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Identification of Therapeutic MicroRNAs in Colorectal Cancer via RNA Interference

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To my parents, Emmanuel and Sandy Perakis, for their compassion, for always helping me put things in perspective, and for their endless loving support.

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ABSTRACT

Colorectal cancer (CRC) is one of the leading causes of death in developed countries and presents a clear demand for the development of novel therapeutic strategies. One potential emerging therapy for the treatment of a heterogenic disease such as cancer involves the use of microRNAs, which are capable of efficiently silencing target genes and which can regulate an array of genes simultaneously. Even though miRNA genes comprise only a small fraction of the genome, recent bioinformatic and experimental evidence suggests that miRNAs might potentially regulate at least one-third of all human genes. The microRNA-mediated RNA interference (RNAi) carried out by endogenous miRNAs in cells can be mimicked for research-related and therapeutic purposes via the use of synthetic miRNA technology. Modulation of these molecules has the potential of altering hundreds of transcripts, which could eventually lead to the controlled regulation of entire deregulated pathways or gene programs. Pharmacological manipulation of the expression levels, particularly of oncogenic miRNAs by cellular transfer of selective RNA-based inhibitors, might lead to the development of novel biomolecule-based therapeutics. We sought out to evaluate the pharmacological outcomes via overexpression and inhibition assays of the following miRNAs in the colorectal carcinoma cell line HCT116: miR-31*, miR-342, miR-215, miR-196b and miR-148a. For further characterization of the mode of action of the selective miRNA inhibitors and mimetics, we also used proliferation, apoptosis and epithelial-mesenchymal transition assays to gain more insight into the biological role of these miRNAs. Overall, data suggested that these miRNA candidates, miR-31* in particular, functioned as pro-apoptotic molecules in this *in vitro* model, with the possible exception of miR-148a. Overexpression of miR-31* led to the suppression of cellular growth and an increase in caspase activity and expression was downregulated in tumor spheres with cancer stem cell-like properties. miR-148a overexpression increased expression levels of the proliferation and EMT markers CCND1 and VIM, respectively, and demonstrated anti-apoptotic activity as observed by an increase in Bcl-2 expression. This study has shed some light onto the complexity of these small RNA molecules in terms of screening for new potential RNAi-based therapeutics which make use of a

highly selective and efficient endogenous process for the treatment of disease via gene silencing.

1 INTRODUCTION

1.1 Overview of colorectal cancer

Colorectal cancer (CRC) is one of the major causes of morbidity and mortality worldwide, accounting for greater than 9% of all incidences of cancer.[1-3] It ranks as the third most common type of cancer in the world and is also the third most common cause of cancer death.[2,4] The worldwide mortality attributed to this disease is estimated to be half of its incidence with approximately 530,000 CRC-related deaths recorded in 2002.[2,5] Although there has been a dramatic decline in the incidence of CRC in the past 10 years due to the more widespread access of screening methods and improvement of treatment options, incidence and mortality still remain high.[6,7] Statistics have also shown that almost 50% of patients initially diagnosed to have a localized form of this cancer eventually develop metastases.[8]

In addition to surgery and radiation therapy for early stage CRC, there are currently several different types of standard treatment used to treat CRC patients. Drug-based therapies include the use of chemotherapeutic agents and targeted therapy including the use of monoclonal antibodies or angiogenesis inhibitors. 5-flourouracil (FU) is a key chemotherapeutic drug used in the treatment of CRC which induces cytotoxicity via the incorporation of fluoronucleotides into RNA and DNA molecules as well as via inhibition of the nucleotide synthetic enzyme thymidylate synthase.[9,10] However, although conventional chemotherapy can be quite powerful as it can disrupt cell organelle function, damage DNA and inhibit enzyme activity involved in DNA replication, mRNA transcription or translation, its mechanism, like that of radiation therapy, does not specifically target cancer cells and can lead to toxicity in normal tissue which results in side effects.[11,12] Another problem surrounding 5-FU-based therapy is the inherent or acquired resistance to the treatment in advanced CRC patients.[13] For these reasons, additional therapeutic agents have been sought after and have led to the development of targeted therapy, which is designed to act specifically on cancer-associated molecular targets, such as those involved in tumor formation and growth. One such targeted agent group, known as monoclonal antibody-based cancer therapy, has turned out to be a relatively safe and efficient strategy in treating cancer.

Monoclonal antibodies are produced by fusing animal spleen cells, typically from a mouse or rabbit which has been stimulated with a particular antigen to produce antibodies against the target, to cultured tumor cells to produce a hybridoma. The hybridoma is cultured further to produce cells which harbor the desired antibody molecule.[14] Examples include the use of agents directed against the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF). Use of these therapeutic mAbs has been shown to have a significant effect on survival when treating patients with solid tumors.[15,16] Anti-VEGF therapy, which was actually the first approved anti-angiogenic therapy for CRC treatment, has also been previously used in combination with chemotherapeutic treatments such as 5-FU and oxaliplatin.[6,17,18] Despite the success of some of these therapies, primary or secondary resistance still remains a limiting factor in the treatment of affected patients. For example, it has even been shown that resistance to anti-EGFR treatment in CRC patients can be attributed to KRAS gene mutations, which demonstrate strong resistance to anti-EGFR antibodies; yet still, this does not explain why CRC patients possessing wildtype KRAS (KRASwt) do not necessarily automatically benefit from this therapy.[13,19] The undesirable side effects of radiotherapy and chemotherapy along with the problems that arise from acquired resistance to treatment pave the way to discover novel, more efficient and safer technologies for the treatment of patients with cancer. This coincides with the fact that cancer is a heterogenic disease and will most likely not be eradicated by simply suppressing just a single target with the use of specific inhibitors or mAbs. One potential emerging therapy for the treatment of a heterogenic disease such as cancer involves the use of microRNAs, which are capable of efficiently silencing target genes and which can regulate an array of genes simultaneously.[11]

1.2 Overview of microRNAs

MicroRNAs (miRNAs) are a class of endogenous small, non-coding RNA molecules consisting of about 22 nucleotides that are able to mediate regulation of gene expression post-transcriptionally.[20,21] Because of their post-transcriptional gene silencing via messenger RNA (mRNA) targeting, miRNAs are involved in numerous

biological processes such as cell proliferation, cell cycle control, apoptosis, DNA repair, metabolism, immunity, stress, aging, developmental timing and modulation of host response to viral infection. [22,23] The database miRBase (Release 21, June 2014) shows that more than 2,500 mature human miRNAs have either been identified or predicted. This number of annotated miRNAs has strikingly increased from the time of the original version's creation as seen in **Figure 1**.[24] Even though miRNA genes comprise only a small fraction of the genome (about 1%), recent bioinformatic and experimental evidence suggests that miRNAs might potentially regulate at least one third of all human genes.[25-27] Given the fact that miRNAs are able to control the regulation of highly important and fundamental biological processes, it seems to make sense that these molecules are also involved in a variety of pathological conditions, including diseases such as cancer and neurological, muscular and cardiovascular disorders. Adding to their complexity, it has been demonstrated that a single miRNA may regulate the gene expression of multiple genes and that a single mRNA might be the target of multiple miRNAs.[11,28] MiRNAs initiate their post-transcriptional regulation of gene expression via base-pairing with partially complementary sequences in their target mRNA, typically in the 3' untranslated region (UTR) of this target mRNA, which facilitates either the degradation of the mRNA or its translational repression, depending on the extent of complementarity between the miRNA and its mRNA target.[26,29,30] In the case of high sequence complementarity, mRNA will be degraded, whereas central mismatches typically lead to translational repression of the mRNA.[29] The degree of mRNA base pairing is determined by the miRNA seed sequence. This sequence found in all miRNAs is strongly conserved among species and is comprised of a critical 7-nucleotide-long region. [26] The short nature of this seed sequence could offer the explanation as to why a given miRNA may target multiple mRNAs and, conversely, that a given mRNA could be the target of more than one miRNA.

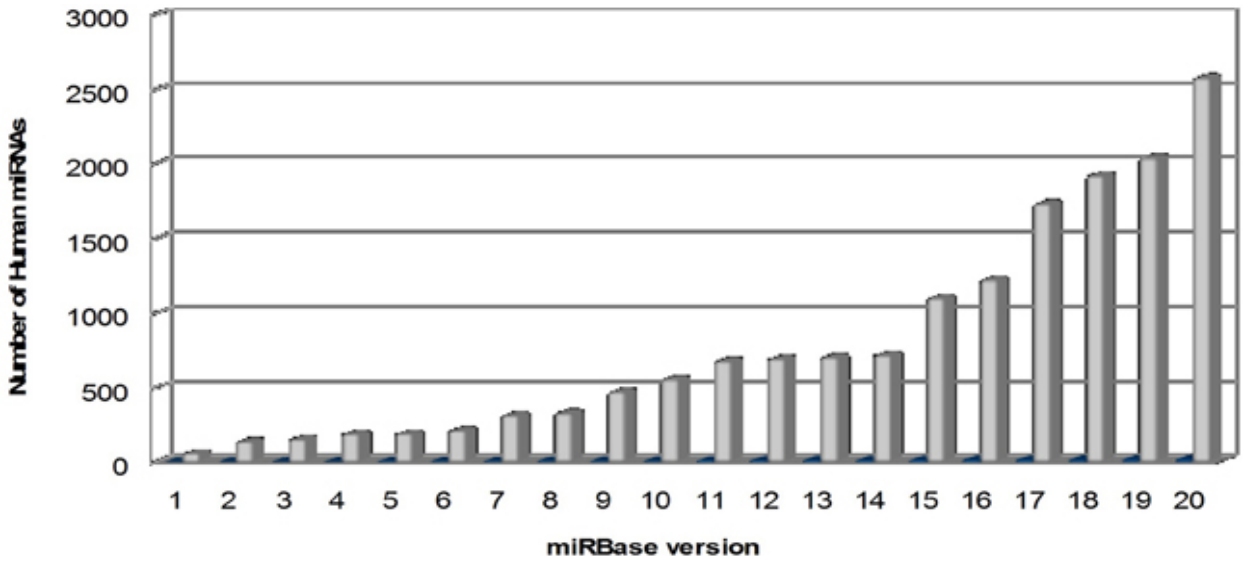


Figure 1. The number of annotated mature human microRNAs in miRBase continues to grow dramatically, beginning with slightly more than 200 entries in the original 2002 release to greater than 2500 annotated microRNAs in the latest 2013 release. [24] (Image taken and caption adapted from Maffioletti et al. 2014)

miRNA dysregulation has been a common attribute in conditions or diseases such as inflammation, fertility dysfunction, diabetes, eye and retinal diseases, CNS disorders, metabolic disorders, cardiovascular diseases and cancer.[31-35] Aberrational miRNA expression has also directly been associated with tumor formation and progression as well as the acquisition of drug resistance due to the influential role of miRNAs in biological processes such as regulation of the cell cycle, apoptosis, metabolism, senescence, angiogenesis and metastasis.[11,36,37] The location of human miRNA genes might also contribute to their association with cancer and its progression. It has been estimated that more than half of these human miRNA genes are located in fragile chromosomal regions or cancer-associated genomic regions which tend to be more susceptible to amplifications, deletions or translocations which occur during the development of cancer.[38] Recent evidence has even suggested that miRNAs might potentially lead the cell to adopt cancer stem cell (CSC) properties such as the ability of self-renewal to contribute to tumorigenicity and drug resistance.[39-41]

Calin and colleagues first reported the link between miRNAs and cancer in 2002 after analysis of a repeated deletion in B cell chronic lymphocytic leukemia (CLL) cells,

which demonstrated that miR-15a and miR-16-1 were located in a small deletion region and that they were downregulated tumor suppressor genes.[42] Later on, Michael et al. provided the first evidence of altered miRNA expression levels in human solid carcinomas and colonic and rectal adenomas in comparison to healthy tissue.[43] Several mechanisms have been suggested which might contribute to these miRNA alterations such as mutation, genomic deletion, epigenetic silencing or changes in miRNA processing.[42,44-46] Experimental evidence evaluating the results of altered miRNA expression has demonstrated that miRNAs can act as tumor suppressors if their loss of function causes a normal cell to undergo malignant transformation or, on the other hand, that they can act as oncogenic miRNAs if they target mRNAs which code for tumor suppressor genes. Data gathered from a variety of studies have demonstrated the role of miRNAs in cancer biology via gene expression regulation of their target mRNAs in facilitating the growth, invasion and angiogenesis of the tumor along with its ability to evade the immune system.[47,48] For these reasons, it is understandable that miRNA research conducted over the past decade has focused on alternative methods of handling and treating cancer. The knowledge regarding the potential therapeutic use of miRNA in cancer continues to increase and has been supplemented with technological advancements in regards to RNA-based therapies. These factors taken together have thus encouraged many efforts towards the development of suitable miRNA therapeutics for the treatment of a multitude of human diseases and especially cancer.

