/F12 (Gibco, Carlsbad, CA.) supplemented with 7.5% $NaHCO_3$, 1% antibiotic antimycotic (Gibco), and 10% Fetal Bovine Serum (19). UtLM cells were cultured in MEM (Sigma, St. Louis, MO) supplemented with 300ug/ml of G418 (Calbiochem, San Diego, CA), 1% essential amino acids (Gibco), 1% non-essential

amino acids (Gibco), 1% L-Glutamine, 15% FBS and 1% vitamins (Gibco). Medium was replaced every 2-3 days and cells were passaged using 1X Trypsin (Gibco) as needed.

Locked nucleic acid (LNA) inhibitors were purchased from Exiqon (http://www.exiqon.com/mirna-inhibitors). The inhibitor specific for miR-21 (LNA-miR-21), has a complementary sequence to miR-21, 5'-**C**AA**C**AT**C**AG**T**CT**G**AT**A**AG**C**T-3' (bolded bases indicate position of locked nucleic acids), which binds to and blocks miR-21 action. The non-specific inhibitor LNA-scramble, which does not recognize any known RNA transcripts, was used as a control. UtM and UtLM cells were transfected in serum free medium (DMEM/F12 or MEM) at 85% confluence with LNA oligonucleotides (40 nM) using lipofectamine 2000 (Life Technologies, Grand Island, NY) per the manufacturer's protocol. Eight hours after transfection, medium was replaced with appropriate culture media for each cell line. Twenty-four hours after transfection, cells were harvested and RNA was isolated using Trizol (Ambion, Carlsbad, CA) per manufacturer's protocol and stored at -80^0 C until use. Cells were lysed in a hyptonic buffer containing 20mM NaCL, 25mM Tris-HCl and 0.1% Triton X-100. Lysates were centrifuged at 10,000g for 10 minutes to pellet cellular debris. The protein supernatant was stored at -80^0 until use.

Western Blotting

Protein concentration was determined for each sample using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules CA). Proteins (15 ug/sample) were denatured by diluting the appropriate volume in 4X Sample Buffer (4ml of glycerol, 0.8g of SDS, 2.5 ml of 1M Tris-HCl, 8ml of H_20, .05% w/v bromophenol blue) and heating at 95^o C for 5 minutes. Proteins were resolved in a 10% SDS PAGE gels in 1X running buffer (4.5g Tris-Base, 21.6 glycine, 2g SDS, 2L dH_2O) and then transferred to PVDF membrane using transfer buffer (6.06g of Tris-Base, 28g glycine, 0.2g SDS, 1600 ml dH_2O, 400 ml MeOH). Membranes were blocked in 5% milk in 1X TBS-T for 1 hour. Primary antibodies were incubated with membranes overnight at 4^0 C at 1:1000 dilution in 5% milk in 1X TBS-T and appropriate secondary antibodies were incubated for 1 hour at room temperature with membranes at 1:10000 dilution in 5% milk in 1X TBS-T. Membranes were washed 3X10 minutes before and after secondary antibody

incubation in 1X TBS-T. West Pico chemiluminescent substrate (Thermo Scientific Waltham, MA) was used to visualize the protein antibody complexes. Primary antibodies included 2-PDCD-4 antibodies (ProSci, Poway, CA and Sigma, St.Louis, MO), actin (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-EF2, and cleaved caspase 3 (Cell Signaling, Danvers, MA). Secondary antibody used for all westerns other than actin was donkey anti-Rabbit IgG-HRP (GE Healthcare, Little Chalftont, UK.). Secondary antibody used for the Actin westerns was donkey anti-Goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA.)

Quantitative RT-PCR

Total RNA (250ng) was reverse transcribed using the miScript Reverse Transcription Kit per the manufacturer's protocol (Qiagen, Hilden, Germany). Samples were diluted 1:10 in dH_20 for qRT-PCR, which was performed on an Applied Biosystems HT7900 sequence detector (Foster City, CA). Primer sets for U6 (forward primer CTC GCT TCG GCA GCA CA, reverse primer AAC GCT TCA CGA ATT TGC GT) and PDCD-4 (forward primer GGC CCG AGG GAT TCT GAA, reverse primer TAT CTG CTC ATT TTC TAC ATC CAT TTT) and the forward primer for miR-21 (TAG CTT ATC AGA CTG AT) were designed using Primer Express 3.0 Software. A universal primer from the miScript Sybr green PCR Kit (Qiagen) along with its forward primer was used to amplify miR-21. Samples were run in triplicates and the □□Ct method was used to calculate the relative fold change between the samples following normalization with U6 (20). The presence of a single dissociation curve confirmed the amplification of a single transcript and lack of primer dimers. Standard RT-PCR using primers (forward primer GAAAATGCTGGGACTGAGGAA, reverse primer GACGACCTCCATCTCCTTCGCT) designed to span the complete PDCD-4 coding sequence was completed to determine if alternative spliced variants exist.

Statistics

Band intensity of each protein was determined using Gel-Pro Analyzer software. The mean amount (n=3) of each protein was determined by normalizing the intensity of the specific protein bands to that of actin. The mean value of each samples' triplicate in qRT-PCR was normalized to U6 using the □□*Ct* method as described previously (20). Student's T-tests were performed to determine if expression levels of protein and/or mRNA of interest was affected by treatment. Statistical significance was considered at $P<0.05$.

4. Results

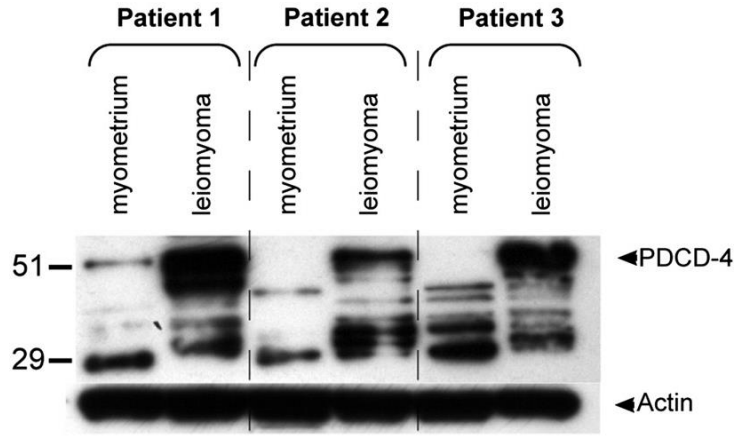
Consistent with elevated miR-21 in leiomyomas in previous studies, mature miR-21 levels in our leiomyoma tissues trended towards a 3.9 ± 2.9-fold increase (P=0.08) compared to normal myometrium (Figure VIII-1). Expression of PDCD-4 mRNA in leiomyoma (4.2 ± 0.8; mean fold ± SEM) and myometrial (3.9 ± 1.1) tissues were not different (P=0.85) (Figure VIII-1). In contrast, full-length 51 kDa PDCD-4 protein levels were markedly different in paired leiomyoma and myometrial tissues with myometrium showing barely detectable levels while leiomyoma tissues exhibited a robust signal (Figure VIII-1). No differences in PDCD-4 or miR-21 by stage of menstrual cycle were noted. Conversely, the paired myometrial tissues exhibited a lower molecular weight immunoreactive band that was not evident in the leiomyoma tissues (Figure VIII-1 and Figure VIII-5). To ensure this lower band was not an artifact, another PDCD-4 antibody (Sigma) was used (Figure VIII-5) and a similar pattern of immunoreactive bands was present, albeit the signal was not as robust for the lower band in the myometrium. In addition, leiomyomas exhibited a number of additional immunoreactive bands below the predicted full length 51 kDa PDCD-4 again with both antibodies (Figure VIII-1 and Figure VIII-5).

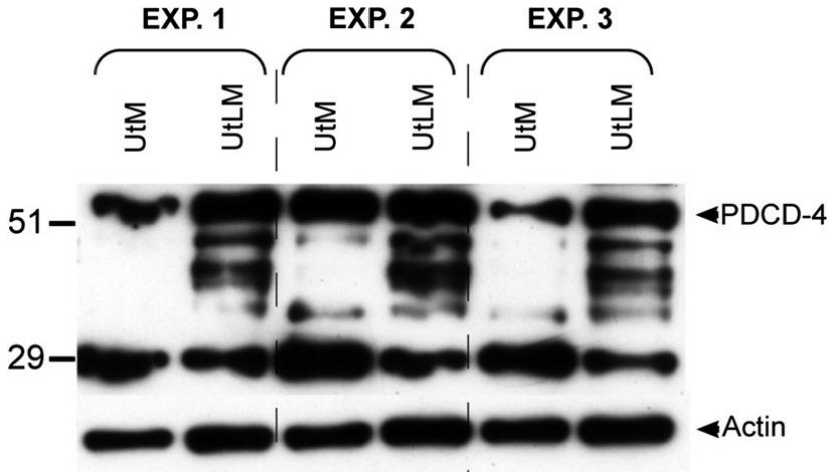
Immortalized myometrial cells (UtM) and leiomyoma cells (UtLM) were assayed for their relative expression of PDCD-4 mRNA/protein and miR-21. Unlike myometrial and leiomyoma tissues obtained from patients, both cell lines expressed the 51 kDa isoform, but consistent with the tissues, the UtLM cells had higher levels (1.4-fold) than UtM cells (Figure VIII-1). Again consistent with tissues, the lower molecular wt immunoreactive band was higher (2-fold) in UtM cells (Figure VIII-1). In addition, similar to patient-derived leiomyomas, a number of immunoreactive bands between 50 and 32 kDa were observed in the UtLM cells with minor evidence of these intermediate bands in the UtM cells (Figure VIII-1). RT-PCR for the full length mRNA failed to show differences between the myometrial and leiomyoma tissues. Direct comparison of PDCD-4 protein expression between tissue samples and immortalized cell lines revealed that the expression pattern of the normal myometrial tissues closely resembles that of the UtM cells while that of the leiomyoma tissues closely resembles that of the UtLM

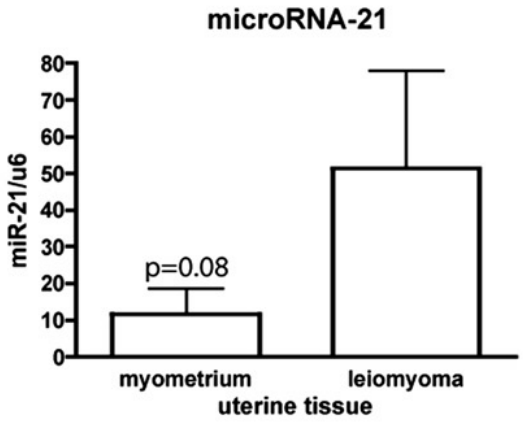
cells. PDCD-4 mRNA expression did not differ significantly between the cell lines (Figure VIII-1), while mature miR-21 expression was 4-fold greater in UtLM cells compared to UtM cells (Figure VIII-1). Since PDCD-4 is a known functional target of miR-21 in other cell lines, we analyzed PDCD-4 expression after inhibition of miR-21 in both the UtM and UtLM cell lines (Figure VIII-6 confirms the loss of mature miR-21 levels following LNA-miR-21 treatment of UtM and UtLM cells). Inhibition of miR-21 in UtM cells increased expression of the 51 kDa PDCD-4 isoform 2.5-fold (Figure VIII-2), whereas inhibition of miR-21 in UtLM cells increased the expression of the 51 kDa PDCD-4 isoform only 1.5-fold (Figure VIII-2). While loss of miR-21 action following LNA-miR-21 treatment impacted PDCD-4 protein synthesis, we observed no effect on PDCD-4 mRNA expression in either UtM or UtLM cells (Figure VIII-6). Consistent with miR-21's anti-apoptotic activity, inhibition of miR-21 activity increased the level of cleaved caspase-3, a marker of apoptosis in both UtM and UtLM cells (Figure VIII-3). In addition to its effects on PDCD-4 and apoptosis, we also examined whether miR-21 could affect global protein translation by measuring a key regulatory step, the phosphorylation of elongation factor-2 (EF2), a protein critical for protein translation (21). Increased levels of phosphorylated EF-2 in myometrial cells compared to the leiomyoma cells (Figure VIII-4) would be consistent with greater overall protein expression by the leiomyoma cells. Inhibition of miR-21 in UtM and UtLM cell lines caused a 6-fold and 3-fold induction of phosphorylated EF-2, respectively, indicating that miR-21 expression can positively affect translation in both cell lines (Figure VIII-4).

Figure VIII-1. Differential expression of PDCD-4 and miR-21 in leiomyoma and myometrial tissues and immortalized cell lines, UtLM (leiomyoma) and UtM (myometrial). A) Western analysis of PDCD-4 isoforms in 3 representative paired leiomyoma and normal myometrial samples of a total of 11 examined are shown. B) Western analysis of PDCD-4 isoforms in UtLM and UtM cell lines. *Means ± SEM (n=3) for the 51 kDa band between cells types are different ($P<0.05$). C) qRT-PCR analysis of miR-21 and PDCD-4 mRNA levels in UtLM and UtM cell lines. *Means ± SEM (n=3) are different ($P<0.05$) between UtLM cells and UtM cells.