1.3 Endogenous microRNA biogenesis

The multistep processing (depicted in **Figure 2**) that begins with an miRNA gene, which can be located in the introns or exons of a protein-coding region or in an intergenic region, and results in the formation of a mature, functional miRNA is known as miRNA biogenesis.[49] Biogenesis begins in the nucleus where miRNAs are transcribed by RNA polymerase II as long primary transcripts known as pri-miRNA and are characterized by their hairpin structure.[50] These pri-miRNAs are then polyadenylated and capped and subsequently further processed in the nucleus by the enzyme RNase III Drosha and cofactor DGCR8 into long precursor miRNAs (pre-

miRNAs) of about 70-100 nucleotides long and have a stem-loop structure.[50,51] The pre-miRNA is then exported by exportin-5 to the cytoplasm where the loop is cleaved by the RNase III Dicer to produce a miRNA:miRNA* duplex consisting of about 20 nucleotides.[50,51] This mature complex is unwound by helicases to produce two strands, one of which is selected to be the guide strand whereas the other, the passenger complementary strand (miRNA*), is typically degraded.[50,52] Although the miRNA* passenger strand has been thought in the past to usually be degraded, there is recent evidence which suggests that it might actually have a function and can quite possibly have a significant biological role.[53,54] The process continues as mature miRNA is incorporated into the RNA-induced silencing complex (RISC) which contains the proteins Argonaute and GW182.[50] This mature miRNA-RISC complex is then able to regulate gene expression either by binding to partially complementary sequences of the target mRNA 3'UTR, which leads to mRNA degradation or translational inhibition.[52] The fate of the mRNA, as described previously (see Overview of microRNAs), is dependent on the degree of base-pairing complementarity between the miRNA seed region and the target mRNA molecule.

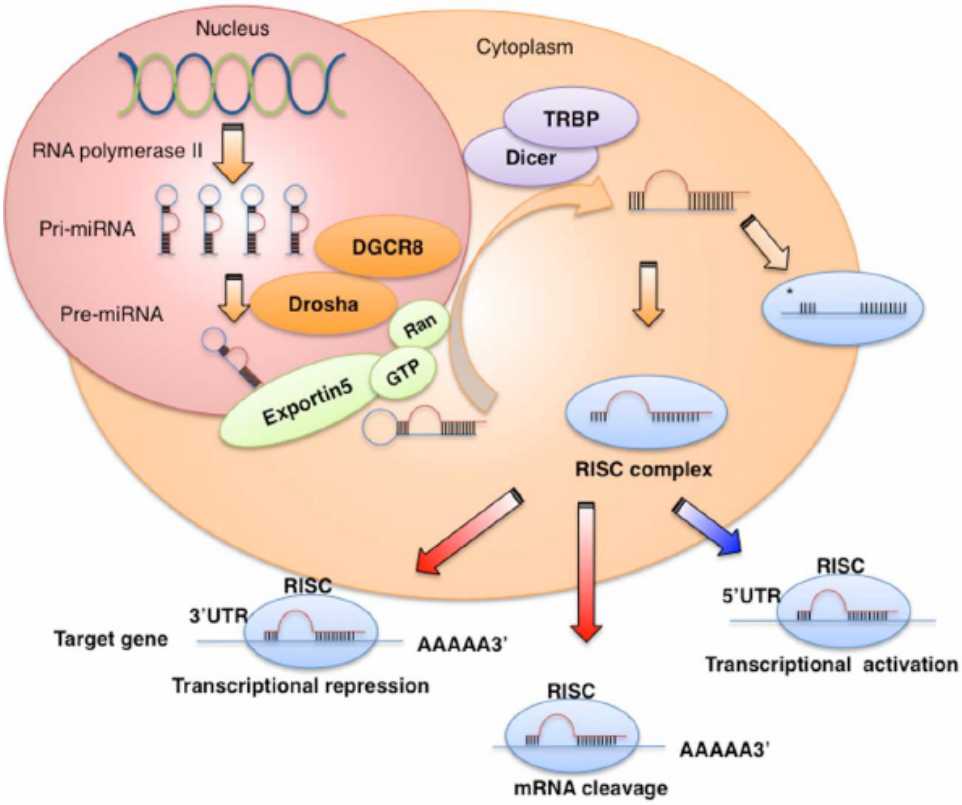


Figure 2. Processing of miRNA into functional molecules. Hairpin-forming pri-miRNAs are transcribed by RNA polymerase II or III and are then processed in the nucleus by Drosha-DGCR8 to form pre-miRNAs. Pre-miRNAs are transported by exportin-5 into the cytoplasm where they are cleaved by the Dicer-TRBP complex to form double-stranded mature miRNAs. The mature miRNA functional strand is subsequently incorporated into the RNA-induced silencing complex (RISC) consisting of the Argonaute and GW182 proteins. The functional miRNA is unwound and anneals to the 3'UTR regions of complementary target mRNAs via imperfect base pairing, resulting in either mRNA cleavage and degradation or translational inhibition. [50] (Image taken and caption adapted from Takahashi et al. 2013)

1.4 Modulation of microRNA activity via synthetic mimic and inhibitor technology

The microRNA-mediated RNA interference (RNAi) carried out by endogenous miRNAs in cells can be mimicked for research-related and therapeutic purposes via the use of synthetic miRNA technology. The fundamental role of miRNAs as oncogenes or tumor suppressors makes them appealing targets to be subjected to manipulation. There are currently two strategies used in the modulation of miRNA activity. One of these strategies applies double-stranded miRNA mimics to restore the function of

miRNA, and the other involves administration of single-stranded antagomir (antimiR) oligonucleotides to inhibit miRNA function. Double-stranded mimics are most often used due to their greater potency in comparison to single-stranded miRNA mimics.[55] The sequence of the guide strand is identical to that of the mature miRNA while the passenger strand has a sequence complementary to this mature miRNA.[56] Both versions of oligonucleotides are chemically synthesized but they differ in structure and goal. miRNA mimics are double-stranded RNAs which can mimic mature endogenous miRNAs after being transfected into cells, whereas miRNA inhibitors are single-stranded in structure and modified in order to specifically inhibit the function of endogenous miRNA **(Figure 3)**. The appropriate therapeutic method, either inhibition via antisense targeting with oligonucleotides or miRNA replacement using viral or liposomal delivery, can be selected depending on the given status of an miRNA, that is, if it is upregulated or downregulated.[57,58]

MiRNA mimics, which behave like endogenous miRNAs, can restore genomic loss of tumor suppressor function. It has also been shown that miRNA mimics are not only beneficial to cancer cells with low expression of tumor suppressor miRNA, but also in cancers with normal expression levels of miRNA. The miR-34 family, for example, has an important role in p53 pathway in the suppression of tumor development and has been shown to be dysregulated in various types of cancer. Delivery of miR-34 in synthetic mimic form to cancer cells with downregulated or normal levels of miR-34 showed growth inhibition in both cell models.[59,60] Furthermore, miRNA mimics can be used to modulate and reactivate unregulated or ablated biological pathways to restore loss-of-function in diseased tissues.[61]

If the expression of an miRNA is increased in a specific state of disease, it could be potentially beneficial to inhibit its function with the goal of returning expression to a more normal state.[62] miRNA antagonist sequences are complementary to their target miRNAs and are designed to interrupt both processing of miRNA and assembly of the RISC complex, thereby disrupting the function of this target miRNA. This in turn prevents the degradation of the corresponding mRNA and the transcript can be translated. An example of the effects of miRNA inhibition were demonstrated in an experiment with miR-21, an miRNA overexpressed in a

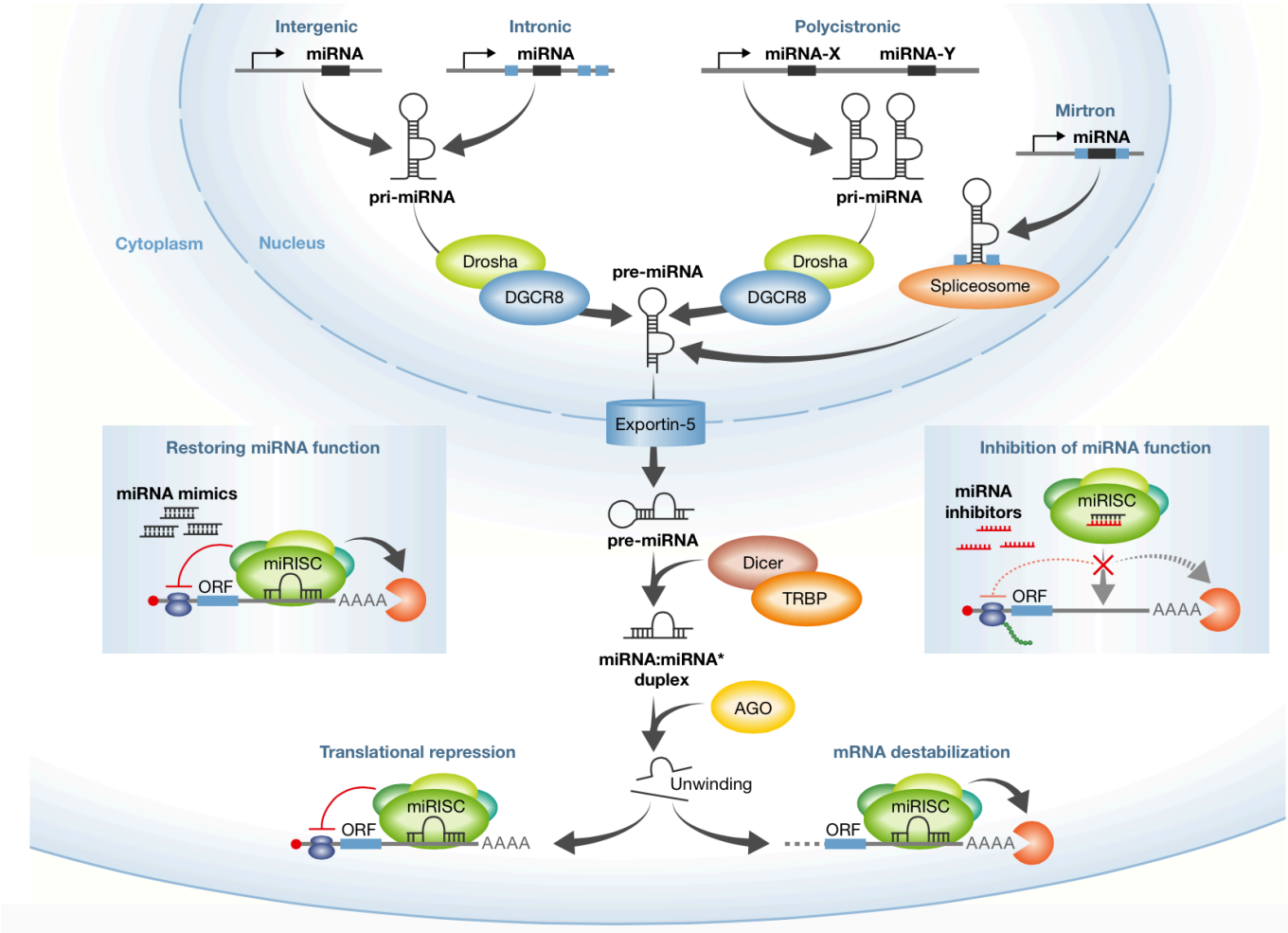


Figure 3. miRNA biogenesis: miRNA mimics and antimiR oligonucleotides can modulate miRNA activity. MiRNA genes can be transcribed from intergenic, intronic or polycistronic loci to form pri-miRNAs which are further processed by the Drosha-DGCR8 complex in the nucleus. This processing results in a pre-miRNA hairpin structure of about 70 nucleotides. An alternative miRNA biogenesis pathway results in the formation of mirtrons, which are short intronic hairpins, that are eventually spliced and deb ranched to create pre-miRNA hairpins. Once pre-miRNAs are exported into the cytoplasm and cleaved by the Dicer complex, they form imperfect miRNA:miRNA* duplexes consisting of about 22 nucleotides. The duplex incorporates into the miRNA-induced silencing complex (miRISC) and the mature miRNA serves as a guide molecule by directing miRISC to partially complementary sites within target mRNAs to induce either mRNA degradation or translational repression. Modulation of miRNA activity is currently conducted via one of two methods: miRNA restoration or miRNA inhibition. To restore lost or dysfunctional miRNA expression, double-stranded mimics are brought into the cell. On the other hand, single-stranded anti-miR oligonucleotides can be used to knock down overexpressed or dysfunctional miRNA expression. [63] (Image taken and caption adapted from van Rooij et al. 2014)

variety of tumors, which happens to downregulate tumor suppressor genes responsible for the regulation of cell proliferation and death, metastasis and chemoresistance. When a miR-21 antagonist is administered to breast cancer cells, the epithelial-to-mesynchymal (EMT) phenotype is reversed and angiogenesis is blocked, as the AKT and MAPK pathways become inactivated.[64,65] AntimiRs are able to inhibit miRNA function because they irreversibly bind to the active target miRNA strand, thereby inhibiting the RISC complex from processing a new miRNA duplex, which is also very resistant to degradation.[61] In vivo studies have also shown that antisense oligonucleotides targeting oncogenic miRNAs were able to significantly reduce their respective expression in various tissues which ultimately led to the upregulation of the related targeted genes.[66]