Figure VIII-1







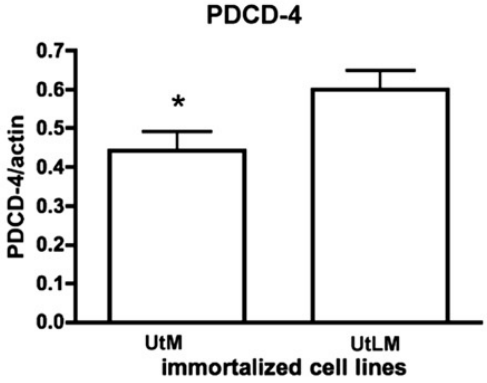
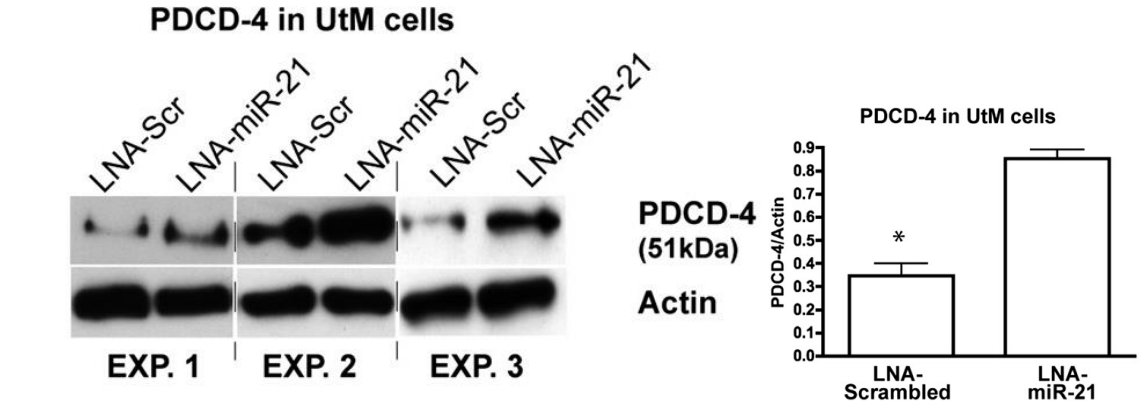


Figure VIII-2. MiR-21 regulation of PDCD-4 in UtM and UtLM cells. Locked nucleic acid (LNA) oligonucleotides complementary to miR-21 (LNA-miR-21) or a scrambled (LNA-scr) control were transfected into UtM cells (A) or UtLM cells (B). *Means ± SEM (n=3) PDCD-4 (51 kDa isoform) levels normalized to actin are different ($P<0.05$) between LNA-scramble and LNA-miR-21 treated cells.

Figure VIII-2



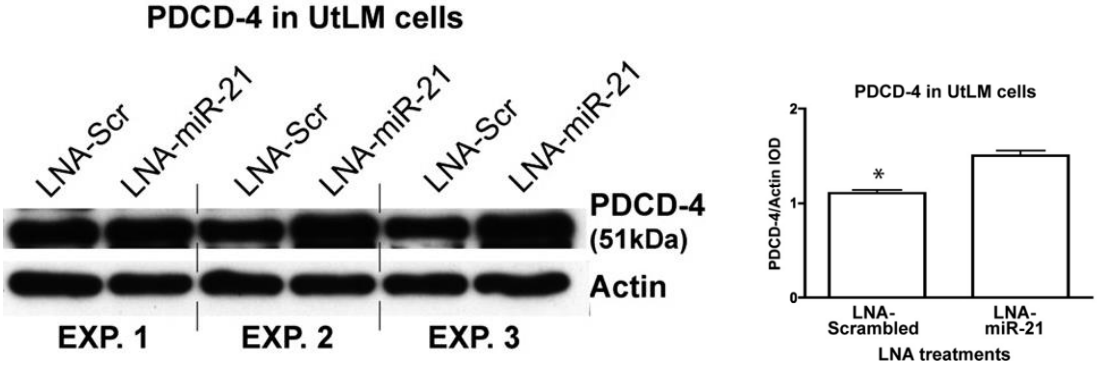


Figure VIII-3. Knockdown of miR-21 in UtM and UtLM cells increases *cleaved caspase-3*. Locked nucleic acid (LNA) oligonucleotides complementary to miR-21 (LNA-miR-21) or a scrambled (LNA-scramble) control were transfected into UtM cells (A) or UtLM cells (B). Western analysis showed a significant induction of cleaved caspase-3 in the LNA-miR-21 treated UtM and UtLM cells across 3 independent experiments.

Figure VIII-3

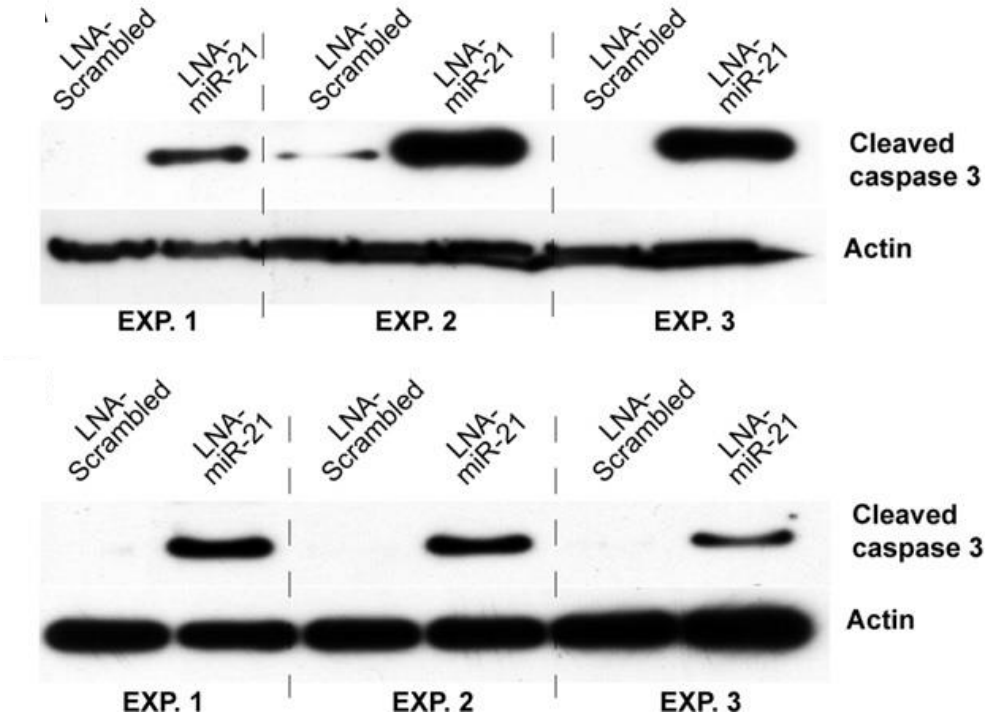
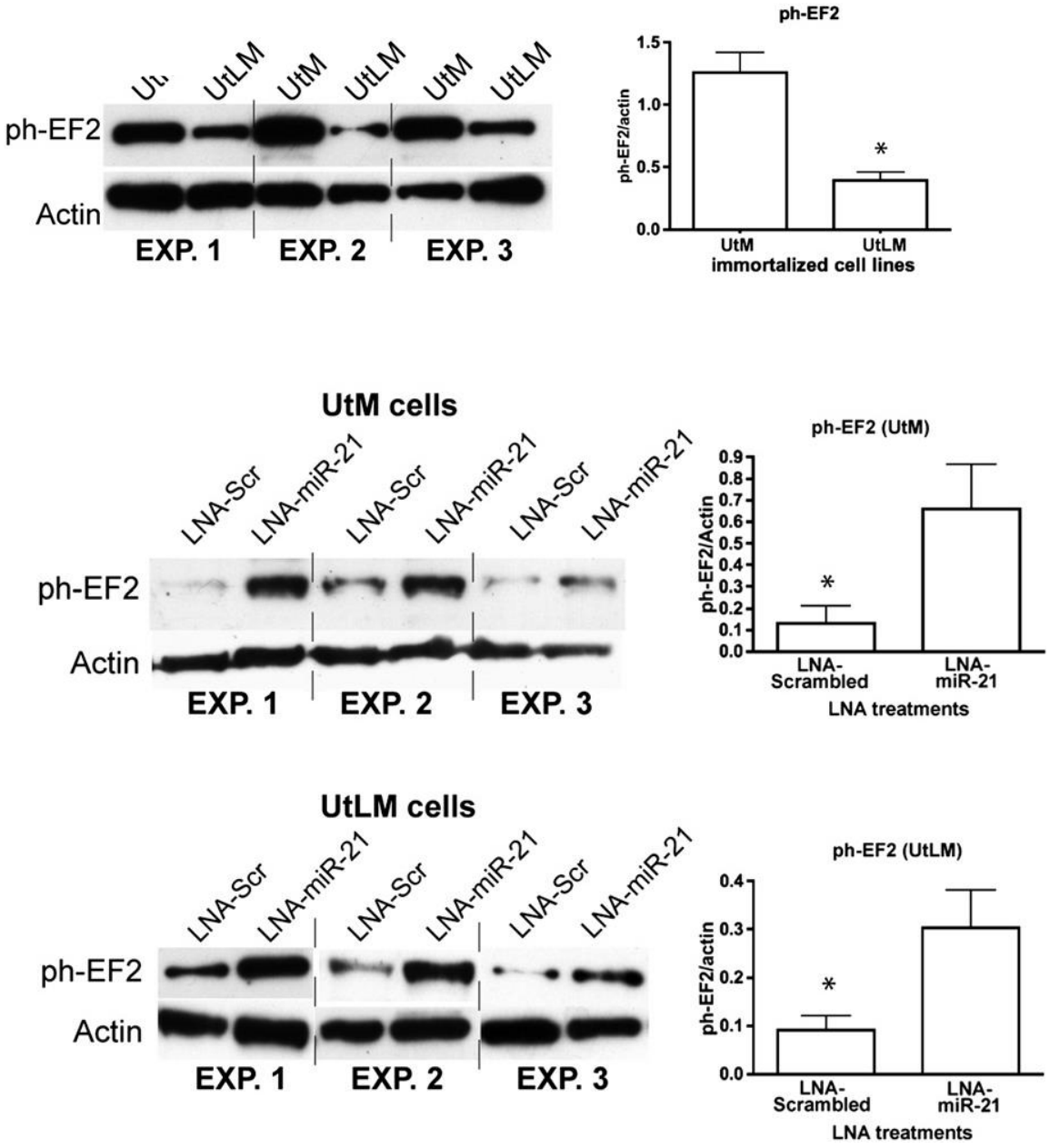


Figure VIII-4. Expression of phosphorylated EF-2 in UtM and UtLM cells following knockdown of miR-21. A) Basal ph-EF2 expression levels in UtM cells and UtLM cells. *Means ± SEM (n=3) ph-EF2 levels normalized to actin are different ($P<0.05$). B) Locked nucleic acid (LNA) oligonucleotides complementary to miR-21 (LNA-miR-21) or a scrambled (LNA-scr) control were transfected into UtM cells or UtLM cells. *Means ± SEM (n=3) ph-EF2 levels normalized to actin are different ($P<0.05$) between LNA-scramble and LNA-miR-21 treated cells.

Figure VIII-4



5. Discussion

As part of our examination of miR-21's role in uterine fibroid pathophysiology, we uncovered that PDCD-4, a potential target of miR-21, exhibited a dramatic increase in protein expression in leiomyoma tissues compared to autologous myometrial tissues, with no corresponding change in mRNA levels. Our results also revealed that the pattern of PDCD-4 expression, as well as that of miR-21 were preserved in immortalized myometrial and leiomyoma cells, enabling us to investigate miR-21's potential role in regulating PDCD-4 protein levels. Using these cells we demonstrated that miR-21 down regulates PDCD-4 protein expression. However, this is likely a minor effect because even though levels of miR-21 were elevated in leiomyoma tissues (our findings and others 9-11), the expression of PDCD-4 protein in leiomyomas was increased as well. We further demonstrated that miR-21 is able to exert independent effects in myometrial/leiomyoma cells (i.e., increased cell growth and blockade of apoptosis) that are consistent with the pathophysiology of this disease.

Loss of PDCD-4 is associated with tumor progression and PDCD-4 reintroduction into cells can block neoplastic growth [221]. Based on these studies we were surprised to detect increased expression of PDCD-4 protein in leiomyoma tissues in our study, particularly, because we expected the elevated levels of miR-21 to decrease protein levels by blocking mRNA translation. Recent studies indicate that PDCD-4 exhibits decreased expression in metastatic tumors in humans and mice and that restoring expression blocks the metastatic (invasive) potential of tumor cells [148, 221]. Thus, our unexpected finding of highly elevated PDCD-4 protein in leiomyomas raises the possibility that its role in these benign tumors may be to prevent malignant progression. Perhaps loss of PDCD-4 may occur in metastasizing leiomyomas or in leiomyosarcomas. What drives the increased PDCD-4 protein expression in leiomyomas remains unknown. While our study indicated that this was not likely a transcriptional response (i.e., equivalent PDCD-4 mRNA in leiomyoma and myometrial tissues), a large expression analysis study [248] of paired human uterine leiomyoma and myometrial tissue was able to show a slight 1.32-fold ($P<0.05$) increase in PDCD-4 mRNA levels in leiomyoma over myometrial tissues

(GSE13319). This slight increase in PDCD-4 mRNA expression, however, does not explain the marked differences in protein levels we observed.

Functional studies of the 51 kDa PDCD-4 have revealed that it can inhibit cap dependent translation as well as regulate RNA metabolism [249]. We were unable to find reported evidence of the low molecular weight band observed in myometrial tissues in any other tissues. We also observed that leiomyoma tissue and immortalized-leiomyoma cells exhibited the presence of numerous immunoreactive bands below the full length 51 kDA isoform. These may be the result of alternative splicing or proteasomal degradation in leiomyoma tissues. Recently, Schmid et al., [250] proposed that proteosomal degradation of PDCD-4 requiring activation of the mTOR pathway is involved in inflammation-mediated tumor progression. The exact nature of these immunoreactive products (alternative spliced protein, degradation product) remains to be determined, yet our simple RT-PCR analysis was unable to detect different mRNA isoforms. Determination of the PDCD-4 isoform composition within biopsies may have clinical value for characterization of the metastatic potential of leiomyomas.

The combined differential expression of miR-21 and PDCD-4 protein in the UtLM (leiomyoma) cells compared to UtM (myometrial) cells in a manner consistent with the expression in autologous pairs of primary tissues indicates that these cell lines are a useful model system for studying miR-21 and PDCD-4 expression and their potential interactions. Previous investigations have revealed that miR-21 post-transcriptionally regulates PDCD-4 in a number of cancer cell lines, including MCF-7, Colo206f and MDA-MB-231 [138, 140, 146]. Inhibition of miR-21 in both UtLM and UtM cells increased expression of the 51 kDa isoform of PDCD-4 protein, while having no effect on the lower immunoreactive molecular weight band. The lack of change in PDCD-4 mRNA levels indicates that miR-21 post-transcriptionally regulates PDCD-4 primarily through a block of translation and not mRNA degradation. The PDCD-4 mRNA results of our study, contrast those of Pan et al [247], who showed that inhibition and overexpression of miR-21 in several uterine smooth muscle cell lines could impact PDCD-4 mRNA levels. Possible differences in experimental systems (i.e., cell lines or type of inhibitors used) may account for these differences, yet our results from the UtM-immortalized cells are consistent with our

tissue-derived data. Inhibition of miR-21 resulted in reduced induction of PDCD-4 protein in UtLM cells compared to UtM cells, possibly due to the higher initial basal levels of PDCD-4 in UtLM cells or because miR-21 levels may have less regulatory impact in UtM cells. The higher basal expression levels of miR-21 in UtLM cells may also explain why inhibition of PDCD-4 with a fixed amount (albeit a saturating amount) of the blocking LNA-miR-21 was less effective. Thus, while showing for the first time that miR-21 can modulate PDCD-4 protein levels in leiomyoma cells in culture, our experiments suggest that miR-21 is in of itself, not a potent regulator of PDCD-4 expression in leiomyoma tissues as the levels of PDCD-4 should have decreased in leiomyoma tissues rather than increased.

Earlier array studies revealed that miR-21 has higher expression levels in leiomyomas when compared to normal myometrium [202-204]. Our study supports these previous observations, although the 3.9-fold induction we observed only trended toward significance (P=0.08). This discrepancy could be a result of our smaller sample sizes (n=12) compared to the larger sample sizes (n>50) used in previous studies where miR-21 was identified as upregulated. Significantly, the higher expression of miR-21 in leiomyoma tissues was consistent with the findings of in vitro cell culture results that showed elevated miR-21 expression levels in UtLM cells. This increased level of expression of miR-21 in leiomyoma tissues may provide limited regulatory control of PDCD-4 expression in vivo as compared to the greater regulation seen in our in vitro results. Since miR-21 overexpression in leiomyoma tissues and immortalized leiomyoma cells did not have a major impact on PDCD-4 expression, we also examined whether it might be influencing other cellular processes that had previously been reported [133, 139, 251]. Studies have shown increased expression of Bcl-2, a cell survival gene, and TNFα, a gene known to induce apoptosis in leiomyomas compared to normal myometrium [252]. Wu et al., found that there was no difference between the apoptotic index of leiomyoma and myometrial tissues [253], suggesting that Bcl-2, TNFα as well as other yet-to-be identified proteins (factors) are functioning to maintain homeostatic relationship in leiomyoma tissues. Our study sought to determine whether miR-21, an oncomir known to inhibit apoptosis, might play a role in regulation of apoptosis in UtLM and/or UtM cells. Knockdown of miR-21 caused a robust increase in cleavage of caspase 3, a marker for programmed

cell death, in both cell lines. This finding indicates that elevated levels of miR-21 may act to prevent programmed cell death in rapidly growing leiomyomas. It is possible that miR-21 contributes to this apoptotic homeostatic balance through indirect regulation of Bcl-2 and/or TNFα. Further studies are needed to determine if these genes may be direct or indirect targets of miR-21, or whether miR-21 impacts other apoptotic genes.

Previous investigations have also shown increased PCNA and Ki67 staining in leiomyomas over that of normal myometrium, indicating that leiomyoma is a more highly proliferative tissue [184, 253, 254]. Studies have also implicated the involvement of the mTOR pathway in the development of leiomyomas. Makker et al., [255] showed that mTOR signaling is increased in leiomyomas, while Yin et al. [256], revealed that estrogen requires mTOR to drive G1 cell cycle progression in leiomyomas. Elongation factor 2 (EF2), a gene that functions in protein synthesis and a downstream mediator of the mTOR pathway, is involved in control of global translation. In its un-phosphorylated form, EF2 is able to bind to the ribosome and translocate the mRNA-tRNA complex from the A site to the P site, and thus promote translation [100, 249]. Our study revealed that inhibition of miR-21 in both UtLM and UtM cells increased phosphorylation levels of EF2, supporting the hypothesis that basal levels of miR-21 in both cell lines are critical for maintaining EF2 in its un-phosphorylated state thereby allowing increased translation. These findings indicate that miR-21 may promote cell proliferation/growth through regulation of upstream mediators of EF2 phosphorylation.

Overall, the findings from our studies indicate that PDCD-4 exhibits a unique expression profile ,with almost complete absence of the full length PDCD-4 protein in normal myometrium, combined with unexpected, high overexpression in leiomyomas. This would suggest that PDCD-4 does not act as a typical tumor suppressor gene in leiomyomas as has been shown for several malignant tumors. Future studies, examining the role that steroids and other key regulatory molecules play in the regulation of PDCD-4 expression may prove to be highly informative. Additionally, a possible beneficial role in maintaining leiomyomas as benign non-metastatic tumors can be envisioned based on previous studies. Our studies also demonstrated that while the elevated levels of miR-21 found in leiomyoma tissues had a

limited effect on PDCD-4 expression; it may yet play key roles in promoting translation to stimulate growth of the leiomyoma cells. Lastly, the similar patterns of expression of miR-21 and PDCD-4 between the immortalized cell lines and in vivo tissues, supports the concept that UtLM and UtM cells are useful model systems for studying miR-21 and PDCD-4 function and regulation.

6 Supplemental Data

Figure VIII-5. PDCD-4 expression across paired normal and leiomyoma tissue using polyclonal anti-PDCD-4 antibody from (A) Sigma and (B) ProSci. P represents individual patients.

Figure VIII-5

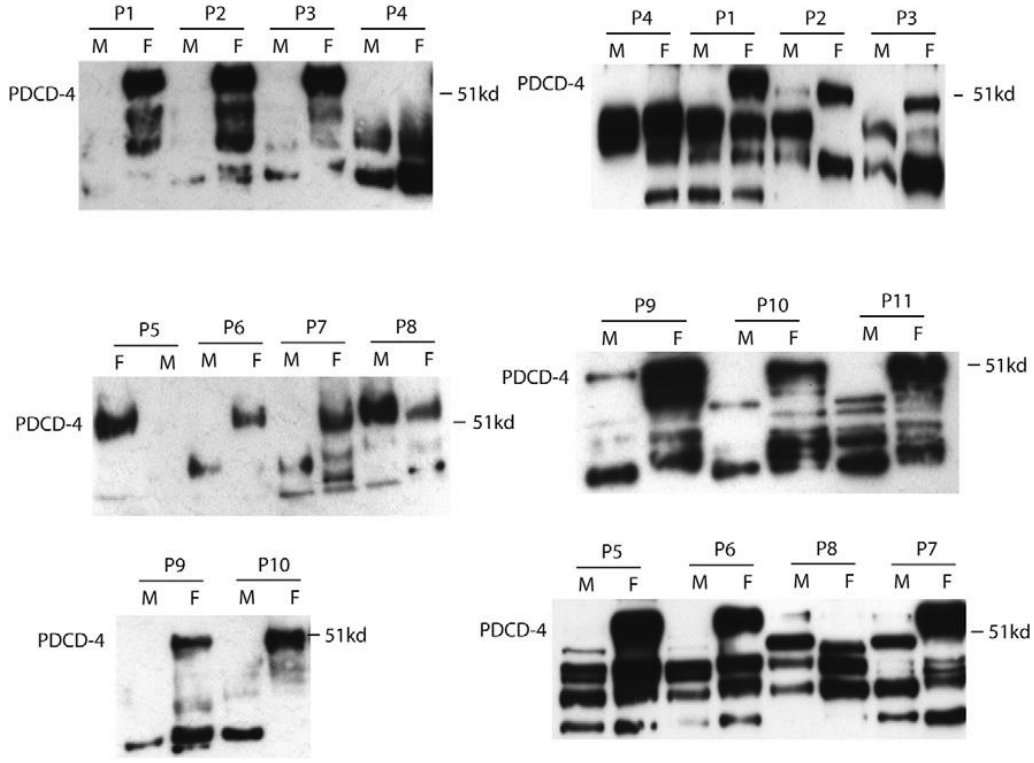
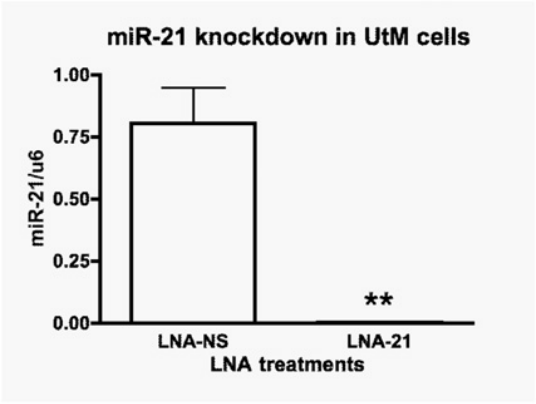
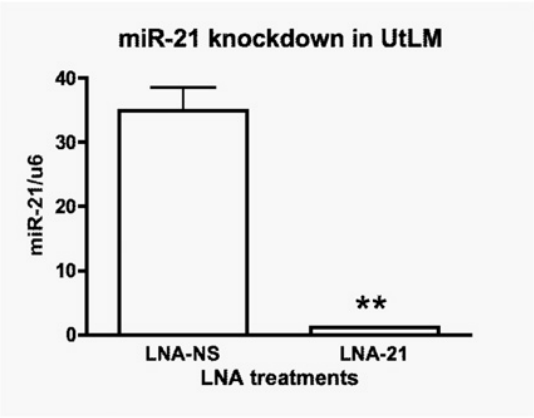
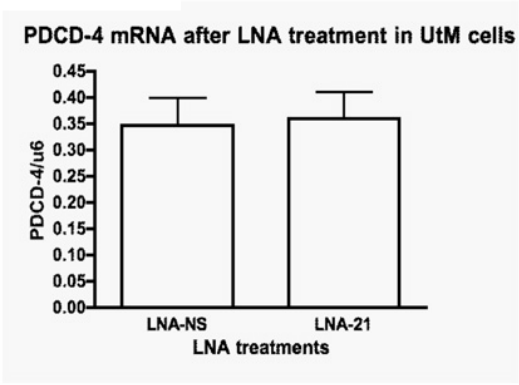


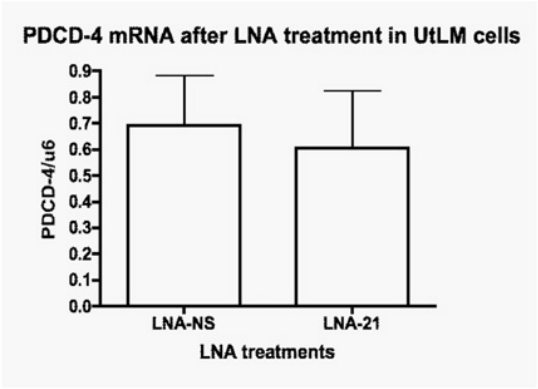
Figure VIII-6. (A) Efficacy of miR-21 knockdown after LNA-21 treatment in UtM and UtLM cells. (B) PDCD-4 mRNA levels after LNA-NS (scrambled) and LNA-21 treatment in UtM and UtLM cells. **means +/- standard error of the mean (n=3) miR-21 levels normalized to U6 are different (P<.01) between LNA-miR-21 (LNA-21) and LNA-scrambled (LNA-NS).

Figure VIII-6









IX. Chapter 5: Concluding Statements

A significant number of studies have investigated the role of microRNA-21 (miR-21) in cancer through identifying its direct targets and investigating its functions. The current investigation extends and broadens the understanding of miR-21 through identifying its direct targets in a physiological context. This study is the first to identify a miR-21 direct target in a physiological cell line and it is the first to implicate Apobec3 and USP30 as potential targets and confirm ISX as miR-21 direct target. Additionally the role of miR-21 and one of its most studied direct targets, PDCD-4, was investigated in ULMs. While miR-21 was capable of modest inhibition of PDCD-4 expression in our in vitro assays, our study revealed that both PDCD-4 and miR-21 were overexpressed in ULMs when compared to healthy myometrial tissue. This is inconsistent with many previous studies that have shown that miR-21 inhibits PDCD-4 expression. Induction of PDCD-4 in ULMs is also atypical as PDCD-4 is consistently shown to be lost in tumors. Together these findings have revealed novel miR-21 direct targets in ovarian granulosa cells and has shownthat cellular and tissue context can have dramatic effects on miR-21 action on target genes, and lastly that PDCD-4 may have unique functional roles in the etiology of ULMs.