Furthermore, it has been demonstrated that aberrant miRNA expression plays a role in the development of resistance to chemotherapy. Restoration of miRNA function via synthetic mimics or inhibition of function via antimiRs could provide the needed therapeutic benefit which could abolish drug resistance in cancer cells or eliminate resistant CSCs as well as help modulate drug sensitivity.[28,40,67-69]

1.5 Potential of microRNA for therapeutic use

There have been multiple studies to date which have shown how application of miRNA mimics and inhibitors could be useful in restoring physiological gene networks in various in vitro and in vivo models, indicating the potential of miRNA as an anti-cancer therapy.[70] Not only can miRNAs potentially directly target tumor cells, but they might also be used to reverse drug resistance, as has already been demonstrated with miR-100 in small cell lung cancer as well as the increase of chemoresistance due to epigenetic silencing of miR-199b-5p in ovarian cancer.[71,72] In cervical cancer, miR-214 is often downregulated, which leads to enhanced proliferation, migration and invasiveness of cancer cells.[73] This particular miRNA could serve as a candidate for miRNA replacement therapy via administration of a miR-214 mimic.

CRC cases mainly arise from dysplastic adenomatous polyps in combination with the activation of oncogenes such as KRAS (Kirsten rat sarcoma viral oncogene

homolog), c-MYC (v-myc avian myelocytomatosis viral oncogene homolog) and NRAS (neuroblastoma RAS viral oncogene homolog) along with inactivation of major tumor suppressor genes such as p53 (tumor protein p53) and APC (adenomatous polyposis coli).[13,74] miRNA mimics and inhibitors are thus being developed accordingly to restore loss-of-function of these tumor suppressors or to inactivate the targeted oncogenes. Development of the appropriate therapeutic miRNA oligonucleotide is also dependent on the status—upregulated or downregulated--of the specific miRNA in the CRC tumor.

Other studies outside of cancer research have also demonstrated promising data regarding therapeutic miRNAs in other diseases. Hepatologic studies have linked inhibition of miR-122 to steatosis improvement in a mouse model investigating obesity, indicating a therapeutic potential of miR-122 antagonism for treating non-alcoholic fatty liver disease.[75] There has also been progress made in the identification of metabolic pathways that are regulated by miRNAs at the level of translation. It has been demonstrated that miR-21, for example, controls the regulation of genes associated with the lipid metabolism pathway and is also involved in kidney scarring progression and increased fibrosis. In one study, overexpression of peroxisome proliferator-activated receptor-α (PPARα), which is a direct target of miR-21 and is known to regulate the lipid metabolic pathway, in combination with miR-21 inhibition led to the prevention of injury and fibrosis caused by ureteral obstruction.[76] miRNAs miR-33a/b have also been attributed to regulation of lipid metabolism along with the regulation of genes involved in export of cholesterol, such as the ABC transporters ABCA1 and ABCG1. One study demonstrated how treatment of LDL receptor-deficient mice with a miR-33 inhibitor was able to enhance reverse cholesterol transport as well as atherosclerotic plaque regression.[77] More examples of current or possible therapeutic applications using miRNA are listed with their corresponding references in **Table 1**.

Table 1. Current and potential applications of miRNAs in therapeutics and the biotech industry.[78] (from Basir et al. 2013)

miRNA	Application(s)	Target mRNA	Reference
miR-21	regulation of genes involved in metabolic pathways	Pparα	Chau et al.[76]
miR-33a/b	Regulation of genes involved in lipid metabolism	Peroxisomal carnitine o-octanoyltransferase (CROT), carnitine palmitoyltransferase 1a (CPT1a), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (HADHB)	Davalos et al.[77]

miR-33a/b	Regulation of genes involved in insulin signaling	Insulin receptor substrate-2 (IRS2)	Davalos et al. [77]
miR-33a/b	Regulation of genes involved in cholesterol export	(ABC) transporters ABCA1 and ABCG1 (cholesterol effluc and HDL biosynthesis)	Davalos et al. [77]
miR-214	Regulation of genes involved in cervical cancer	UDP-N-acetyl-alpha-Dgalactosamin:polypeptide N-acetylgalactosaminyltransferase 7 (GALNT7)	Peng et al. [73]
miR-211	Regulation of genes involved in colorectal cancer	Chromodomain-helicase-DNA-binding protein 5 (CHD5)	Cai et al. [79]
miR-375, miR-9 and miR-124a	Control glucose homeostasis and diabetes	Regulating B-cell function and insulin secretion	Shantikumar et al. [80]
miR-21	Associated in cardiac disease including hypertrophy, fibrosis and impaired cardiac function		Thum et al. [81]
miR-26a	Hepatocellular carcinoma (HCC)	Increase cell cycle arrest by targeting cyclins D2 and E2	Kota et al. [82]
miR-122	Liver-specific miRNA	Hepatitis C virus	Elmen et al. [83]

1.6 Existing examples of promising microRNA therapeutics

Because miRNAs are frequently upregulated or underexpressed in cancer, companies have been working on the development of appropriate therapeutics to improve this aberrant expression. Santaris Pharma A/S, for example, is a biopharmaceutical company that mainly focuses on the discovery and development of human RNA-based therapies and has developed methods that harness miRNA antagonist technology. Their main product, SPC3649 is in phase II clinical trials as of August 2013, making it the first drug to ever reach this phase for the treatment of hepatitis C virus (HCV) infections.[84] SPC3649, also known as miravirsen, is a locked nucleic acid (LNA) molecule that specifically targets and inhibits miR-122 for the effective reduction of viral HCV RNA with no evidence of resistance.[61,85] The miravirsen antisense oligonucleotide is not only complementary to the 5′ region of mature miR-122, but it also has a high affinity and specificity for the region, thus enabling the drug to sequester and inhibit miR-122 as shown in **Figure 4**.[23] Although its intended target is

mature miRNA, recent studies have also demonstrated that miravirsen has affinity for both pri- and pre-miR-122 as well, which in turn enhances its therapeutic effect.[85] Mirna Therapeutics, Inc. is another biopharmaceutical frontrunner in the development of miRNA-based human therapies. In April 2013, the Austin, Texas-based company reached phase I clinical trials with the first miRNA mimic, MRX34.[86]

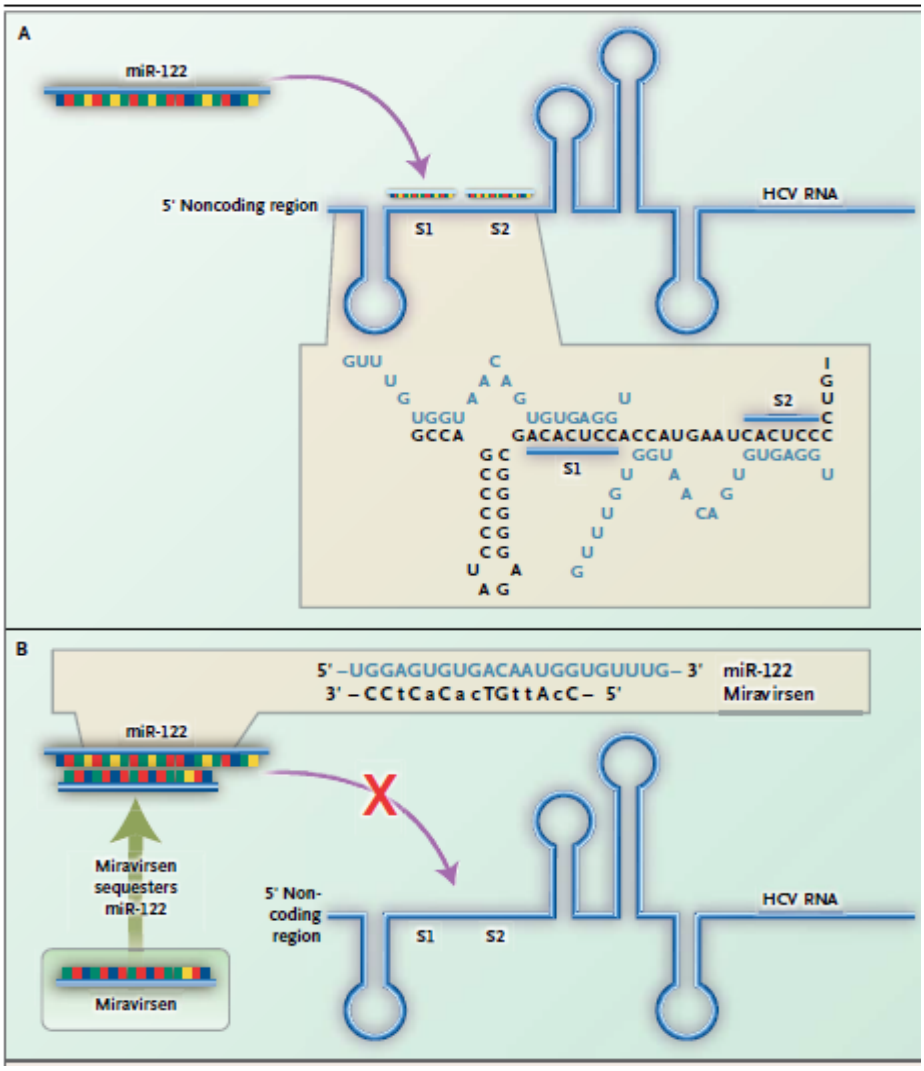


Figure 4. Inhibition of functional miR-122 by miravirsen. (A) MicroRNA-122 (miR-122) binds to two target sites located in the 5' noncoding region of the hepatitis C virus (HCV) genome, S1 and S2, which are situated closely to one another. This promotes HCV RNA propagation. (B) The locked nucleic acid-modified antisense oligonucleotide known as miravirsen is able to capture mature miR-122 to form a very stable heteroduplex, resulting in the inhibition of miR-122 function. [23] (Image taken and caption adapted from Janssen et al. 2013)

This miRNA mimic is based on the miR-34 family, which is reportedly a direct transcriptional target of the tumor suppressor p53 and induces apoptosis and cell cycle arrest when overexpressed in cells.[87,88] It has been demonstrated in vitro and in cancer mouse models that MRX34 is able to restore the tumor suppressor pathway back to normal, thereby inducing apoptosis in tumor cells.[86] The synthetic mimic, which is known to directly regulate at least 24 oncogenes involved in proliferation, the cell cycle, anti-apoptosis mechanisms, metastasis and chemoresistance, is currently being used in phase I trials for treating patients with primary liver cancer or metastatic cancer involving the liver.[86] This replacement therapy represents the first miRNA-based therapy used for cancer treatment.[86]

Recently, in September 2014, researchers from Mirna Therapeutics published data suggesting the potential of a "tumor-suppressing microRNA cocktail" for the treatment of non-small cell lung cancer (NSCLC)[89] The cocktail contains synthetic mimic versions of miR-34a (MRX34) and let-7b and was able to suppress tumor growth in an animal model of NSCLC.[89] This drug combination has entered preclinical trials to assess its potential as the company's next clinical candidate.