Identification of miR-21 Direct Targets in Granulosa Cells

The foundational work that drove much of the studies in this dissertation was the findings that miR-21was LH regulated and a critical factor in maintaining optimal ovulation rates and preventing cell death in granulosa cells. What was unknown was the mechanism by which miR-21 carried out these functions. The purpose of this study was to begin to elucidate this mechanism through identification of miR-21 direct targets. The methodological approach used was to identify candidate genes through examining mRNA that are under miR-21 regulation that also have potential miR-21 binding sites in their 3'UTRs. Once candidate genes were identified, a select number were screened, using 3'UTR luciferase reporter assays, to determine if miR-21 could modulate luciferase levels. Luciferase activity for the

3'UTRs from the apolipoprotein mRNA editing enzyme catalytic polypeptide 3 (Apobec3), Ubiquitin-specific protease 30 (USP30) and intestinal-specific homeobox (ISX) genes were shown to be regulated by miR-21 in mouse granulosa cells.

Each of these genes has been shown to function in roles that may have significance in granulosa cells. USP30 plays a role in mitochondrial morphology and its deletion leads to interconnected and elongated microtubular structures [208]. Studies in the ovary have shown that appropriate mitochondrial morphology is critical for optimal steroid output [209-211]. Apobec3 functions in innate immunity against retroviruses and is known to regulate many different immune markers [212]. A variety of immunological markers are also under LH regulation in granulosa cells following the LH surge [213-214]. Intestinal-specific homeobox (ISX) has been shown to regulate scavenger receptor class B type 1 (SRB1) in the intestine [215]. Previous studies have revealed that SRB1 is regulated by LH and is a critical factor in steroidogenesis [216-217]. It is possible that miR-21 is directly targeting Apobec3, USP30 and ISX to regulate these functions in granulosa cells. In this way, this project has not only identified unique miR-21 direct targets but it also implicated this microRNA in novel functional roles, already known to be important for normal ovarian physiology.

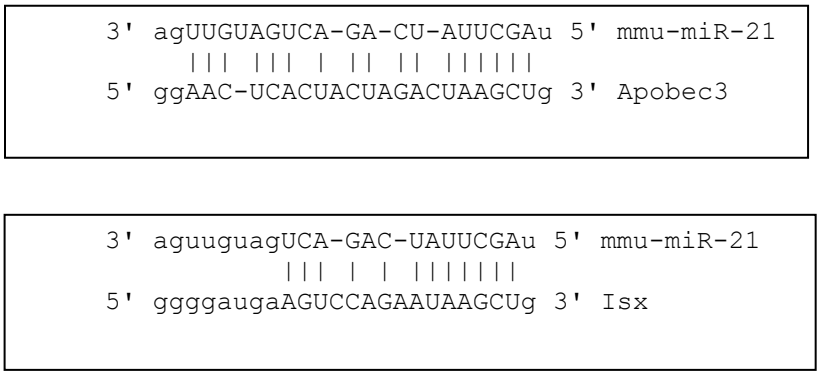
Additional research needs to be performed to determine if Apobec3, USP30 and/or ISX are bona fide miR-21 direct targets. Specifically, while this project revealed that miR-21 does regulate each genes' 3'UTR, further study is necessary to determine if this regulation is direct or indirect. Future studies should also include the identification of additional miR-21 direct targets. This project revealed the efficacy of examining steady-state mRNA as a means to identify miR-21 direct targets. Examination of the candidate genes identified in this study should, therefore, reveal more miR-21 direct targets. Sequence analysis from the bioinformatic algorithm, miRanda, identified a miR-21 binding site on the 3'UTR of Apobec3 and ISX (miR-21 direct targets identified in this study), which had 6 and 7 consecutive bases, respectively, that had perfect complementarity to the 5' end of miR-21 (Figure IX-1). Perfect complementary base pairing at the 5' end of the microRNA is this most prominent mechanism of

microRNA/mRNA interaction and sequence analysis of the putative miR-21 binding site of Apobec3 and ISX is supportive of these findings. It may, therefore, be useful for future studies to test genes that have the 5' dominant base pairing with miR-21 as top candidates for miR-21 direct targets.

Since it is likely that miR-21 directly targets multiple genes in granulosa cells, identification of additional direct targets is critical for fully elucidating the fundamental molecular events under miR-21 regulation. Identification of these targets is important for understanding the mechanism by which miR-21 carries out its anti-apoptotic and pro-ovulatory functions in granulosa cells.

Figure IX-1. Miranda sequence analysis for putative miR-21 binding sites for Apobec3 and ISX. Apobec3 and ISX contain 6 and 7 consecutive bases, respectively, that have perfect complementarity with the 5' end of miR-21.

Figure IX-1


3' agUUGUAGUCA-GA-CU-AUUCGAu 5' mmu-miR-21
5' ggAAC-UCACUACUAGACUAAGCUg 3' Apobec3

3' aguuguagUCA-GAC-UAUUCGAu 5' mmu-miR-21
5' ggggaugaAGUCCAGAAUAAGCUg 3' Isx

Identification of intestinal-specific homeobox (ISX) as a miR-21 direct target

The luciferase assay screen from chapter 2 implicated ISX as miR-21 direct target. Results in chapter 3 showed that miR-21 regulation of the ISX 3'UTR was direct and that miR-21 regulated ISX protein expression in granulosa cells. Together these findings show that ISX is a bona fide miR-21 direct in granulosa cells.

Intestinal-specific homeobox is a transcription factor originally identified in the intestinal epithelium [227]. An ISX knockout study in which β-galactosidase was knocked in to the ISX locus revealed that ISX expression exists throughout the epithelial cells from the duodenum to the proximal colon [228]. Expression analysis from this same study revealed that there was significant induction of scavenger receptor class B type (SRB1) and β,β-carotene 15,15′-monooxygenase 1 (BCMO1) mRNA levels throughout the duodenum and jejunum of the ISX knockout mice [228]. In a subsequent study it was shown that retinoic acid signaling lead to upregulation of ISX expression, which caused inhibition of retinoic acid receptor α (RARα) and BCMO1 [215]. Withing the intestine, SRB1, BCMO1 and RARα are important factors in lipid uptake and vitamin A metabolism in the intestine [257-259], so ISX regulation of these genes suggests that this transcription factor is regulating these functions in the intestine.

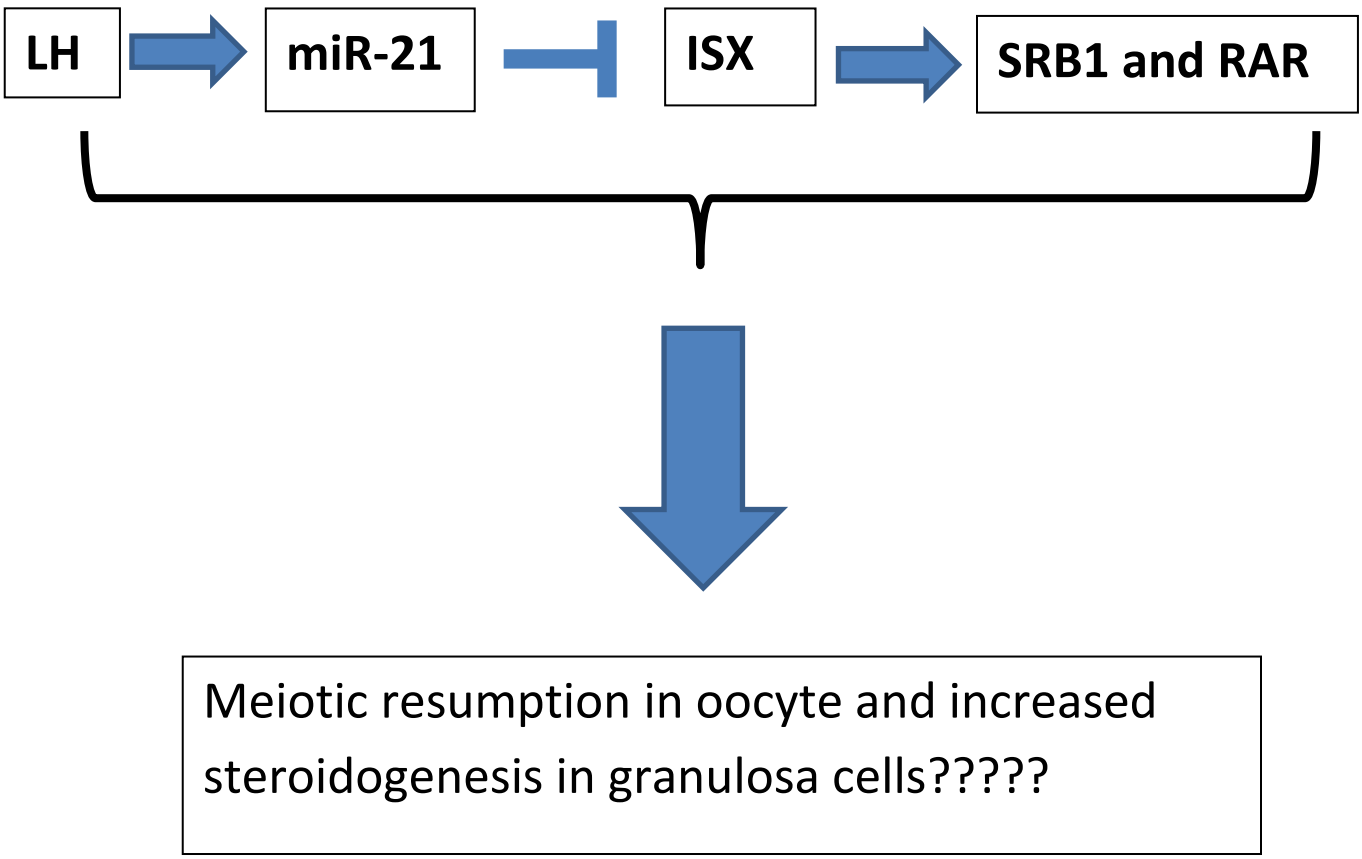
In addition to their roles in the intestine SRB1, BCMO1 and RARα have been implicated in roles in the ovary. Highly expressed in both the liver and rodent steroidogenic cells, SRB1 is known to mediate cholesterol uptake [223, 224, 260]. SRB1 is also capable of recognizing both negatively charged liposomes and apoptotic cells [260]. β,β-carotene 15,15′-monooxygenase 1 (BCMO1) functions in many tissues as the key enzyme for beta carotene metabolism [228, 262-264]. Reduced vitamin A causes induction of BCMO1 expression [265]. Studies in trout have shown that BCMO1 is expressed in the ovary and that it has higher expression in juvenile ovaries compared to adult [236]. β,β-carotene 15,15′-monooxygenase 1 has also been shown to function development and differentiation as well as embryogenesis [266]. Retinoic acid receptor α (RARα) has high expression in both the human and rat

ovary, mostly being localized to nuclear compartments within cells [267]. It plays an essential role in cellular growth regulation, differentiation and cell death across many tissues during fetal development. It is also important for maintaining the viability of germ cells during fetal oogenesis in the mouse [235]. Also retinoic acid signaling via regulation of Stra8 is essential for meiotic re-entry of ovarian germ cells [268] [225].

In this study each of these genes, SRB1, BCMO1 and RARα were all shown to be negatively by ISX in granulosa cells. This suggests the possibility that ISX is regulating, in granulosa cells, the functions under SRB1, BCMO1 and RARα control. Since miR-21 directly targets ISX, miR-21 may be the master regulator of SRB1, BCMO1 and RARα as well as their functions. Studies done prior to this investigation showed that miR-21 is regulated by the LH surge. If the gene pathway identified in cultured granulosa cells in this project is at play in vivo, the LH surge may function to upregulate SRB1, BCMO1 and RARα in granulosa cells through induction of miR-21 and subsequent inhibition of ISX (Figure IX-2). Previous research has already shown that LH causes induction of SRB1 in granulosa cells and this regulatory event is important for increased steroidogenesis. Revealed in this work, is the potential mechanism by which LH might upregulate SRB1 and induce steroidogenesis in granulosa cells. The LH surge also functions to cause meiotic resumption in the oocyte. Retinoic acid receptor α is known to play a critical role in this process. Potentially LH, through induction and inhibition of miR-21 and ISX, respectively, upregulates RARα in granulosa cells and through paracrine interaction with the oocyte (a well-known signaling mechanism between granulosa cells and the oocyte) leads to meiotic re-entry. Shown in figure IX-2 is a diagram of this proposed gene regulatory mechanism in granulosa cells. BCMO1 may also play a role in carrying out LH function. However, since what, if any, function it carries out in the periovulatory period is not known, it is difficult to hypothesize its potential LH mediated role.

Figure IX-2. Proposed LH pathway in granulosa cells. The LH surge causes induction of miR-21 leading to repression of ISX. Inhibition of ISX causes induction of SRB1 and RAR, genes known to function in steroidogenesis and meiotic resumption in the ovary.

Figure IX-2.


LH
miR-21
ISX
SRB1 and RAR
Meiotic resumption in oocyte and increased steroidogenesis in granulosa cells?????

Further research could be pursued to test this proposed model. First it should be determined if ISX is an in-vivo miR-21 direct target in granulosa cells specifically after the LH surge. Additional studies could also analyze the expression of BCMO1 and RAR after the LH surge (LH regulation of SRB1 has already been determined) and if these genes including SRB1 are regulated by miR-21 and/or ISX during the periovulatory period. Together these assays would determine if the pathway identified in cultured granulosa cells in this study also exists in granulosa cells in-vivo.

Subsequent studies could then be performed to identify the functional consequences of this gene pathway in the granulosa cells and the ovary. Assays in cultured granulosa cells could test, for instance, if dysregulation of ISX affects SRB1 and/or steroidogenesis. Culture studies could also be pursued to determine if ISX, through regulation of SRB1, affects vitamin A uptake as this is a known mechanism of vitamin A uptake in the intestine. In-vivo studies could be performed to determine if ISX, through regulation of RAR in granulosa cells, impacts meiosis in the oocyte through paracrine signaling between the oocyte and granulosa cells. And while investigating the potential impact on embryogenesis of ISX through regulation of BCMO1 is outside the scope of this project it is useful to keep in mind that this control mechanism may exist in the ovary. More generally miR-21, ISX and the genes it regulates may have important functions outside of the window of events that occur in cultured granulosa cells and after the LH surge.

Ultimately these studies will determine the functional consequences of genes regulated by miR-21. This will tie together the regulatory pathway under miR-21 control with its impact on granulosa cell and ovarian physiology. Together findings from this project combined with additional mechanistic and functional studies suggested above will elucidate the molecular and physiological events critical for understanding the impact of miR-21 in granulosa cells and the ovary.

MicroRNA-21 and programmed cell death 4 in Human Uterine Leiomyomas

Human Uterine Leiomyomas (ULMs) are benign tumors of the myometrium that can cause abnormal bleeding, pelvic pressure/pain and can lead to reproductive dysfunction. There is a 70-80% lifetime incidence rate for women throughout their reproductive life and they are the leading cause of hysterectomies causing over 200,000 annually in the United States. Hormones, growth factors and genetics are known to play a role in ULMs but the etiology and biological mechanisms that give rise to the disease are not well understood. Profiling experiments have shown that ULMs have a distinct microRNA profile and that miR-21 is highly overexpressed in this pathology. Based upon this latter finding, this project sought to investigate the role of miR-21 in ULMs.

This study showed that knockdown of miR-21 in UtLM cells (a cell line derived from ULMs) caused induction of cleaved caspase 3 and phospho-EF2. Together these findings indicate that miR-21 is playing an anti-apoptotic role and promoting global translation. MiR-21 has been shown to function in these roles in other tissues and studies in ULMs have shown that the tissue is highly proliferative. Together these findings suggest that miR-21 may have an anti-apoptotic role and promote global translation in ULMs.

Further study in this investigation that miR-21 inhibited expression of PDCD-4 (one of miR-21's most functionally relevant direct targets) in the UtLM cell line. However miR-21's regulatory control over PDCD-4 was minor and it was significantly less than miR-21's inhibition in UtM cells (a cell line derived from healthy myometrial tissue). If these cell lines are adequate model systems for studying miR-21 and PDCD-4 interaction in the tissues from which they derive, this finding means that miR-21 is exerting less regulatory control over PDCD-4 in ULMs when compared to healthy myometrial tissue. Most significantly this study revealed that PDCD-4 is highly upregulated in ULMs when compared to healthy myometrial tissue. This finding is quite unique as PDCD-4 is a well-studied tumor suppressor that is inhibited in metastatic tumors. Functional studies in mouse models have shown that PDCD-4

inhibits metastases. Potentially overexpression of PDCD-4 in ULMs, a benign tumor, is functioning in this same capacity. Another unique finding from this study is that overexpression of PDCD-4 in ULMs is commensurate with the induction of miR-21. PDCD-4 and miR-21 are inversely expressed across many cancers and miR-21 directly targets PDCD-4 in many cancer cell lines. So comcomitant induction of both PDCD-4 and miR-21 in ULMs indicates that PDCD-4 is outside of the regulatory control of miR-21 as it is upregulated in this uterine disease.

Future studies should test both PDCD-4's functional role and its mechanism of induction in ULMs. To test this hypothesis that PDCD-4 is preventing metastasis in ULMs, assays could be performed in UtLM cells to determine if PDCD-4 regulates migration in this cell line. If PDCD-4 does regulate cell migration in UtLM cells, one could hypothesize that PDCD-4 is carrying out this same function in ULMs. A further study that could be pursued to test PDCD-4's potential impact on migration is to compare its expression patterns between ULMs and uterine leiomyomsarcomas, which are metastatic uterine tumors. If, for example, PDCD-4 expression is inhibited in leiomyosarcomas vs. ULMs, this finding would be consistent with the hypothesis that PDCD-4 is inhibiting migration in ULMs.

To identify the mechanism of PDCD-4 induction in ULMs microRNA, other than miR-21, could be examined. Potential candidates would include those that are inhibited in ULMs vs. healthy myometrium and are complementary to a sequence on the 3'UTR of PDCD-4. Once candidate microRNA were identified, 3'UTR luciferase analysis, similar to those performed in this study, could be performed in UtLM cells to determine if the microRNA could bind the PDCD-4 3'UTR. Also, polysomal gradient analysis could be performed on UtM and UtLM cell lines (cell lines that showed differential PDCD-4 expression patterns in this study) to determine if PDCD-4 migration patterns within the gradient were different between the cell lines. This approach would determine if the mechanism of PDCD-4 induction in UtLM cells is via translation control. Together these studies would reveal the functional importance of PDCD-4 and the regulatory pathway of PDCD-4 induction in ULMs.

Results from these future studies may, in addition to adding to our understanding of the role of PDCD-4 ULMs, impact our understanding of tumor biology. As previously mentioned PDCD-4 is inhibited in metastatic tumors. What is not known though is expression patterns of PDCD-4 across benign tumors. This study has shown that PDCD-4 is highly upregulated in ULMs, a benign tumor. Potentially this PDCD-4 expression pattern persists across other benign tumors. If so, PDCD-4 may be functioning as a metastatic inhibitor in tumors, a role that it plays in healthy tissue.

This study has identified novel miR-21 direct targets in granulosa cells and identified novel cell lines (UtM and UtLM cell lines) in which microRNA regulates one of its most well-studied direct targets, PDCD-4. This work has also revealed potential novel functional roles under miR-21 regulation in granulosa cells, which may be mediated through its regulatory control of the miR-21 direct targets identified in this study. Additionally this study has revealed that miR-21 regulation of PDCD-4 (One of the most well-studied mechanisms of miR-21 control) is not a control mechanism in the development of ULMs and it is implicated PDCD-4 in the etiology of ULMs. Together these findings have revealed novel direct targets and functional roles for both miR-21 and PDCD-4 in granulosa cells and ULMs.

X. Chapter 6: References

1. Tomkins, G.M., et al., *Control of specific gene expression in higher organisms. Expression of mammalian genes may be controlled by repressors acting on the translation of messenger RNA.* Science (New York, NY), 1969. **166**(3912): p. 1474.
2. Ideker, T., et al., *Integrated genomic and proteomic analyses of a systematically perturbed metabolic network.* Science, 2001. **292**(5518): p. 929-934.
3. Gygi, S.P., et al., *Correlation between protein and mRNA abundance in yeast.* Molecular and cellular biology, 1999. **19**(3): p. 1720-1730.
4. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-854.
5. Wightman, B., I. Ha, and G. Ruvkun, *Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans.* Cell, 1993. **75**(5): p. 855-862.
6. Abbott, A.L., et al., *The< i> let-7</i> MicroRNA Family Members< i> mir-48</i>,< i> mir-84</i>, and< i> mir-241</i> Function Together to Regulate Developmental Timing in< i> Caenorhabditis elegans</i>.* Developmental cell, 2005. **9**(3): p. 403-414.
7. Li, M., et al., *Regulatory mutations of mir-48, a C. elegans let-7 family MicroRNA, cause developmental timing defects.* Developmental cell, 2005. **9**(3): p. 415.
8. Reinhart, B.J., et al., *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans.* Nature, 2000. **403**(6772): p. 901-906.
9. Johnson, S.M., et al., *RAS is regulated by the let-7 microRNA family.* Cell, 2005. **120**(5): p. 635-647.
10. Davison, T.S., C.D. Johnson, and B.F. Andruss, *[2] Analyzing Micro-RNA Expression Using Microarrays.* Methods in enzymology, 2006. **411**: p. 14-34.
11. Zhao, Y., E. Samal, and D. Srivastava, *Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis.* Nature, 2005. **436**(7048): p. 214-220.
12. Chen, C.Z., et al., *MicroRNAs modulate hematopoietic lineage differentiation.* Science, 2004. **303**(5654): p. 83-86.
13. Johnston, R.J. and O. Hobert, *A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans.* Nature, 2003. **426**(6968): p. 845-849.
14. Chang, J., et al., *miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1.* RNA biology, 2004. **1**(2): p. 106.
15. Giraldez, A.J., et al., *MicroRNAs regulate brain morphogenesis in zebrafish.* Science, 2005. **308**(5723): p. 833-838.
16. Xu, P., et al., *The< i> Drosophila</i> MicroRNA Mir-14 Suppresses Cell Death and Is Required for Normal Fat Metabolism.* Current Biology, 2003. **13**(9): p. 790-795.
17. LEAMAN, D., et al., *Antisense-mediated depletion reveals essential and specific functions of MicroRNAs in drosophila development.* Cell, 2007. **131**(4): p. 89-101.
18. Brennecke, J., et al., *< i> bantam</i> Encodes a Developmentally Regulated microRNA that Controls Cell Proliferation and Regulates the Proapoptotic Gene< i> hid</i> in< i> Drosophila</i>.* Cell, 2003. **113**(1): p. 25-36.
19. Brennecke, J., et al., *Principles of microRNA–target recognition.* PLoS biology, 2005. **3**(3): p. e85.
20. Lai, E.C., *Notch signaling: control of cell communication and cell fate.* Development, 2004. **131**(5): p. 965-973.
21. Stark, A., et al., *Identification of Drosophila microRNA targets.* PLoS biology, 2003. **1**(3): p. e60.

22. Poy, M.N., et al., *A pancreatic islet-specific microRNA regulates insulin secretion.* Nature, 2004. **432**(7014): p. 226-230.
23. He, L., et al., *A microRNA polycistron as a potential human oncogene.* Nature, 2005. **435**(7043): p. 828-833.
24. O'Donnell, K.A., et al., *c-Myc-regulated microRNAs modulate E2F1 expression.* Nature, 2005. **435**(7043): p. 839-843.
25. Lecellier, C.H., et al., *A cellular microRNA mediates antiviral defense in human cells.* Science Signalling, 2005. **308**(5721): p. 557.
26. Cai, X., C.H. Hagedorn, and B.R. Cullen, *Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs.* Rna, 2004. **10**(12): p. 1957-1966.
27. Lee, Y., et al., *MicroRNA genes are transcribed by RNA polymerase II.* The EMBO journal, 2004. **23**(20): p. 4051-4060.
28. Bracht, J., et al., *Trans-splicing and polyadenylation of let-7 microRNA primary transcripts.* Rna, 2004. **10**(10): p. 1586-1594.
29. Denli, A.M., et al., *Processing of primary microRNAs by the Microprocessor complex.* Nature, 2004. **432**(7014): p. 231-235.
30. Bohnsack, M.T., K. Czaplinski, and D. GÖRLICH, *Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs.* Rna, 2004. **10**(2): p. 185-191.
31. Jiang, F., et al., *Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila.* Genes & development, 2005. **19**(14): p. 1674-1679.
32. Ketting, R.F., et al., *Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans.* Genes & development, 2001. **15**(20): p. 2654-2659.
33. Gregory, R.I., et al., *Human RISC couples microRNA biogenesis and posttranscriptional gene silencing.* Cell, 2005. **123**(4): p. 631-640.
34. Du, T., et al., *Asymmetry in the assembly of the RNAi enzyme complex.* Cell, 2003. **115**: p. 199-208.
35. Du, T. and P.D. Zamore, *microPrimer: the biogenesis and function of microRNA.* Development, 2005. **132**(21): p. 4645-4652.
36. Bushati, N. and S.M. Cohen, *microRNA functions.* Annu. Rev. Cell Dev. Biol., 2007. **23**: p. 175-205.
37. Carthew, R.W., *Gene regulation by microRNAs.* Current opinion in genetics & development, 2006. **16**(2): p. 203-208.
38. Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg, *Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?* Nature Reviews Genetics, 2008. **9**(2): p. 102-114.
39. Pillai, R.S., et al., *Inhibition of translational initiation by Let-7 MicroRNA in human cells.* Science, 2005. **309**(5740): p. 1573.
40. Baek, D., et al., *The impact of microRNAs on protein output.* Nature, 2008. **455**(7209): p. 64.
41. Selbach, M., et al., *Widespread changes in protein synthesis induced by microRNAs.* Nature, 2008. **455**(7209): p. 58-63.
42. Lim, L.P., et al., *Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs.* Nature, 2005. **433**(7027): p. 769-773.
43. Doench, J.G. and P.A. Sharp, *Specificity of microRNA target selection in translational repression.* Genes & development, 2004. **18**(5): p. 504-511.
44. Olsen, P.H. and V. Ambros, *The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation.* Developmental biology, 1999. **216**(2): p. 671-680.
45. Seggerson, K., L. Tang, and E.G. Moss, *Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation.* Developmental biology, 2002. **243**(2): p. 215-225.