Table 2. Current cancer-related clinical trials with a major microRNA component. [90,91] (from Hayes et al. 2014 and Kasinski et al. 2014)

MicroRNA	Trial reference	Disease	Trial/Status
let-7	Preclinical trials	Cancer	In preclinical development
miR-34a	NCT01829971	Liver cancers and liver metastases	Interventional phase I multicenter study to investigate the safety, pharmacokinetics and pharmacodynamics of MRX34
Numerous	NCT01964508	Thyroid cancer	Observational study of microRNA expression in fine needle aspirates
Circulating	NCT01722851	Breast cancer	Observational studies of microRNA expression levels as biomarkers of response to treatment in the tumors and circulation
miR-10b	NCT01849952	Glioma	Observational studies of miR-10b expression levels as biomarkers of tumor grade, survival and genotypic variation
miR-29b	NCT02009852	Oral squamous cell carcinoma	Observational study to explore the prognostic value of miR-29b in tissue, blood and saliva
Circulating	NCT01595139	Low-grade glioma	Observational study of the circulating microRNA expression patterns in low-grade glioma as early predictors of cancer and as a marker of response to therapy
Numerous	NCT01828918	Colorectal carcinoma	Observational study identifying biomarkers for patient stratification in tissue samples

Numerous	NCt01119573	Endometrial cancer	Observational study of association of microRNA expression and lymph node metastasis in tissue samples
miR-29 family	NCT01927354	Head and neck squamous cell carcinoma	Observational study to investigate the role of microRNA in Twist1-mediated cancer metastasis
Numerous	NCT00864266	Non-small-cell lung cancer	Interventional study to identify a signature of response to chemotherapy
Circulating	NCT01595126	Central nervous system cancer	Observational study of microRNA expression in the blood, cerebrospinal fluid and urine of patients through the course of their treatment
Circulating	NCT01391351	Ovarian cancer	Observational studies of biomarker response to treatment
Circulating	NCT01505699	B-cell acute lymphocytic leukemia	Observational studies of biomarkers of clinical outcomes
Numerous	NCT01050296	Pediatric solid tumors	Observational study of microRNA expression profiles in different tumor types

Other research groups and pharmaceutical companies have taken a step towards preclinical and clinical trials as well in order to evaluate the use of miRNA mimics and antagonists for cancer therapy. An overview of some further currently open cancer-related clinical trials involving the utilization of miRNA is listed in Table 2.[90-92]

1.7 Current challenges in microRNA-based therapy

The application of RNAi therapeutics offers the opportunity to make use of a highly selective and efficient endogenous process for the treatment of disease via gene silencing.[93] Furthermore, because miRNAs possess multiple targets, often within the context of a common network, development of treatments could potentially be not only faster, but also cost-effective. This targeting of multiple genes in a single pathway could in turn lead to a stronger and more amassed effect on the respective target proteins at multiple levels.[94] In fact, it has been suggested that the sense and antisense strands of an individual miRNA could potentially regulate different mRNAs, thus enabling the targeting of a wide array of gene products.[95] RNAi therapeutics is a genetic technology, which means it can also be adjusted for the development of treatments for genetic rare, orphan diseases.[96] The motivation for the use of miRNAs as therapeutic agents in the treatment of a heterogeneous disease such as cancer is based on the fact that expression is dysregulated in cancer and that this phenotype can be altered by targeting

the dysregulated miRNA expression.[39,42,97-103] Another main advantage of miRNA-based therapy owes to the point that when compared to protein-based drugs or plasmid DNA-based gene therapy, miRNAs demonstrate a lower immune response and minimal toxicity. This reduced immune response can be attributed to the fact that miRNAs are natural antisense molecules and are therefore not recognized as foreign by the immune system.[94] Furthermore, both miRNA mimic and inhibitor oligonucleotides have a low molecular weight, which offers the advantage of eased delivery to the target cells in comparison to larger molecules often used in gene therapy, such as viral vectors or plasmids.[11]

Despite these advantages and successes regarding a miRNA-based therapeutic approach, the application of miRNAs in the treatment of disease still presents quite an array of limitations, some of which are illustrated in **Figure 5**. As with other similar therapeutic oligonucleotides, the delivery of miRNAs is confronted with problems such as poor bioavailability and limited tissue permeability as well as instability.[11] Low permeability is a result of poor penetration of miRNA mimics or antagonists into the targeted tumor tissue due to both mechanical and biological barriers.[11] Furthermore, vessels within tumors are abnormally compressed and leaky in structure, leading to poor blood perfusion and reduction of efficacy of naked miRNA delivery.[104] In addition, high interstitial fluid pressure slows the diffusion of miRNA into solid tumors.[105] The tumor microenvironment has also been attributed as a barrier for successful delivery of miRNA. Even nonmalignant cells present within the microenvironment have proven to be an extracellular barrier, as macrophages and neutrophils, for example, can nonspecifically uptake the therapeutic miRNAs and prevent them from reaching target tissue.[106]

In order to reach systemic circulation, oral administration of miRNA therapeutics would have to be formulated in such a way that the drug survives the harsh conditions in the digestive track.[96] Unmodified miRNA mimics and inhibitors also present a problem as they are degraded quickly by nucleases in the bloodstream and are cleared via renal excretion, leading to a short half-life within the circulation.[107,108] Even though some miRNAs are in fact able to successfully reach the target tumor tissue, inefficient delivery into the cells can in turn lead to inefficient silencing of the target genes. MiRNA

uptake must be increased so that cancer cells can optimally uptake the oligonucleotides and the subsequent effects of gene silencing must be prolonged long enough in order to reach the maximum therapeutic benefit.[90]

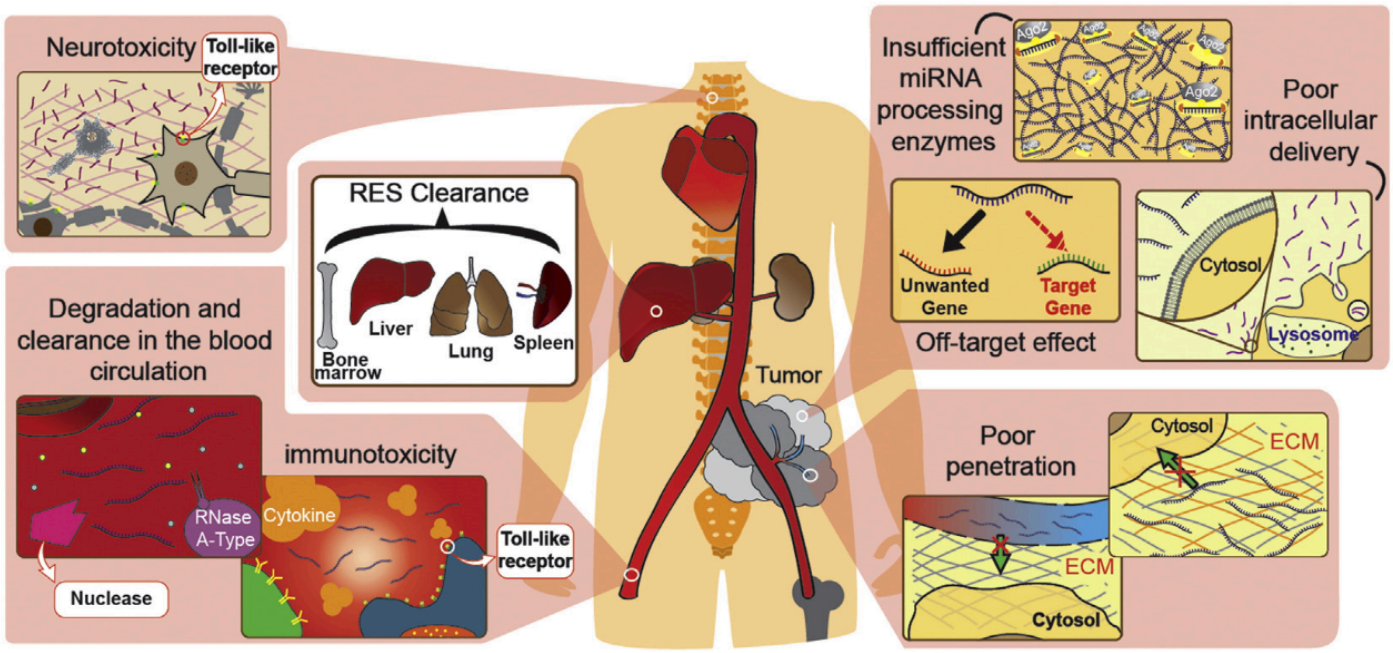


Figure 5. Barriers of in vivo delivery of miRNA for cancer therapy.[11] The efficacy of naked miRNA delivery is hindered by poor blood perfusion as a result of the leaky structure and compression of the tumor vessels. The extracellular matrix prevents miRNAs from penetrating into the tumor whereas unmodified miRNAs can encounter intravascular barriers such as degradation by enzymes. Furthermore, binding of miRNAs to nanoparticles with a diameter above 100nm can increase clearance by the liver and other organs and can potentially result in non-specific uptake of the miRNAs into undesired cells. If naked miRNAs do manage to reach the target tumor cells, they could potentially be taken up by endosomes destined for degradation. Inadequate or saturated processing enzymes may result in miRNA deficiency. Most importantly, as miRNA therapy has the ability to interact with multiple targets, a miRNA may produce off-target effects in non-target tissue. (Image taken and caption adapted from Chen et al. 2015)

Quite possibly the most concerning disadvantage of miRNA-based therapy is the unwanted off-target effect that miRNAs can cause due to their innate ability of regulating multiple mRNA targets. Due to their imperfect matching within the 3'UTR regions of mRNA, miRNAs could potentially warrant undesired gene silencing of normally expressed tumor suppressor genes, in turn causing possible toxicity as well as a

reduction in therapeutic efficacy. To ensure tumor-specific delivery and to avoid substantial complications of off-target effects, miRNAs encapsulated in nanoparticles can be bound to tumor-specific ligands to increase affinity of the drug to the tumor.[101]

As is the case with many current cancer therapies, particularly those involved in the treatment of CRC, resistance may also become a limiting factor for miRNA-based therapeutics. It has been suggested that potential subsequent resistance to therapy could be overcome by taking a combinatorial miRNA-based approach.[90] Furthermore, the effects of miRNA replacement therapy and overloading miRNA networks with synthetic mimics are still questionable.[48] However, because the focus of miRNA-based therapies has just recently come into focus, these barriers and hypothetical unwanted side effects still need to be evaluated, especially in clinical trials, to assess any potential systemic effects in cancer patients.[90]

1.8 Background of thesis

CRC is one of the leading causes of death in developed countries and presents a clear demand for developing novel therapeutic strategies. MiRNAs are small RNA molecules that regulate tumor growth, progression and metastatic spread. The discovery of miRNAs and their roles as regulators of many crucial biological processes allows for the exploration of new approaches for the management of CRC and other cancers, especially considering that inherent or acquired resistance to therapy still remains a major problem in many treatment plans. It is now evident that miRNAs are central to the post-transcriptional regulation of gene expression and are critical to the normal functioning of biological processes. The regulatory potential of miRNAs is thus an attractive concept for the development of new biotechnological and therapeutic applications.[78] Pharmacological manipulation of the expression levels, particularly of oncogenic miRNAs by cellular transfer of selective RNA-based inhibitors, might lead to the development of novel biomolecule-based therapeutics. A collaborative initiative between Dr. Martin Pichler (Division of Oncology, Medical University of Graz) and Dr. Marcel Scheideler (Institute of Genomics and Bioinformatics, Technical University of Graz) resulted in a comprehensive miRNA microarray analysis regarding the expression

of hundreds of miRNAs in different CRC cell lines. Several interesting candidates for selective cellular silencing and overexpression experiments have been identified that provide the basis for further functional cellular experiments proposed in the following section.