46. Baek, D. and J. VillÃ, *The impact of microRNAs on protein output.* Nature, 2008. **455**(7209): p. 64-71.
47. Kiriakidou, M., et al., *A combined computational-experimental approach predicts human microRNA targets.* Genes & development, 2004. **18**(10): p. 1165-1178.
48. Enright, A.J., et al., *MicroRNA targets in Drosophila.* Genome biology, 2004. **5**(1): p. 1-1.
49. Grün, D., et al., *microRNA target predictions across seven Drosophila species and comparison to mammalian targets.* PLoS computational biology, 2005. **1**(1): p. e13.
50. Krek, A., et al., *Combinatorial microRNA target predictions.* Nature genetics, 2005. **37**(5): p. 495-500.
51. Lewis, B.P., C.B. Burge, and D.P. Bartel, *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets.* Cell, 2005. **120**(1): p. 15-20.
52. Xie, X., et al., *Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals.* Nature, 2005. **434**(7031): p. 338-345.
53. Bentwich, I., *Prediction and validation of microRNAs and their targets.* FEBS letters, 2005. **579**(26): p. 5904-5910.
54. Lewis, B.P., et al., *Prediction of mammalian microRNA targets.* Cell, 2003. **115**(7): p. 787-798.
55. Rajewsky, N., *microRNA target predictions in animals.* Nature genetics, 2006. **38**: p. S8-S13.
56. Miranda, K.C., et al., *A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes.* Cell, 2006. **126**(6): p. 1203-1217.
57. Brenner, R.M. and N.B. West, *Hormonal regulation of the reproductive tract in female mammals.* Annual review of physiology, 1975. **37**(1): p. 273-302.
58. Brenner, R., N. West, and M. McClellan, *Estrogen and progestin receptors in the reproductive tract of male and female primates.* Biology of reproduction, 1990. **42**(1): p. 11-19.
59. Hyder, S.M. and G.M. Stancel, *Regulation of angiogenic growth factors in the female reproductive tract by estrogens and progestins.* Molecular Endocrinology, 1999. **13**(6): p. 806-811.
60. Hyder, S.M., et al., *Regulation of vascular endothelial growth factor expression by estrogens and progestins.* Environmental health perspectives, 2000: p. 785-790.
61. Miller, C. and D.A. Sassoon, *Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract.* Development, 1998. **125**(16): p. 3201-3211.
62. Miller, C., A. Pavlova, and D.A. Sassoon, *Differential expression patterns of Wnt genes in the murine female reproductive tract during development and the estrous cycle.* Mechanisms of development, 1998. **76**(1): p. 91-99.
63. Wang, H., H. Eriksson, and L. Sahlin, *Estrogen receptors α and ß in the female reproductive tract of the rat during the estrous cycle.* Biology of reproduction, 2000. **63**(5): p. 1331-1340.
64. Kurita, T., et al., *Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract.* Biology of reproduction, 2000. **62**(4): p. 821-830.
65. Waters, K.M., S. Safe, and K.W. Gaido, *Differential gene expression in response to methoxychlor and estradiol through ERα, ERß, and AR in reproductive tissues of female mice.* Toxicological Sciences, 2001. **63**(1): p. 47-56.
66. Yen, S., P. Vela, and J. Rankin, *Inappropriate secretion of follicle-stimulating hormone and luteinizing hormone in polycystic ovarian disease.* Journal of Clinical Endocrinology & Metabolism, 1970. **30**(4): p. 435-442.
67. Crain, D.A., et al., *Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing.* Fertility and sterility, 2008. **90**(4): p. 911-940.
68. Shifren, J.L., et al., *Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis.* Journal of Clinical Endocrinology & Metabolism, 1996. **81**(8): p. 3112-3118.

69. Nozaki, M. and Y. Ito, *Changes in physiological properties of rabbit oviduct by ovarian steroids.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 1987. **252**(6): p. R1059-R1065.
70. Kaji, K. and A. Kudo, *The mechanism of sperm–oocyte fusion in mammals.* Reproduction, 2004. **127**(4): p. 423-429.
71. Stewart, C.L., et al., *Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor.* Nature, 1992. **359**(6390): p. 76-79.
72. Carson, D.D., et al., *Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening.* Molecular human reproduction, 2002. **8**(9): p. 871-879.
73. Carson, D.D., et al., *Embryo implantation.* Developmental biology, 2000. **223**(2): p. 217-237.
74. RICHARDS, J.S., et al., *Ovarian follicular development in the rat: hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone.* Endocrinology, 1976. **99**(6): p. 1562-1570.
75. Richards, J.S., *Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation.* Physiological reviews, 1980. **60**(1): p. 51-89.
76. Richards, J.S., *Hormonal control of gene expression in the ovary.* Endocrine reviews, 1994. **15**(6): p. 725-751.
77. Richards, J., et al., *Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes.* Recent progress in hormone research, 1995. **50**: p. 223.
78. Duggavathi, R. and B.D. Murphy, *Ovulation signals.* Science Signaling, 2009. **324**(5929): p. 890.
79. McNEILLY, A.S., M. O'CONNELL, and D.T. BAIRD, *Induction of ovulation and normal luteal function by pulsed injections of luteinizing hormone in anestrous ewes.* Endocrinology, 1982. **110**(4): p. 1292-1299.
80. Corner, G.W. and W.M. Allen, *Physiology of the corpus luteum II. Production of a special uterine reaction (progestational proliferation) by extracts of the corpus luteum.* American Journal of Physiology--Legacy Content, 1929. **88**(2): p. 326-339.
81. Eppig, J.J., et al., *Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells.* Biology of reproduction, 1997. **56**(4): p. 976-984.
82. Davis, J.S., *Mechanisms of hormone action: luteinizing hormone receptors and second-messenger pathways.* Current Opinion in Obstetrics and Gynecology, 1994. **6**(3): p. 254-261.
83. Marsh, J.M., et al., *The stimulatory effect of luteinizing hormone on adenosine 3', 5'-monophosphate accumulation in corpus luteum slices.* Journal of Biological Chemistry, 1966. **241**(22): p. 5436-5440.
84. Babu, P.S., et al., *Activation of Extracellular-regulated Kinase Pathways in Ovarian Granulosa Cells by the Novel Growth Factor Type 1 Follicle-stimulating Hormone Receptor ROLE IN HORMONE SIGNALING AND CELL PROLIFERATION.* Journal of Biological Chemistry, 2000. **275**(36): p. 27615-27626.
85. Bachelot, A. and N. Binart, *Corpus luteum development: lessons from genetic models in mice.* Current topics in developmental biology, 2005. **68**: p. 49-84.
86. Cameron, M.R., et al., *Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5'-monophosphates in porcine granulosa cells.* Biology of reproduction, 1996. **55**(1): p. 111-119.
87. Fan, H.-Y., et al., *MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility.* Science Signaling, 2009. **324**(5929): p. 938.
88. Joyce, I.M., et al., *Oocyte regulation of kit ligand expression in mouse ovarian follicles.* Developmental biology, 1999. **214**(2): p. 342-353.

89. Robker, R.L. and J.S. Richards, *Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1.* Molecular Endocrinology, 1998. **12**(7): p. 924-940.
90. Richards, J.A.S., et al., *Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins.* Molecular Endocrinology, 2002. **16**(3): p. 580.
91. Park, J.Y., et al., *EGF-like growth factors as mediators of LH action in the ovulatory follicle.* Science, 2004. **303**(5658): p. 682.
92. Conti, M., et al., *Role of the epidermal growth factor network in ovarian follicles.* Molecular Endocrinology, 2006. **20**(4): p. 715-723.
93. Gonzalez, G. and R.R. Behringer, *Dicer is required for female reproductive tract development and fertility in the mouse.* Molecular reproduction and development, 2009. **76**(7): p. 678-688.
94. Hong, X., et al., *Dicer1 is essential for female fertility and normal development of the female reproductive system.* Endocrinology, 2008. **149**(12): p. 6207-6212.
95. Nagaraja, A.K., et al., *Deletion of Dicer in somatic cells of the female reproductive tract causes sterility.* Molecular Endocrinology, 2008. **22**(10): p. 2336-2352.
96. Otsuka, M., et al., *Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice.* The Journal of clinical investigation, 2008. **118**(5): p. 1944.
97. Fiedler, S.D., et al., *Hormonal regulation of MicroRNA expression in periovulatory mouse mural granulosa cells.* Biology of reproduction, 2008. **79**(6): p. 1030-1037.
98. Fiedler, S.D., et al., *Hormonal regulation of MicroRNA expression in periovulatory mouse mural granulosa cells.* Biology of reproduction, 2008. **79**(6): p. 1030.
99. Carletti, M.Z., S.D. Fiedler, and L.K. Christenson, *MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells.* Biology of reproduction, 2010. **83**(2): p. 286.
100. Ryazanov, A.G., E.A. Shestakova, and P.G. Natapov, *Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation.* 1988.
101. Nairn, A. and H. Palfrey, *Identification of the major Mr 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2.* Journal of Biological Chemistry, 1987. **262**(36): p. 17299-17303.
102. Chan, J.A., A.M. Krichevsky, and K.S. Kosik, *MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells.* Cancer research, 2005. **65**(14): p. 6029.
103. Kutay, H., et al., *Downregulation of miR-122 in the rodent and human hepatocellular carcinomas.* Journal of cellular biochemistry, 2006. **99**(3): p. 671-678.
104. Zhang, Z., et al., *miR-21 plays a pivotal role in gastric cancer pathogenesis and progression.* Laboratory investigation, 2008. **88**(12): p. 1358-1366.
105. Iorio, M.V., et al., *MicroRNA signatures in human ovarian cancer.* Cancer research, 2007. **67**(18): p. 8699.
106. Nam, E.J., et al., *MicroRNA expression profiles in serous ovarian carcinoma.* Clinical Cancer Research, 2008. **14**(9): p. 2690-2695.
107. Lui, W.O., et al., *Patterns of known and novel small RNAs in human cervical cancer.* Cancer research, 2007. **67**(13): p. 6031.
108. Tran, N., et al., *MicroRNA expression profiles in head and neck cancer cell lines.* Biochemical and biophysical research communications, 2007. **358**(1): p. 12-17.
109. Tetzlaff, M.T., et al., *Differential expression of miRNAs in papillary thyroid carcinoma compared to multinodular goiter using formalin fixed paraffin embedded tissues.* Endocrine pathology, 2007. **18**(3): p. 163-173.
110. Fulci, V., et al., *Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia.* Blood, 2007. **109**(11): p. 4944-4951.

111. Lawrie, C.H., et al., *Detection of elevated levels of tumour associated microRNAs in serum of patients with diffuse large B cell lymphoma.* British journal of haematology, 2008. **141**(5): p. 672-675.
112. Calin, G.A. and C.M. Croce, *MicroRNA signatures in human cancers.* Nature Reviews Cancer, 2006. **6**(11): p. 857-866.
113. Fujita, S., et al., *< i> miR-21</i> Gene Expression Triggered by AP-1 Is Sustained through a Double-Negative Feedback Mechanism.* Journal of molecular biology, 2008. **378**(3): p. 492-504.
114. Wu, G.J., et al., *17q23 amplifications in breast cancer involve the PAT1, RAD51C, PS6K, and SIGma1B genes.* Cancer research, 2000. **60**(19): p. 5371-5375.
115. Chui, D.T.Y., et al., *Classical Hodgkin lymphoma is associated with frequent gains of 17q.* Genes, Chromosomes and Cancer, 2003. **38**(2): p. 126-136.
116. Griffin, C.A., et al., *Chromosome abnormalities in pediatric brain tumors.* Cancer research, 1988. **48**(1): p. 175-180.
117. Kasahara, K., et al., *Detection of genetic alterations in advanced prostate cancer by comparative genomic hybridization.* Cancer genetics and cytogenetics, 2002. **137**(1): p. 59-63.
118. Roversi, G., et al., *Identification of novel genomic markers related to progression to glioblastoma through genomic profiling of 25 primary glioma cell lines.* Oncogene, 2005. **25**(10): p. 1571-1583.
119. Löffler, D., et al., *Interleukin-6–dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer.* Blood, 2007. **110**(4): p. 1330-1333.
120. Piper, M., et al. *Emx and Nfi genes regulate cortical development and axon guidance in the telencephalon*. in *Cortical Development: Genes and Genetic Abnormalities: Novartis Foundation Symposium 288*. 2008: Wiley Online Library.
121. Landgraf, P., et al., *A mammalian microRNA expression atlas based on small RNA library sequencing.* Cell, 2007. **129**(7): p. 1401-1414.
122. Singh, S.K., et al., *REST maintains self-renewal and pluripotency of embryonic stem cells.* Nature, 2008. **453**(7192): p. 223-227.
123. Johnson, R., et al., *A microRNA-based gene dysregulation pathway in Huntington's disease.* Neurobiology of disease, 2008. **29**(3): p. 438-445.
124. Lu, J., et al., *MicroRNA expression profiles classify human cancers.* Nature, 2005. **435**(7043): p. 834-838.
125. Thomson, J.M., et al., *Extensive post-transcriptional regulation of microRNAs and its implications for cancer.* Genes & development, 2006. **20**(16): p. 2202.
126. Roy, S., et al., *MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue.* Cardiovascular research, 2009. **82**(1): p. 21.
127. Zhang, J., et al., *MicroRNA-21 (miR-21) represses tumor suppressor PTEN and promotes growth and invasion in non-small cell lung cancer (NSCLC).* Clinica Chimica Acta, 2010. **411**(11): p. 846-852.
128. Gabriely, G., et al., *MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators.* Molecular and cellular biology, 2008. **28**(17): p. 5369.
129. Li, Y., et al., *MicroRNA-21 targets LRRFIP1 and contributes to VM-26 resistance in glioblastoma multiforme.* Brain research, 2009. **1286**: p. 13-18.
130. Qin, W., et al., *BMPRII is a direct target of miR-21.* Acta biochimica et biophysica Sinica, 2009. **41**(7): p. 618.
131. Connolly, E.C., et al., *Overexpression of miR-21 promotes an in vitro metastatic phenotype by targeting the tumor suppressor RHOB.* Molecular Cancer Research, 2010. **8**(5): p. 691-700.
132. Sayed, D., et al., *MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths.* Molecular Biology of the Cell, 2008. **19**(8): p. 3272-3282.