1.9 Aims of thesis

The main goal of this diploma thesis is to identify miRNAs in CRC cells that might provide a novel target for pharmacological intervention and consequently potential biotechnological production. Based on the microarray data generated by Dr. Marcel Scheideler, we selected the top 5 most differentially expressed miRNAs (up- and down-regulated in the highly aggressive HRT-18 CRC cell line) for cellular manipulation experiments. For this purpose, we transfected cancer cells with selective inhibitors and mimetics of these miRNAs and studied their time and dose-dependent effects on viability of cancer cells. For further characterization of the mode of action of the selective miRNA inhibitors/mimetics, we also used proliferation, apoptosis and epithelial-mesenchymal transition assays to gain more insight into the biological role of these miRNAs. Thus, we hoped that the results of these knockdown and overexpression studies might be helpful to characterize these miRNAs and in identifying novel targets for future biopharmaceutical interventions in CRC.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and solutions

Chemicals	Company
FBS serum GOLD	PAA Laboratories
McCoy's 5A medium	Gibco
Pen Strep	Gibco
1x PBS, pH 7.4	Gibco
Trypsin	Gibco
HiPerFect transfection reagent	Qiagen
TRIzol	Invitrogen
Bromochloropropane	Sigma-Aldrich
Isopropanol	Millipore
RNase-free water	Qiagen
Triton X-100	Thermo Scientific
Tris-HCl, pH 7.4	Bio-Rad
NaCl	Sigma-Aldrich
Sodium deoxycholate	Sigma-Aldrich
SDS	Sigma-Aldrich
1mM DTT	Qiagen
Protease inhibitor cocktail	Roche
100mM PMSF	Roche
Pierce 660nm Protein Assay	Thermo Scientific
2x sample buffer	Bio-Rad
1x SeeBlue Plus2 Prestained Standard	Invitrogen
10x Tris/Glycine/SDS	Bio-Rad
Distilled water	Millipore
10x Tris/Glycine Buffer	Bio-Rad
100% ethanol	Millipore
Ponceau S solution	Sigma-Aldrich
10x TBS	Bio-Rad
Tween-20	Millipore
PARP rabbit polyclonal antibody	Cell Signaling
LGR5 rabbit polyclonal antibody	Millipore
Nanog mouse monoclonal antibody	Cell Signaling
Oct-4 mouse monoclonal antibody	Cell Signaling
Beta-actin mouse monoclonal antibody	Cell Signaling
Polyclonal rabbit anti-mouse HRP	Dako
Polyclonal goat anti-rabbit HRP	Dako
SuperSignal West Pico Chemiluminescent Substrates	Thermo Scientific
Restore Western Blot Stripping Buffer	Thermo Scientific
miScript miRNA mimics and inhibitors	Qiagen
miScript Primer Assays	Qiagen
QuantiTect Primer Assays	Qiagen

2.1.2 Self-made solutions

RIPA buffer
5ml 1M Tris-Cl, pH 7.4
30ml 5M NaCl
5ml 20% Triton-X 100
5ml 10 % sodium deoxycholate
0.5ml 20% SDS
50ml Millipore H_2O

Lysis buffer
1ml RIPA buffer
10µl 1mM DTT
10µl protease inhibitor cocktail
10µl 100mM PMSF

1x running buffer
100ml 10x Tris/Glycine/SDS
900ml Millipore H_2O

1x transfer buffer
100ml 10x Tris/Glycine buffer
200ml 100% ethanol
700ml Millipore H_2O

1x TBS-T
100ml 10x TBS
900ml Millipore H_2O
1ml Tween-20

3% blocking buffer
1.5g milk powder
50ml 1x TBS-T

1% antibody solution
10ml 3% blocking buffer
20ml 1x TBS-T

2.1.3 Oligonucleotides

miScript Primer Assay	Mature miRNA sequence 5'
Hs_miR-31*	UGCUAUGCCAACAUAUUGCCAU
Hs_miR-342	AGGGGUGCUAUCUGUGAUUGA
Hs_miR-148a	AAAGUUCUGAGACACUCCGACU
Hs_miR-196b	UAGGUAGUUUCCUGUUGUUGGG
Hs_miR-215	AUGACCUAUGAAUUGACAGAC
Hs_RNU6-2_11	ACGCAAATTCGTGAAGCGTT

miScript miRNA	Mature miRNA sequence/guide strand	Anti-hsa-miR (standard, 2'Ome, same sequence)
Syn-hsa-miR-31-3p	5'-UGCUAUGCCAACAUAUUGCCAU-3'	Anti-hsa-miR-31-3p
Syn-hsa-miR-342-5p	5'-AGGGGUGCUAUCUGUGAUUGA-3	Anti-hsa-miR-342-5p
Syn-hsa-miR-148a-5p	5'-AAAGUUCUGAGACACUCCGACU-3'	Anti-hsa-miR-148a-5p
Syn-hsa-miR-196b-5p	5'-UAGGUAGUUUCCUGUUGUUGGG-3'	Anti-hsa-miR-196b-5p
Syn-hsa-miR-215-5p	5'-AUGACCUAUGAAUUGACAGAC-3'	Anti-hsa-miR-215-5p
miScript Inhibitor Negative Control	Targets AllStars Negative Control siRNA	
AllStars Hs Cell Death Control siRNA	Positive cell death phenotype control; targets ubiquitous human cell survival genes	

QuantiTect Primer Assay	Genes
Hs_CCND1_1_SG	CCND1
Hs_CDKN1B_2_SG	CDKN1B (p27)
Hs_VIM_1_SG	VIM
Hs_CDH1_1_SG	CDH1
Hs_GAPDH_1_SG	GAPDH

Eurofins Primer Assay	Customized sequence	Genes
BAX forward	CCTTTTCTACTTTGCCAGCAAAC	BAX
BAX reverse	GAGGCCGTCCCAACCAC	
Bcl-2 forward	GGAGGATTGTGGCCTTCTTTG	Bcl-2
Bcl-2 reverse	GCCGGTTCAGGTACTCAGTCAT	

2.1.4 Commercial Kits

Kit	Company
miScript II RT Kit	Qiagen
QuantiTect SYBR Green PCR Kit	Qiagen
miScript SYBR Green PCR Kit	Qiagen
Pierce Pre-Diluted Protein Assay Standard BSA Kit	Thermo Scientific
WST-1 Proliferation Assay	Roche
Caspase-Glo 3/7 Assay	Promega

2.1.5 Materials and instrumentation

Cell culture	
Laminar flow hood for cell culture	
Incubator	
Casy Cell Counter	Innovatis
Falcon tissue culture plates; 6-well	Fisher Scientific
Falcon tissue culture plates; 96-well, flat bottom	Fisher Scientific
Falcon tissue culture plates; 96-well, flat bottom, solid, opaque	Fisher Scientific
RNA and protein isolation	
Hereaus Fresco 17 centrifuge	Thermo Scientific
SONOPULS ultrasonic homogenizer	Bandelin
BioPhotometer	Eppendorf
cDNA preparation	
MyCycler thermal cycler	Bio-Rad
RT-PCR	
96-well PCR plate	Life Technologies
LightCycler 480 system	Roche
Assays	
SpectraMax Plus Absorbance Microplate Reader	Molecular Devices
LUMIstar OPTIMA microplate luminometer	BMG Labtech
Western blotting	
Mini-PROTEAN Tetra blotting system	Bio-Rad
Mini-PROTEAN TBE Precast gel	Bio-Rad
Nitrocellulose transfer membrane	Bio-Rad
Film sheets	
Curix 60 film processor	Agfa HealthCare

2.2 Methods

2.2.1 Measurement of endogenous miRNA expression in model HCT116 cell line

Cell culture

HCT116 cells were purchased from American Type Culture Collection and were cultured in McCoy's 5A modified Medium supplemented with 10% FBS serum GOLD (PAA Laboratories) and 1% pen-strep. Cells were harvested at passage #6 and plated into 6-well plates (Fisher Scientific) with a final concentration of 250,000 cells per 2.5ml in each well. Plates were incubated for 48 hours at 37°C and 5% CO_2.

2.2.1.1 RNA isolation

After 48 hours of incubation, medium from each well was removed and cells were rinsed twice with 1x PBS (Gibco) followed by the addition of1ml of TRIzol (Invitrogen) to each well to lyse the cells. Lysates were incubated for 10 minutes at room temperature and then homogenized with a needle (type) 5 times. 200µl of BCP was added to the lysate and mixed before centrifuging for 15 minutes at 4°C and 13000rpm. The supernatant was moved to a fresh tube and then mixed with 500µl of isopropanol before centrifugation for 30 minutes at 4°C and 13000rpm. Alcohol was removed without disturbing the pellet followed by two wash steps using 75% ethanol and centrifugation for 5 minutes at 4°C and 13,000rpm. After removal of ethanol, the pellet was dried and then resuspended in 23µl of RNase-free H_2O. RNA concentrations were measured on an Eppendorf BioPhotometer.

2.2.1.2 cDNA preparation

1000ng of isolated template RNA was reverse transcribed into cDNA using the Qiagen miScript II RT Kit with Hi-Flex buffer in a 20µl reaction as described in the manufacturer's instructions. Samples were incubated for 1 hour at 37°C followed by 5 minutes at 95°C. The resulting cDNA was diluted with RNase-free water to a final concentration of 1ng/µl.

2.2.1.3 qRT-PCR

*q*RT-PCR was set up in a 96-well plate for quantification of the miRNAs according to Qiagen's miScript SYBR Green PCR Kit protocol. 1ng of cDNA was used per well and each primer assay reaction was performed in triplicate. The miScript Primer Assays used corresponded to the microRNAs miR-31, miR-148a, miR-215, miR-196b and miR-342 and targeted mature miRNA. The plate was run on the Roche LightCycler 480 System using the following thermocycler settings: 15 minutes at 95°C (initial activation step) followed by 40 cycles of 15 seconds at 94°C (denaturation), 30 seconds at 55°C (annealing) and 30 seconds at 70°C (extension). Analysis was performed to determine relative miRNA expression in untreated HCT116 cells by normalizing sample Ct values to the RNU6B internal housekeeping gene.

2.2.2 Measurement of miRNAs after transient transfection of mimics and inhibitors

2.2.2.1 Transfection of miRNA mimics and inhibitors

HCT116 cells cultured as described previously were harvested at passage #12 and plated into 6-well plates at a concentration of 250,000 cells/2.3ml of medium containing serum. Plates were temporarily incubated on a warming plate at 37°C prior to transfection. To prepare the transfection complex, miRNA mimics and inhibitors were first diluted to the following series of concentrations: 1, 5 and 10nM in 100µl of serum-free McCoy's medium. The miScript Inhibitor Negative Control (Qiagen) was used as a control, as it has no homology to any known mammalian gene. 6µl of HiPerFect transfection reagent was added to each of the miRNA dilutions followed by vortexing and incubation for 10 minutes at room temperature. Transfection complexes were added to the designated wells in a drop-wise fashion and plates were incubated for 48 hours at 37°C and 5% CO_2. RNA was isolated and transcribed into cDNA as described previously. All 5 miScript miRNA mimics and corresponding inhibitors were used for transfection.

2.2.2.2 qRT-PCR

To test the transfection efficiency using the miRNA mimics and inhibitors, quantification of miRNAs was performed as described above using the corresponding miScript Primer Assay for each miRNA target with the miScript SYBR Green PCR Kit (Qiagen). Sample Ct values were

normalized to the RNU6B housekeeping gene. Differences in relative expression were calculated using the averaged Ct value from the triplicate samples according to the $2^{-\Delta\Delta CT}$ method.

2.2.3 WST-1 cell proliferation assay

A set of 2 independent WST-1 (Roche) proliferation assay biological repeats was performed to evaluate the influence of individual miRNA expression and inhibition on the cellular growth rate of HCT116. Six technical replicates of all miRNA mimics and inhibitors corresponding to miR-148a, miR-215, miR-31, miR-196b and miR-342 were performed at a time for each of the experiments. miRNA constructs were brought into the cells via reverse transfection. miRNA mimics and inhibitors were diluted with water to a volume of 25µl so that the final concentration per well after seeding cells was 10nM for mimics and 50nM for inhibitors. 10nM of the miScript Inhibitor Negative Control and 10nM of the AllStars Hs Cell Death Control siRNA (Qiagen) were used as negative and positive controls, respectively. HiPerFect transfection reagent (Qiagen) was diluted by adding 0.75µl to 24.25µl of serum-free medium for a final volume of 25µl per reaction. The 25µl miRNA constructs were first spotted onto the bottom of each well in a clear, flat-bottom 96-well culture plate and then overlaid with 25µl of diluted HiPerFect transfection reagent followed by incubation at room temperature for 10 minutes to allow the complex to form. HCT116 cells were harvested and resuspended in McCoy's medium containing serum to a concentration of $2x10^4$ cells/150µl. After the 10 minute incubation step, this 150µl cell mixture was added on top of the transfection complex. Plates were incubated for 48 hours at 37°C and 5% CO_2 prior to measurement.

To measure proliferation trends 48 hours post-transfection, medium was first removed from each well via aspiration. The WST-1 reagent was diluted 1:10 in McCoy's medium without serum and 100µl of this mixture was added to each well. Colormetric changes were measured on a SpectraMax Plus Absorbance Microplate Reader (Molecular Devices) at 450nm with a reference wavelength of 620nm at the following time points after addition of substrate: 0 minutes, 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours.

To analyze the data collected from the WST-1 assay, averages and standard deviations were calculated for the 6 technical replicates for each of the 6 time points in each individual experimental repeat. From these calculations, it was determined that the 4 hour time point represented the point at which values reached a plateau and were therefore at their highest at the 3 hour time point. For this reason, data from this time point was used for further analysis of

cellular growth activity. First, all calculations were performed separately for each of the 2 independent experiments. The average value for colorimetric change was calculated for the 6 technical replicates for the negative inhibitor control. This average was set to 100%, to which the standard deviation was normalized. These calculations for the negative inhibitor control were used to normalize values for both mimic and inhibitors. This process of averaging and normalizing was repeated individually for both experimental repeats. Data for the averages and standard deviations for the negative inhibitor control across the 2 experiments were then averaged and set to 100% to be used as a reference control to which the mimics and inhibitors could be normalized. Values for mimics and inhibitors were averaged across the 2 experiments and normalized to the determined value of the negative inhibitor reference control. Significance was calculated using the student's t-test ($p<0.05$). If the miRNA mimic or inhibitor produced the same significant trend in both independent experimental repeats, the results were considered to be significant.

2.2.4 Caspase-Glo 3/7 Assay

The luminescent Caspase-Glo 3/7 Assay (Promega) was performed in a set of 2 independent assay repeats to measure caspase-3 and -7 activities in HCT-116 cells (all between passages 3-6). Transfection setup was identical to the WST-1 assay described above, but this time reactions were performed in triplicate rather than in six technical replicates. Unlike for the WST-1 assay, a white, opaque flat-bottomed 96-well plate was used in order to measure luminescence activity and maximize light output signal. Mir-342 and miR-196b were excluded from these experiments, as assay reagents were expensive and miR-215, miR-31* and miR-196b were of highest priority. After cell seeding and 24 hours of incubation, plates were removed from the incubator and allowed to equilibrate to room temperature for 1 hour. During this time, the caspase reagent was prepared by mixing 50µl of serum-free medium with 50µl of Caspase-Glo reagent for one reaction and was kept protected from light. Media from the wells was removed via aspiration and 100µl of the caspase reagent mixture was added to each well. The plate was put on a plate shaker for 30 seconds at 650rpm to mix the contents. The plate was protected from light exposure and incubated for 1 hour at room temperature followed by luminescence measurement on a LUMIstar OPTIMA microplate luminometer (BMG Labtech).

For analysis of the apoptosis data, average signals of the negative inhibitor control were calculated for each individual experiment. The same was done for miRNA mimic and inhibitor data which was then normalized to this calculated reference control within the experiment.

Averages of normalized values between the 2 biological repeats were then calculated to determine the average relative luminescence for HCT-116 cells transfected with the respective individual miRNA mimics and inhibitors. Significance was calculated using the student's t-test ($p<0.05$). If the miRNA mimic or inhibitor produced the same significant trend in both independent experimental repeats, the results were considered to be significant.

2.2.5 Measurement of proliferation, EMT and apoptosis gene markers

RT-PCR was performed with cDNA from HCT-116 cells transfected individually with each of the miRNA mimics and inhibitors of the 5 miRNAs being studied to assess expression of various proliferation, EMT and apoptosis markers. Mimics and inhibitors were transfected in cells at concentrations of 1, 5 and 10nM and these samples were investigated separately in RT-PCR. The miScript Inhibitor Negative Control (Qiagen) was introduced into the cells at a final concentration of 10nM to be run with all primer assays. RNA was collected and reverse transcribed as described above and diluted to a final concentration of 5ng/µl. Reactions were set up in a 96-well plate according to Qiagen's QuantiTect SYBR Green PCR Kit for a 25µl reaction. 10ng of cDNA was used per well and each QuantiTect primer assay reaction was performed in triplicate. The plate was run on the Roche LightCycler 480 System using the following thermocycler settings: using the following thermocycler settings:
15 minutes at 95°C (initial activation step) followed by 40 cycles of 15 seconds at 94°C (denaturation), 30 seconds at 55°C (annealing) and 30 seconds at 72°C (extension).

Analysis was performed to determine relative marker expression in transfected HCT-116 cells by normalizing the sample Ct values to the GAPDH internal housekeeping gene. Differences in relative expression were calculated using the averaged Ct value from the triplicate samples according to the $2^{-\Delta\Delta CT}$ method. Fold changes were calculated after normalizing to the GAPDH housekeeping gene and negative control via the ddCt method. Fold changes above or equal to 1.2 were considered significant and upregulated whereas fold changes equal to or below 0.8 were considered significant and downregulated after performing a student's t-test.

2.2.6 Western blot for PARP detection

A western blot was performed to examine poly ADP ribose polymerase (PARP) expression in its cleaved and uncleaved form in HCT-116 cells after manipulation with the miRNA mimics and corresponding inhibitors for miR-31, miR-148a and miR-215. miR-342 and

miR-196b were ruled to be insignificant as determined by RT-PCR and were therefore excluded from protein expression analysis.

2.2.6.1 Protein collection

Transfection of miR-31, miR-148a and miR-215 mimics and inhibitors as well as the miScript Inhibitor Negative Control and AllStars Hs Cell Death Control siRNA was set up in a 6-well plate. HCT-116 cells were trypsinized and resuspended in McCoy medium containing serum and pen-strep at a concentration of 250,000 cells/2.3ml. 2.3ml of this suspension was added to each well and the plate was briefly incubated at 37°C on a heat plate just prior to transfection. miRNA mimics and inhibitors were diluted in 100ul of serum-free McCoy medium to a final concentration of 10nM and 50nM, respectively. Both negative and positive controls had a final concentration of 10nM. 6ul of HiPerFect transfection reagent was mixed with each complex and complexes were allowed to form for 10 minutes at room temperature. After this incubation step, transfection complexes were added in a drop-wise fashion to each corresponding well. The plate was incubated for 48 hours at 37°C and 5% CO2.

48 hours post-transfection, cells were lysed in RIPA buffer. Just prior to lysis, 10µl of 1mM DTT, 10µl of protease inhibitor cocktail and 10µl of 100mM PMSF were added to each ml of RIPA buffer. Detached cells were first collected from each well in the plate to be included in the analysis. To do this, medium was collected from each well into 1.5ml Eppendorf tubes and centrifuged at max speed to obtain a cell pellet. The cell pellet was washed twice in 1x PBS and afterwards kept on ice. Each well was then washed twice with cold PBS. With the 6-well plate on ice, 200µl of ice-cold lysis buffer was added to each well. Cells were lysed by pipetting up and down and collected in 1.5ml Eppendorf tubes. Cell pellets previously collected were then resuspended into each corresponding sample containing lysis buffer. Samples were then kept on ice for 30 minutes and were vortexed every 5 minutes. After 30 minutes, each sample was sonicated with a Bandelin Sonopuls homogenizer for 15 seconds. Lysates were then centrifuged for 15 minutes at 13000rpm at 4°C. Supernatants were transferred to a new tube and the pellets were discarded.

Protein concentrations were determined using the Pierce Pre-Diluted Protein Assay Standard BSA kit (Thermo Scientific) in combination with the Thermo Scientific Pierce 660nm Protein Assay in a clear 96-well flat-bottom plate in accordance with the manufacturer's instructions. Samples were diluted 1:10 in NaCl by adding 5µl of sample to 45µl of NaCl. A pseudo-sample was also prepared containing 10µl of RIPA buffer and 90µl of NaCl. 10µl of diluted sample was added to each well and topped off with 150µl of the Pierce 660nm Protein

Assay reagent. The plate was shaken at medium speed for 1 minute and incubated at room temperature for 5 minutes prior to being read on a SpectraMax Plus Absorbance Microplate Reader at 660nm. The plate reader program used the standard curve to determine each sample concentration in µg/ml.

2.2.6.2 Sample preparation and gel electrophoresis

Samples were prepared for use with the BioRad Mini-PROTEAN Tetra system. 25µg of protein was used for each sample and RIPA buffer was added to make up a final volume of 25µl. 25µl of 2x sample buffer was mixed to each lysate and samples were incubated at 95°C for 5 minutes and then kept on ice. The entire 50µl of the samples was added to the wells of a Mini-PROTEAN TBE Precast gel (Bio-Rad). 10µl of 1x SeeBlue Plus2 Prestained Standard (Invitrogen) was added to the first and last wells of the gel to serve as a ladder. The running buffer was prepared by diluting 100ml of 10x Tris/Glycine/SDS (Bio-Rad) with 900ml of Millipore distilled water and then added to the gel box about halfway. The run was set to 60V and once the samples aligned linearly, the voltage was increased to 160V until samples reached the bottom of the gel.

2.2.6.3 Gel transfer

Transfer buffer was prepared by mixing 100ml of 10x Transfer Buffer (Bio-Rad) with 200ml of 100% ethanol and 700ml of Millipore water and kept in the freezer until use. The gel was removed from its casting tray and transferred onto a nitrocellulose membrane (Bio-Rad) and set up in the transfer apparatus with cold transfer buffer. The transfer was run for 1.5 hours at 90V at 4°C.

2.2.6.4 Blocking of membrane and addition of primary antibody

After completion of the transfer run, the membrane was immersed in Ponceau buffer for 5 minutes to confirm that the proteins transferred successfully from the gel and was then rinsed in water. 1x TBS-T buffer was prepared by diluting 100ml of 10x TBS in 900ml of Millipore water and then adding 1ml of Tween-20. 3% blocking buffer was then prepared by mixing 1.5g of powdered milk in 50ml of TBS-T. 10ml of this 3% blocking buffer was added to the membrane tray and rocked for 1 hour at room temperature before being discarded.

1% primary antibody solution was prepared by adding 10ml of 3% blocking buffer to 20ml of TBS-T. 7µl of the PARP polyclonal antibody (Cell Signaling) was added to 7ml of 1% blocking buffer for a 1:000 dilution and then added to the tray with the membrane. The tray was rocked overnight at 4°C to allow the antibody to bind.

2.2.6.5 Washing steps and addition of secondary antibody

The primary antibody solution was discarded from the membrane tray. The membrane was washed with TBS-T on a rocker for 15 minutes for a total of 3 washes. After the final wash, 2µl of polyclonal goat anti-rabbit HRP secondary antibody (Dako) was added to 10ml of 1% blocking buffer for a 1:5000 dilution and added to the membrane tray. The membrane was allowed to rock for 1.5 hours at room temperature. After this incubation period, the membrane was washed again 3 times as described above.

2.2.6.6 Developing of membrane

The following steps were carried out in a dark room after the final wash. The membrane was drip dried from the buffer and placed back into a tray containing 4ml of mixed SuperSignal West Pico Chemiluminescent Substrates (Thermo Scientific) and was incubated for 5 minutes at room temperature for low-picogram-level detection of the proteins. The membrane was drip dried again by tapping onto a paper towel and then placed between two transparency sheets. Individual film sheets were pressed onto the membrane for various amounts of time to achieve the optimal development of images. Films were processed in a Curix 60 film developer (Agfa HealthCare).

2.2.6.7 Stripping membrane for detection of beta-actin control

The membrane was kept in fresh TBS-T buffer at 4°C prior to stripping and reprobing. To strip the immunoblot, Restore Western Blot Stripping Buffer (Thermo Scientific) was poured into the membrane tray so that the blot was completely immersed. The membrane was rocked for 15 minutes at room temperature to allow for the removal of bound primary and secondary antibodies. The blot was then washed 3 times in 15-minute intervals using TBS-T. After the last wash, the membrane was blocked for 1 hour with 3% blocking buffer and then washed again 3 times prior to addition of the primary antibody. 2µl of the mouse monoclonal beta-actin antibody (Cell Signaling) was diluted in 10ml of 1% blocking solution and added to the membrane. The

blot was rocked overnight at 4°C. 24 hours after the addition of the primary antibody solution, the membrane was washed and probed with polyclonal rabbit anti-mouse HRP (Dako) using a 1:1000 dilution. Subsequent washing and developing steps were performed as described above.