133. Zhu, S., et al., *MicroRNA-21 targets tumor suppressor genes in invasion and metastasis.* Cell research, 2008. **18**(3): p. 350-359.
134. Zhu, S., et al., *MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1).* Journal of Biological Chemistry, 2007. **282**(19): p. 14328.
135. Frankel, L.B., et al., *Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells.* Journal of Biological Chemistry, 2008. **283**(2): p. 1026.
136. Cheng, Y., et al., *MicroRNA-21 protects against the H (2) O (2)-induced injury on cardiac myocytes via its target gene PDCD4.* Journal of molecular and cellular cardiology, 2009. **47**(1): p. 5.
137. Meng, F., et al., *MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer.* Gastroenterology, 2007. **133**(2): p. 647-658.
138. Lu, Z., et al., *MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene.* Oncogene, 2008. **27**(31): p. 4373-4379.
139. Si, M., et al., *miR-21-mediated tumor growth.* ONCOGENE-BASINGSTOKE-, 2007. **26**(19): p. 2799.
140. Asangani, I., et al., *MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer.* Oncogene, 2007. **27**(15): p. 2128-2136.
141. Connolly, D.C., et al., *Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer.* Cancer research, 2003. **63**(6): p. 1389.
142. Haenisch, S. and I. Cascorbi, *miRNAs as mediators of drug resistance.* Epigenomics, 2012. **4**(4): p. 369-381.
143. Buscaglia, L.E.B. and Y. Li, *Apoptosis and the target genes of miR-21.* Chinese Journal of Cancer, 2011. **30**(6): p. 371.
144. Talotta, F., et al., *An autoregulatory loop mediated by miR-21 and PDCD4 controls the AP-1 activity in RAS transformation.* Oncogene, 2008. **28**(1): p. 73-84.
145. Yao, Q., et al., *MicroRNA-21 promotes cell proliferation and down-regulates the expression of programmed cell death 4 (PDCD4) in HeLa cervical carcinoma cells.* Biochemical and biophysical research communications, 2009. **388**(3): p. 539-542.
146. Allgayer, H., *Pdcd4, a colon cancer prognostic that is regulated by a microRNA.* Critical reviews in oncology/hematology, 2010. **73**(3): p. 185-191.
147. Selaru, F.M., et al., *MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3.* Hepatology, 2009. **49**(5): p. 1595-1601.
148. Lankat-Buttgereit, B. and R. Goke, *The tumour suppressor Pdcd4: recent advances in the elucidation of function and regulation.* Biology of the Cell, 2009. **101**: p. 309-317.
149. ZAKOWICZ, H., et al., *Mutational analysis of the DEAD-box RNA helicase eIF4AII characterizes its interaction with transformation suppressor Pdcd4 and eIF4GI.* Rna, 2005. **11**(3): p. 261-274.
150. Yang, H.S., et al., *A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A.* Molecular and cellular biology, 2004. **24**(9): p. 3894.
151. Böhm, M., et al., *The transformation suppressor protein Pdcd4 shuttles between nucleus and cytoplasm and binds RNA.* Oncogene, 2003. **22**(31): p. 4905-4910.
152. Yang, H.S., et al., *A novel transformation suppressor, Pdcd4, inhibits AP-1 transactivation but not NF-kappaB or ODC transactivation.* Oncogene, 2001. **20**(6): p. 669.
153. Jansen, A.P., C.E. Camalier, and N.H. Colburn, *Epidermal expression of the translation inhibitor programmed cell death 4 suppresses tumorigenesis.* Cancer research, 2005. **65**(14): p. 6034.
154. Schmid, T., et al., *Translation inhibitor Pdcd4 is targeted for degradation during tumor promotion.* Cancer research, 2008. **68**(5): p. 1254.

155. Hilliard, A., et al., *Translational regulation of autoimmune inflammation and lymphoma genesis by programmed cell death 4.* The Journal of Immunology, 2006. **177**(11): p. 8095-8102.
156. Day Baird, D., et al., *High cumulative incidence of uterine leiomyoma in black and white women: Ultrasound evidence* 1.* American Journal of Obstetrics and Gynecology, 2003. **188**(1): p. 100-107.
157. Lippman, S.A., et al., *Uterine fibroids and gynecologic pain symptoms in a population-based study.* Fertility and sterility, 2003. **80**(6): p. 1488-1494.
158. Payson, M., P. Leppert, and J. Segars, *Epidemiology of myomas.* Obstetrics and gynecology clinics of North America, 2006. **33**(1): p. 1.
159. Pritts, E.A., *Fibroids and infertility: a systematic review of the evidence.* Obstetrical & gynecological survey, 2001. **56**(8): p. 483-491.
160. Farquhar, C.M. and C.A. Steiner, *Hysterectomy rates in the United States 1990–1997.* Obstetrics & Gynecology, 2002. **99**(2): p. 229.
161. Ligon, A.H. and C.C. Morton, *Leiomyomata: heritability and cytogenetic studies.* Human reproduction update, 2001. **7**(1): p. 8.
162. Marshall, L.M., et al., *A prospective study of reproductive factors and oral contraceptive use in relation to the risk of uterine leiomyomata.* Fertility and sterility, 1998. **70**(3): p. 432.
163. Parazzini, F., et al., *Epidemiologic characteristics of women with uterine fibroids: a case-control study.* Obstetrics & Gynecology, 1988. **72**(6): p. 853-857.
164. Lumbiganon, P., et al., *Protective effect of depot-medroxyprogesterone acetate on surgically treated uterine leiomyomas: a multicentre case-control study.* BJOG: An International Journal of Obstetrics & Gynaecology, 1996. **103**(9): p. 909-914.
165. Ross, R.K., et al., *Risk factors for uterine fibroids: reduced risk associated with oral contraceptives.* British medical journal (Clinical research ed.), 1986. **293**(6543): p. 359.
166. Sato, F., et al., *Fertility and uterine size among Asian women undergoing hysterectomy for leiomyomas.* International journal of fertility and women's medicine, 2000. **45**(1): p. 34-37.
167. COLDITZ, G.A., et al., *Variation in the incidence of uterine leiomyoma among premenopausal women by age and race.* Obstetrics & Gynecology, 1997. **90**(6): p. 967-973.
168. Velebil, P., et al., *Rate of hospitalization for gynecologic disorders among reproductive-age women in the United States.* Obstetrics & Gynecology, 1995. **86**(5): p. 764-769.
169. Wilcox, L.S., et al., *Hysterectomy in the United States, 1988-1990.* Obstetrics & Gynecology, 1994. **83**(4): p. 549&hyhen.
170. Samadi, A.R., et al., *Risk factors for self-reported uterine fibroids: a case-control study.* American journal of public health, 1996. **86**(6): p. 858-862.
171. Marshall, L.M., et al., *Risk of uterine leiomyomata among premenopausal women in relation to body size and cigarette smoking.* Epidemiology, 1998. **9**(5): p. 511-517.
172. Sato, F., et al., *Familial aggregation of uterine myomas in Japanese women.* Journal of epidemiology, 2002. **12**(3): p. 249-253.
173. Sato, F., et al., *Body fat distribution and uterine leiomyomas.* Journal of epidemiology/Japan Epidemiological Association, 1998. **8**(3): p. 176.
174. Abraham, S. and C.L. Johnson, *Prevalence of severe obesity in adults in the United States.* American journal of clinical nutrition, 1980. **33**.
175. Flegal, K.M., et al., *Overweight and obesity in the United States: prevalence and trends, 1960-1994.* International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity, 1998. **22**(1): p. 39.
176. Witherspoon, J.T. and V. Butler, *The etiology of uterine fibroids with special reference to the frequency of their occurrence in the Negro: an hypothesis.* Surg Gynecol Obstet, 1934. **58**: p. 57-61.
177. Buttram Jr, V.C., *Uterine leiomyomata--aetiology, symptomatology and management.* Progress in clinical and biological research, 1986. **225**: p. 275.

178. Vollenhoven, B.J., A. Lawrence, and D. Healy, *Uterine fibroids: a clinical review.* BJOG: An International Journal of Obstetrics & Gynaecology, 1990. **97**(4): p. 285-298.
179. Baird, D., et al., *African Americans at higher risk than whites for uterine fibroids: ultrasound evidence.* Am J Epidemiol, 1998. **147**(11): p. S90.
180. Kjerulff, K., et al., *Uterine leiomyomas. Racial differences in severity, symptoms and age at diagnosis.* The Journal of reproductive medicine, 1996. **41**(7): p. 483.
181. Rein, M.S., R.L. Barbieri, and A.J. Friedman, *Progesterone: a critical role in the pathogenesis of uterine myomas.* American Journal of Obstetrics and Gynecology, 1995. **172**(1): p. 14-18.
182. Friedman, A., et al., *Efficacy and safety considerations in women with uterine leiomyomas treated with gonadotropin-releasing hormone agonists: the estrogen threshold hypothesis.* American Journal of Obstetrics and Gynecology, 1990. **163**(4 Pt 1): p. 1114.
183. Benassayag, C., et al., *Estrogen receptors (ERα/ERβ) in normal and pathological growth of the human myometrium: pregnancy and leiomyoma.* American Journal of Physiology-Endocrinology And Metabolism, 1999. **276**(6): p. E1112-E1118.
184. Maruo, T., et al., *Effects of progesterone on uterine leiomyoma growth and apoptosis.* Steroids, 2000. **65**(10-11): p. 585-592.
185. Brandon, D., et al., *Progesterone receptor messenger ribonucleic acid and protein are overexpressed in human uterine leiomyomas.* American Journal of Obstetrics and Gynecology, 1993. **169**(1): p. 78-85.
186. Kettel, L.M., et al., *Rapid regression of uterine leiomyomas in response to daily administration of gonadotropin-releasing hormone antagonist.* Obstetrical & gynecological survey, 1994. **49**(3): p. 191-193.
187. Steinauer, J., et al., *Systematic review of mifepristone for the treatment of uterine leiomyomata.* Obstetrics & Gynecology, 2004. **103**(6): p. 1331-1336.
188. Vollenhoven, B.J., A. Herington, and D. Healy, *Messenger ribonucleic acid expression of the insulin-like growth factors and their binding proteins in uterine fibroids and myometrium.* Journal of Clinical Endocrinology & Metabolism, 1993. **76**(5): p. 1106-1110.
189. Mangrulkar, R.S., et al., *Isolation and characterization of heparin-binding growth factors in human leiomyomas and normal myometrium.* Biology of reproduction, 1995. **53**(3): p. 636-646.
190. Fayed, Y., et al., *Human uterine leiomyoma cells: binding and growth responses to epidermal growth factor, platelet-derived growth factor, and insulin.* Laboratory investigation; a journal of technical methods and pathology, 1989. **60**(1): p. 30.
191. Boehm, K.D., et al., *Expression of the insulin-like and platelet-derived growth factor genes in human uterine tissues.* Molecular reproduction and development, 1990. **27**(2): p. 93-101.
192. Murphy, L.J., et al., *Growth factors in normal and malignant uterine tissue.* Annals of the New York Academy of Sciences, 1991. **622**(1): p. 383-391.
193. Charnock-Jones, D.S., et al., *Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines.* Biology of reproduction, 1993. **48**(5): p. 1120-1128.
194. Cullinan-Bove, K. and R.D. Koos, *Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth.* Endocrinology, 1993. **133**(2): p. 829-837.
195. Fujimoto, J., et al., *Ovarian steroids regulate the expression of basic fibroblast growth factor and its mRNA in fibroblasts derived from uterine endometrium.* Annals of clinical biochemistry, 1997. **34**: p. 91.
196. Harrison-Woolrych, M., et al., *Localization and quantification of vascular endothelial growth factor messenger ribonucleic acid in human myometrium and leiomyomata.* Journal of Clinical Endocrinology & Metabolism, 1995. **80**(6): p. 1853-1858.

197. Lumsden, M., et al., *The binding of epidermal growth factor to the human uterus and leiomyomata in women rendered hypooestrogenic by continuous administration of an LHRH agonist.* BJOG: An International Journal of Obstetrics & Gynaecology, 1988. **95**(12): p. 1299-1304.
198. Gloudemans, T., et al., *Insulin-like growth factor gene expression in human smooth muscle tumors.* Cancer research, 1990. **50**(20): p. 6689-6695.
199. Giudice, L., et al., *Physiology: Insulin-like growth factor (IGF), IGF binding protein (IGFBP), and IGF receptor gene expression and IGFBP synthesis in human uterine leiomyomata.* Human Reproduction, 1993. **8**(11): p. 1796-1806.
200. Fejzo, M.S., et al., *Identification of a YAC spanning the translocation breakpoints in uterine leiomyomata, pulmonary chondroid hamartoma, and lipoma: physical mapping of the 12q14–q15 breakpoint region in uterine leiomyomata.* Genomics, 1995. **26**(2): p. 265-271.
201. Vikhlyaeva, E., Z. Khodzhaeva, and N. Fantschenko, *Familial predisposition to uterine leiomyomas.* International Journal of Gynecology & Obstetrics, 1995. **51**(2): p. 127-131.
202. Marsh, E.E., et al., *Differential expression of microRNA species in human uterine leiomyoma versus normal myometrium.* Fertility and sterility, 2008. **89**(6): p. 1771-1776.
203. Pan, Q., X. Luo, and N. Chegini, *In Focus: Differential expression of microRNAs in myometrium and leiomyomas and regulation by ovarian steroids.* Journal of cellular and molecular medicine, 2008. **12**(1): p. 227-240.
204. Wang, T., et al., *A micro RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas.* Genes, Chromosomes and Cancer, 2007. **46**(4): p. 336-347.
205. He, L. and G.J. Hannon, *MicroRNAs: small RNAs with a big role in gene regulation.* Nature Reviews Genetics, 2004. **5**(7): p. 522-531.
206. Lewis, B.P., et al., *Prediction of mammalian microRNA targets.* Cell, 2003. **115**(7): p. 787-798.
207. Bartel, D.P., *MicroRNAs:: Genomics, Biogenesis, Mechanism, and Function.* Cell, 2004. **116**(2): p. 281-297.
208. Nakamura, N. and S. Hirose, *Regulation of mitochondrial morphology by USP30, a deubiquitinating enzyme present in the mitochondrial outer membrane.* Molecular Biology of the Cell, 2008. **19**(5): p. 1903-1911.
209. Ronen-Fuhrmann, T., et al., *Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary.* Endocrinology, 1998. **139**(1): p. 303-315.
210. Guraya, S.S., *Morphology, histochemistry, and biochemistry of human ovarian compartments and steroid hormone synthesis.* Physiological reviews, 1971. **51**(4): p. 785-807.
211. Murdoch, W., *Microtubular dynamics in granulosa cells of periovulatory follicles and granulosa-derived (large) lutein cells of sheep: relationships to the steroidogenic folliculo-luteal shift and functional luteolysis.* Biology of reproduction, 1996. **54**(5): p. 1135-1140.
212. Cullen, B.R., *Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors.* Journal of virology, 2006. **80**(3): p. 1067-1076.
213. Brännström, M., G. Mayrhofer, and S.A. Robertson, *Localization of leukocyte subsets in the rat ovary during the periovulatory period.* Biology of reproduction, 1993. **48**(2): p. 277-286.
214. Brännström, M. and A. Enskog, *Leukocyte networks and ovulation.* Journal of reproductive immunology, 2002. **57**(1): p. 47-60.
215. Lobo, G.P., et al., *ISX is a retinoic acid-sensitive gatekeeper that controls intestinal β, β-carotene absorption and vitamin A production.* The FASEB Journal, 2010. **24**(6): p. 1656-1666.
216. Li, X., H. Peegel, and K. Menon, *In situ hybridization of high density lipoprotein (scavenger, type 1) receptor messenger ribonucleic acid (mRNA) during folliculogenesis and luteinization: evidence for mRNA expression and induction by human chorionic gonadotropin specifically in cell types that use cholesterol for steroidogenesis.* Endocrinology, 1998. **139**(7): p. 3043-3049.

217. Cao, G., et al., *Developmental and hormonal regulation of murine scavenger receptor, class B, type 1.* Molecular Endocrinology, 1999. **13**(9): p. 1460-1473.
218. Cottonham, C.L., S. Kaneko, and L. Xu, *miR-21 and miR-31 converge on TIAM1 to regulate migration and invasion of colon carcinoma cells.* Journal of Biological Chemistry, 2010. **285**(46): p. 35293-35302.
219. Carletti, M.Z., S.D. Fiedler, and L.K. Christenson, *MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells.* Biology of reproduction, 2010. **83**(2): p. 286-295.
220. Krichevsky, A.M. and G. Gabriely, *miR-21: a small multi-faceted RNA.* Journal of cellular and molecular medicine, 2009. **13**(1): p. 39-53.
221. Young, M.R., et al., *Have Tumor Suppressor PDCD4 and its Counteragent Oncogenic miR-21 Gone Rogue?* Molecular Interventions, 2010. **10**(2): p. 76.
222. Chang, J., et al., *miR-122, a Mammalian Liver-Specific microRNA, is Processed from hcr mRNA and MayDownregulate the High Affinity Cationic Amino Acid Transporter CAT-1.* RNA biology, 2004. **1**(2): p. 106-113.
223. Trigatti, B., A. Rigotti, and M. Krieger, *The role of the high-density lipoprotein receptor SR-BI in cholesterol metabolism.* Current opinion in lipidology, 2000. **11**(2): p. 123-131.
224. Rao, R.M., et al., *Differential regulation of steroid hormone biosynthesis in R2C and MA-10 Leydig tumor cells: role of SR-B1-mediated selective cholesteryl ester transport.* Biology of reproduction, 2003. **68**(1): p. 114-121.
225. Koubova, J., et al., *Retinoic acid regulates sex-specific timing of meiotic initiation in mice.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(8): p. 2474-2479.
226. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative CT method.* Nature protocols, 2008. **3**(6): p. 1101-1108.
227. Choi, M.Y., et al., *A dynamic expression survey identifies transcription factors relevant in mouse digestive tract development.* Development, 2006. **133**(20): p. 4119-4129.
228. Seino, Y., et al., *ISX participates in the maintenance of vitamin A metabolism by regulation of β-carotene 15, 15'-monooxygenase (Bcmo1) expression.* Journal of Biological Chemistry, 2008. **283**(8): p. 4905-4911.
229. Hsu, S.-H., et al., *Proinflammatory Homeobox Gene, ISX, Regulates Tumor Growth and Survival in Hepatocellular Carcinoma.* Cancer research, 2013. **73**(2): p. 508-518.
230. Grier, D., et al., *The pathophysiology of HOX genes and their role in cancer.* The Journal of pathology, 2005. **205**(2): p. 154-171.
231. Murphy, B.D., *Models of luteinization.* Biology of reproduction, 2000. **63**(1): p. 2-11.
232. Fiedler, S.D., *MicroRNA expression within periovulatory mural granulosa cells.* 2008.
233. Gerster, H., *Vitamin A-functions, dietary requirements and safety in humans.* International journal for vitamin and nutrition research, 1997. **67**(2): p. 71-90.
234. Borel, P., et al., *Human plasma levels of vitamin E and carotenoids are associated with genetic polymorphisms in genes involved in lipid metabolism.* The Journal of nutrition, 2007. **137**(12): p. 2653-2659.
235. Morita, Y. and J.L. Tilly, *Segregation of retinoic acid effects on fetal ovarian germ cell mitosis versus apoptosis by requirement for new macromolecular synthesis.* Endocrinology, 1999. **140**(6): p. 2696-2703.
236. Levi, L., B. Levavi-Sivan, and E. Lubzens, *Expression of genes associated with retinoid metabolism in the trout ovarian follicle.* Biology of reproduction, 2008. **79**(3): p. 570-577.
237. Griswold, M.D., et al., *Initiating meiosis: the case for retinoic acid.* Biology of reproduction, 2012. **86**(2): p. 35.

238. Whaley, S., et al., *Influence of vitamin A injection before mating on oocyte development, follicular hormones, and ovulation in gilts fed high-energy diets.* Journal of animal science, 2000. **78**(6): p. 1598-1607.
239. Li, H. and M. Clagett-Dame, *Vitamin A deficiency blocks the initiation of meiosis of germ cells in the developing rat ovary in vivo.* Biology of reproduction, 2009. **81**(5): p. 996-1001.
240. IKEDA, S., et al., *The roles of vitamin A for cytoplasmic maturation of bovine oocytes.* Journal of Reproduction and Development, 2005. **51**(1): p. 23-35.
241. Matzuk, M.M., et al., *Intercellular communication in the mammalian ovary: oocytes carry the conversation.* Science Signaling, 2002. **296**(5576): p. 2178.
242. Stewart, E.A. and A.J. Friedman. *Steroidal treatment of myomas: preoperative and long-term medical therapy*. 1992: Thieme.
243. Luoto, R., et al., *Heritability and risk factors of uterine fibroids--the Finnish Twin Cohort study.* Maturitas, 2000. **37**(1): p. 15-26.
244. Esquela-Kerscher, A. and F.J. Slack, *Oncomirs—microRNAs with a role in cancer.* Nature Reviews Cancer, 2006. **6**(4): p. 259.
245. Lankat-Buttgereit, B., et al., *Pdcd4 inhibits growth of tumor cells by suppression of carbonic anhydrase type II.* Molecular and cellular endocrinology, 2004. **214**(1-2): p. 149-153.
246. Wei, N., et al., *Tumour Suppressive Function and Modulation of Programmed Cell Death 4 (PDCD4) in Ovarian Cancer.* PloS one, 2012. **7**(1): p. e30311.
247. Pan, Q., X. Luo, and N. Chegini, *microRNA 21: response to hormonal therapies and regulatory function in leiomyoma, transformed leiomyoma and leiomyosarcoma cells.* Molecular human reproduction, 2010. **16**(3): p. 215-227.
248. Crabtree, J.S., et al., *Comparison of human and rat uterine leiomyomata: identification of a dysregulated mammalian target of rapamycin pathway.* Cancer research, 2009. **69**(15): p. 6171.
249. Yang, H.S., et al., *The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation.* Molecular and cellular biology, 2003. **23**(1): p. 26.
250. Schmid, T., et al., *Inflammation-induced loss of Pdcd4 is mediated by phosphorylation-dependent degradation.* Carcinogenesis, 2011. **32**(10): p. 1427-1433.
251. Cheng, Y., et al., *MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy?* The American journal of pathology, 2007. **170**(6): p. 1831.
252. Kurachi, O., et al., *Tumor necrosis factor-α expression in human uterine leiomyoma and its down-regulation by progesterone.* Journal of Clinical Endocrinology & Metabolism, 2001. **86**(5): p. 2275.
253. Wu, X., et al., *Apoptosis, cellular proliferation and expression of p53 in human uterine leiomyomas and myometrium during the menstrual cycle and after menopause.* Acta obstetricia et gynecologica Scandinavica, 2000. **79**(5): p. 397-404.
254. Maruo, T., et al., *Sex steroidal regulation of uterine leiomyoma growth and apoptosis.* Human reproduction update, 2004. **10**(3): p. 207-220.
255. Makker, A., et al., *PI3K-Akt-mTOR and MAPK signaling pathways in polycystic ovarian syndrome, uterine leiomyomas and endometriosis: an update.* Gynecological Endocrinology, 2011(00): p. 1-7.
256. Yin, X.J., G. Wang, and F.S. Khan-Dawood, *Requirements of phosphatidylinositol-3 kinase and mammalian target of rapamycin for estrogen-induced proliferation in uterine leiomyoma-and myometrium-derived cell lines.* American Journal of Obstetrics and Gynecology, 2007. **196**(2): p. 176. e1-176. e5.
257. Hauser, H., et al., *Identification of a receptor mediating absorption of dietary cholesterol in the intestine.* Biochemistry, 1998. **37**(51): p. 17843-17850.
258. Reboul, E., et al., *Scavenger receptor class B type I (SR-BI) is involved in vitamin E transport across the enterocyte.* Journal of Biological Chemistry, 2006. **281**(8): p. 4739-4745.

259. Bietrix, F., et al., *Accelerated lipid absorption in mice overexpressing intestinal SR-BI.* Journal of Biological Chemistry, 2006. **281**(11): p. 7214-7219.
260. Fukasawa, M., et al., *SRB1, a class B scavenger receptor, recognizes both negatively charged liposomes and apoptotic cells.* Experimental cell research, 1996. **222**(1): p. 246-250.
261. Li, X., H. Peegel, and K. Menon, *Regulation of high density lipoprotein receptor messenger ribonucleic acid expression and cholesterol transport in theca-interstitial cells by insulin and human chorionic gonadotropin.* Endocrinology, 2001. **142**(1): p. 174-181.
262. Amengual, J., et al., *Beta-carotene reduces body adiposity of mice via BCMO1.* PloS one, 2011. **6**(6): p. e20644.
263. Borel, P., et al., *Genetic variants in BCMO1 and CD36 are associated with plasma lutein concentrations and macular pigment optical density in humans.* Annals of medicine, 2011. **43**(1): p. 47-59.
264. van Helden, Y.G., et al., *Gene expression response of mouse lung, liver and white adipose tissue to β-carotene supplementation, knockout of Bcmo1 and sex.* Molecular nutrition & food research, 2011. **55**(10): p. 1466-1474.
265. Lietz, G., J. Lange, and G. Rimbach, *Molecular and dietary regulation of β, β-carotene 15, 15'-monooxygenase 1 (BCMO1).* Archives of biochemistry and biophysics, 2010. **502**(1): p. 8-16.
266. Lietz, G., et al., *Importance of β, β-carotene 15, 15'-monooxygenase 1 (BCMO1) and β, β-carotene 9', 10'-dioxygenase 2 (BCDO2) in nutrition and health.* Molecular nutrition & food research, 2012. **56**(2): p. 241-250.
267. Zhuang, Y.-H., et al., *Immunolocalization of retinoic acid receptors in rat, mouse and human ovary and uterus.* The Journal of Steroid Biochemistry and Molecular Biology, 1994. **48**(1): p. 61-68.
268. Bowles, J. and P. Koopman, *Retinoic acid, meiosis and germ cell fate in mammals.* Development, 2007. **134**(19): p. 3401-3411.