2.2.7 Quantification of miRNA expression in adherent cells and tumor spheres

A previous study done within this research group involved the enrichment and maintenance of cancer stem cells from several cell lines for collection and analysis of tumor spheres and their attached cells. In this particular study, RNA from adherent and sphere-derived cells of the following 3 CRC cell lines was isolated: Caco-2, HCT-116 and HT-29. This RNA was used in the presently described study and reverse transcribed into cDNA as explained above. RT-PCR was performed in order to obtain additional information regarding the expression of miR-31, miR-342, miR-196b, miR-148a and miR-215 in these cell lines previously used as a cancer stem cell model. The miScript Primer Assays were used once again along with Qiagen's miScript SYBR Green PCR protocol according to the manufacturer's instructions to detect these levels of miRNA expression. Values obtained for the adherent cells were used as a reference to compare with values from those of the tumor spheres. All results were first normalized to the RNU6B internal housekeeping gene. Differences in relative expression were calculated using the averaged Ct value from the triplicate samples according to the $2^{-\Delta\Delta CT}$ method. Fold changes were calculated after normalizing to the GAPDH housekeeping gene and negative control via the ddCt method. Fold changes above or equal to 1.2 were considered significant and upregulated whereas fold changes equal to or below 0.8 were considered significant and downregulated after performing a student's t-test.

2.2.8 Western blot for detection of stem cell markers

The same western blotting procedure described above for detection of PARP and cleaved PARP was used to determined protein expression of the following human stem cell markers associated with cancer: Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), Nanog homeobox (Nanog) and octamer-binding transcription factor 4 (Oct-4).

2.2.8.1 Primary and secondary antibody dilutions

3.5µl of the LGR5 rabbit polyclonal antibody (Millipore) was added to 7ml of 1% blocking buffer for a 1:2000 dilution. The polyclonal goat anti-rabbit HRP (Dako) was used for its detection by diluting 2µl of the secondary antibody in 10ml of 1% blocking buffer. The For Nanog detection, both primary and secondary antibody solutions using the mouse monoclonal antibody (Cell Signaling) and polyclonal rabbit anti-mouse HRP (Dako), respectively, were prepared by diluting 7µl of antibody in 7ml of 1% blocking buffer. The Oct-4 mouse monoclonal antibody (Cell Signaling) was diluted the same as for Nanog and also detected with the polyclonal rabbit anti-mouse HRP (Dako). Membranes were stripped and reprobed for beta-actin as described above.

3 RESULTS

3.1 Endogenous miRNA expression levels in model HCT-116 cell line

In order to assess the biological functions and potential therapeutic effects of pharmacological manipulation of specific mature miRNAs, it was first necessary to determine their endogenous expression in untreated cells and to find an optimal experimental model system for miRNA manipulations. Previous studies conducted in the lab of my advisor (Dr. Martin Pichler) clearly demonstrated that the CRC cell line HCT-116 has favorable properties in terms of introduction of miRNA mimetics/inhibitors (in other words, they have a high transfection efficacy). Using the human colorectal carcinoma HCT-116 cell line as a model, quantitative RT-PCR was performed to measure basal levels of the selected miRNAs prior to cellular manipulation. With the exception of miR-215, 4 of the 5 tested miRNAs were detected at threshold cycles values between 27 and 31, whereas endogenous RNU6B, which was used as a control for normalization of the miRNA gene expression data, was detected at a C_T value around 19 (**Figure 6**). Based on the results after normalization (**Figure 7**), it was assumed that an inhibitor construct for miR-215 would not work in this cell line, as endogenous expression of this miRNA was not detectable and would not produce a significant effect upon inhibition.

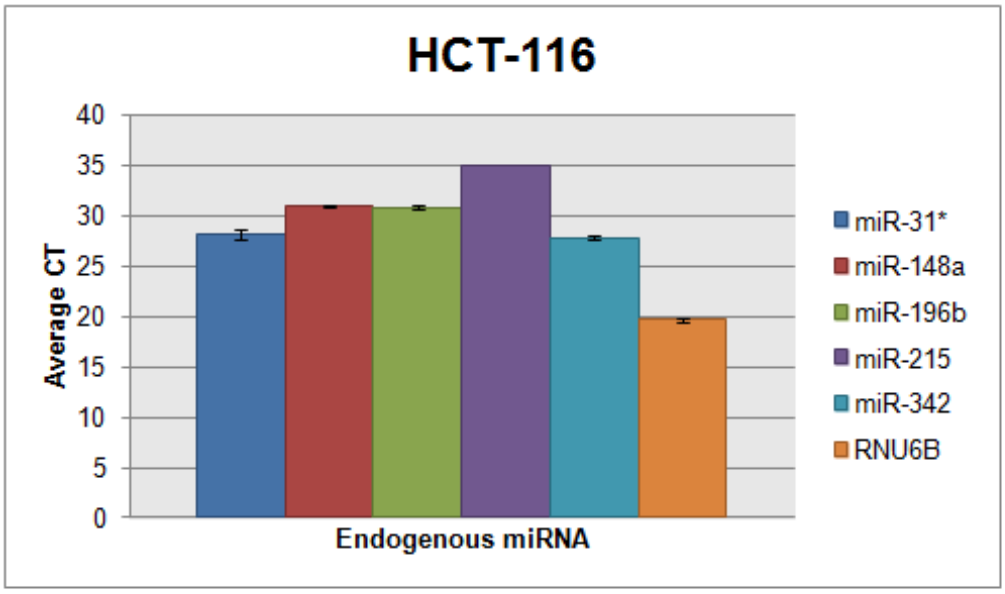


Figure 6. Average threshold cycle (C_T) in untreated HCT-116 cells. 1ng of cDNA was used in each miScript Primer Assay reaction and averages were taken among triplicates. miR-215 was not detected by quantitative RT-PCR and thus excluded from subsequent inhibition experiments. miR-31* and miR-342 demonstrated the highest levels of endogenous expression in untreated HCT-116.

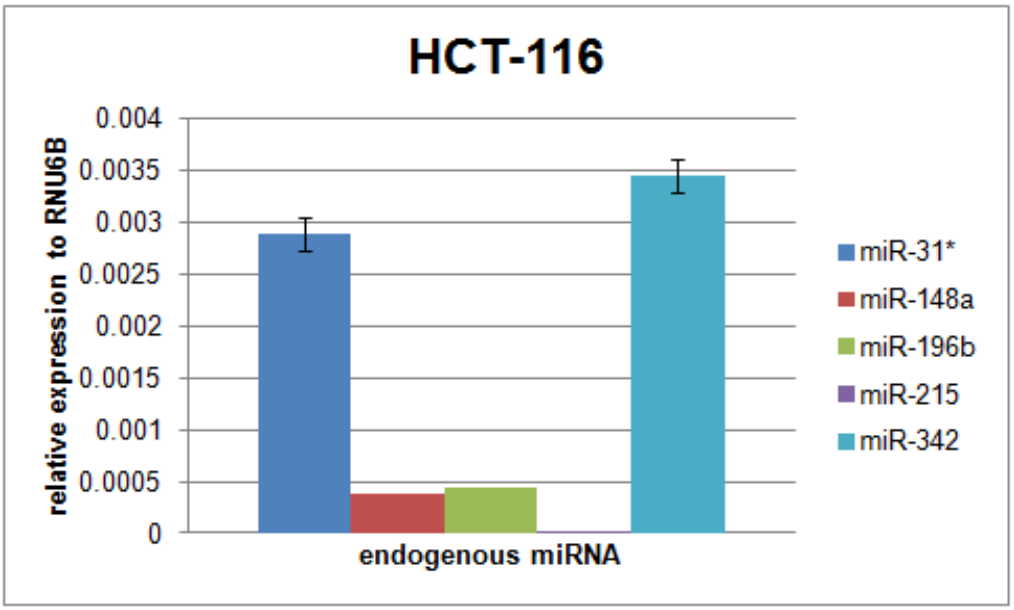
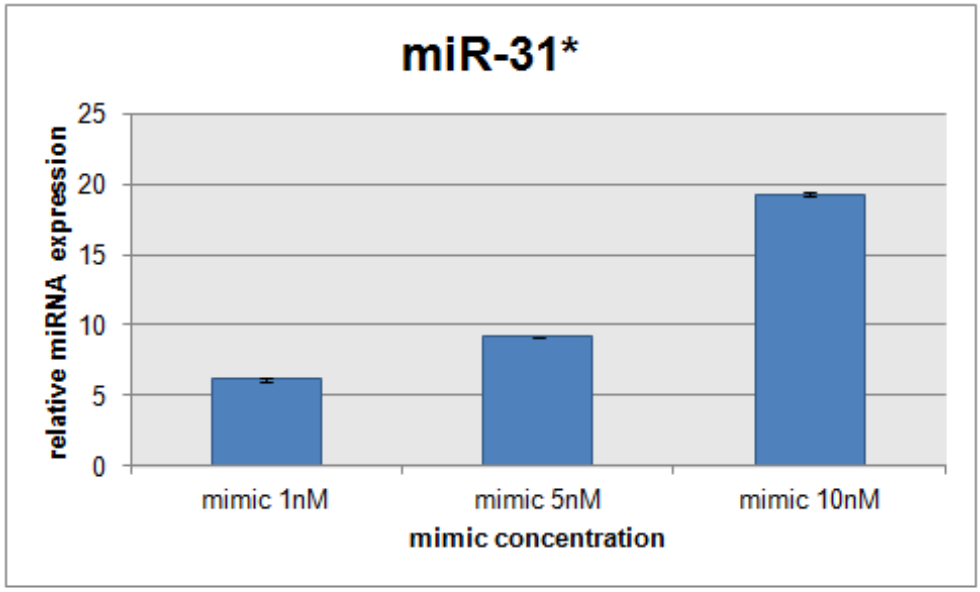


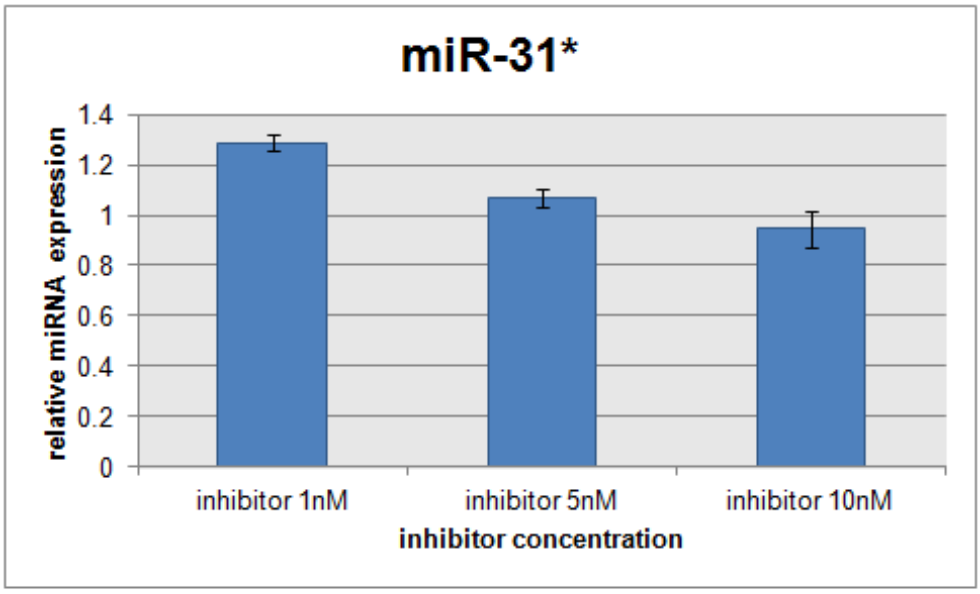
Figure 7. Relative expression of target miRNAs in untreated HCT-116 cells. Ct values were normalized to RNU6B expression, which was used as an internal housekeeping control using the formula $2^{-(\text{Ct target miRNA-Ct RNU6B})}$. After normalization, averages were calculated among triplicates for each miRNA. Endogenous expression levels of miR-31* and miR-342 were highest among the 5 miRNAs and were therefore used as the main candidates for inhibition in subsequent studies.

3.2 Effects of mimics and inhibitors on miRNA levels

A series of transfections was performed in order to investigate the effects of miRNA gain or loss of function and to determine whether miRNA overexpression or inhibition of CRC cells could be successfully established in our model HCT-116 system. miRNA mimics (for all 5 selected miRNAs) and inhibitors (miR-31* and miR-342) or a non-specific control miRNA (NC inhibitor, which is known to have no effect on cellular functions in cells) were transfected individually in HCT-116 cells. Quantitative RT-PCR was used to confirm the success of these transfections by assessing their relative miRNA expression levels after comparison with the negative control and RNU6B reference gene. Transfections were performed using three different final concentrations of mimics and inhibitors of 1, 5 and 10nM to determine the optimal level of expression or suppression in the cells. In some cases, the expression levels of miRNA increased roughly linearly with increasing concentration of introduced mimic construct.



A

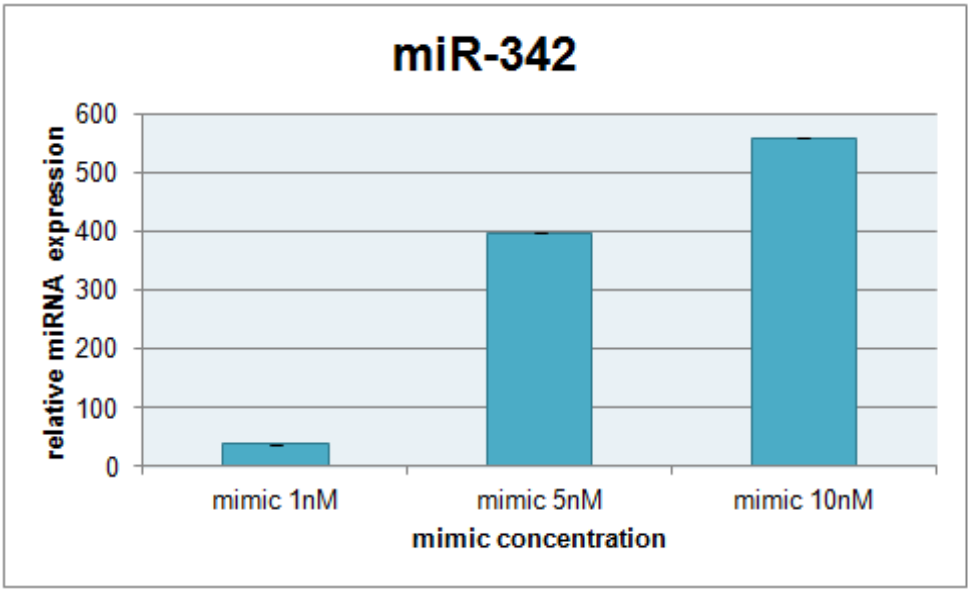


B

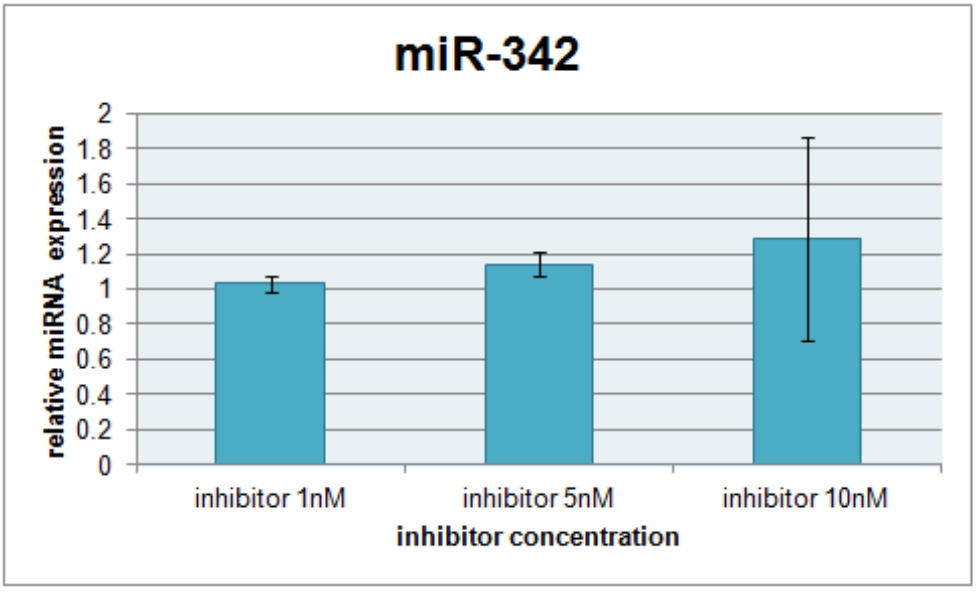
Figure 8. Relative changes in miRNA expression after transfection with miR-31* mimic and inhibitor. Drastic increases in expression can be seen upon introduction of mimic miR-31* (A) and inhibitor of miR-31* (B). Inhibitors were successfully able to suppress miRNA levels with no significant difference among the varying concentrations. 1ng of cDNA was used per reaction for all assays.

With the exception of miR-148a, miRNA mimic transfections pertaining to 10nM provided the highest expression in HCT-116 cells (**Figures 8-10**). For this reason, a final mimic concentration of 10nM was used in transfections for all subsequent experiments. miRNA mimics miR-148a and miR-215 were able to induce the highest level of miRNA overexpression after fold change calculations (**Figure 10**). Decreasing levels of miRNA expression were observed with increasing final concentration of introduced miRNA inhibitors for miR-31* but not for miR-

342 (**Figures 8 and 9**). However, in order to ensure appreciable inhibitory effects of the corresponding inhibitors on miR-31* and miR-342, subsequent transfections for miRNA inhibitors were performed at a final concentration of 50nM, as often recommended by the manufacturer and previously described in the literature.[109-111]

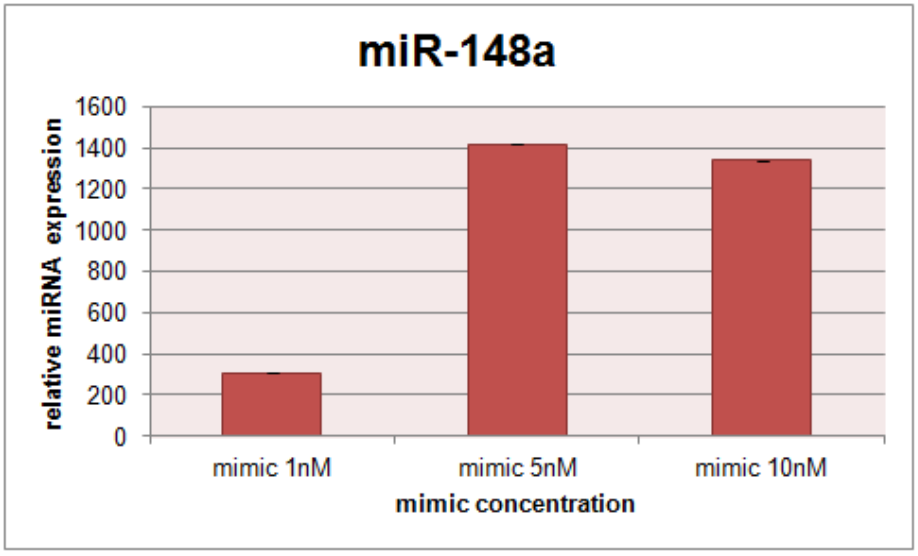


A

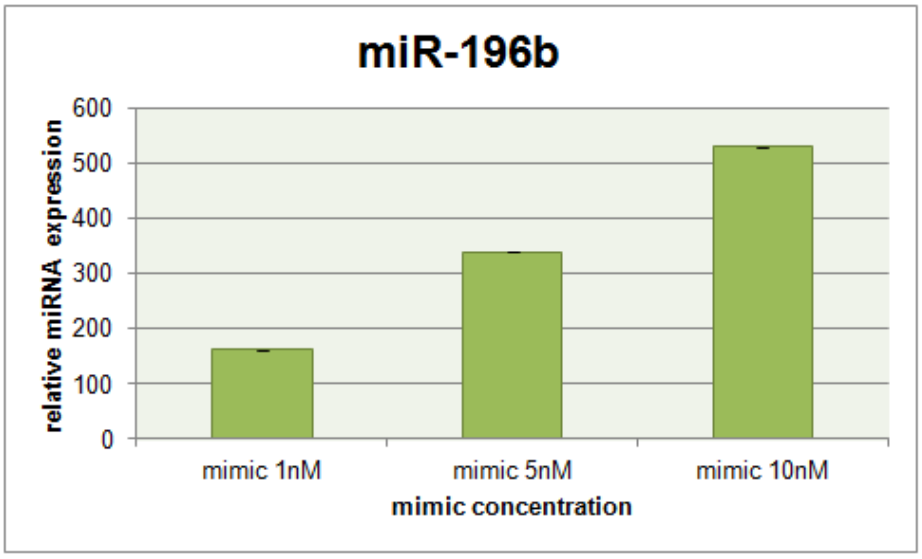


B

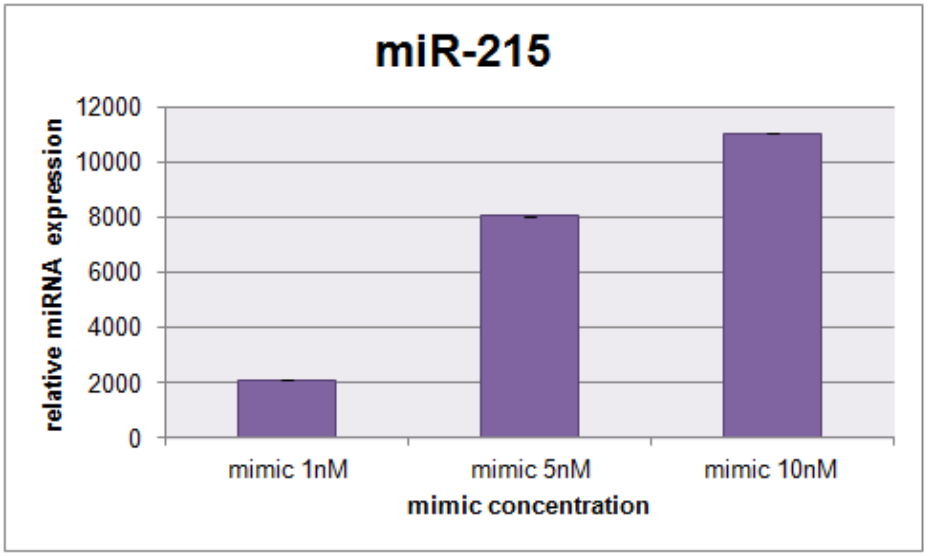
Figure 9. Relative changes in miRNA expression after transfection with miR-342 mimics and inhibitors. Drastic increases in expression can be seen upon introduction of mimic miR-342 (A). Inhibitors led to reduced miRNA levels with no drastic difference among the varying concentrations (B). 1ng of cDNA was used per reaction for all assays.


miR-148a
relative miRNA expression
1600
1400
1200
1000
800
600
400
200
0
mimic 1nM
mimic 5nM
mimic 10nM
mimic concentration

A


miR-196b
relative miRNA expression
600
500
400
300
200
100
0
mimic 1nM
mimic 5nM
mimic 10nM
mimic concentration

B

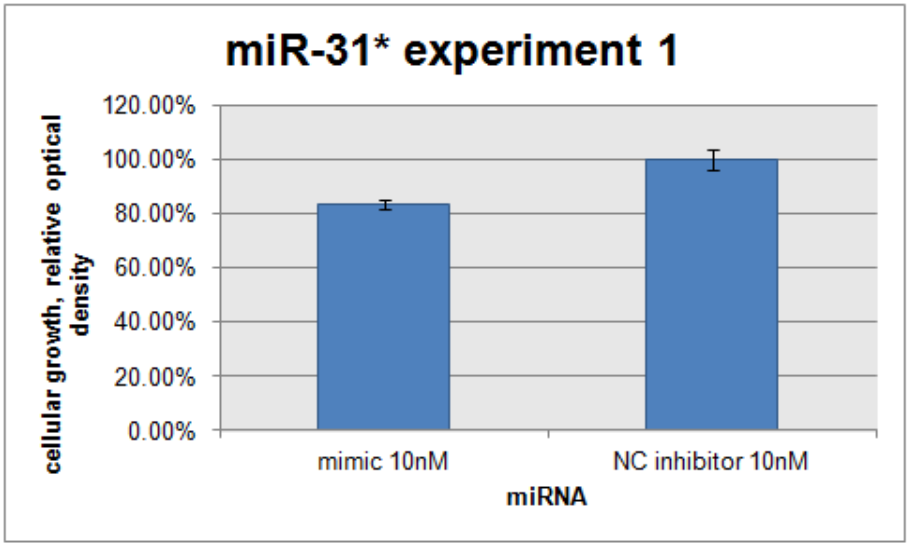


C

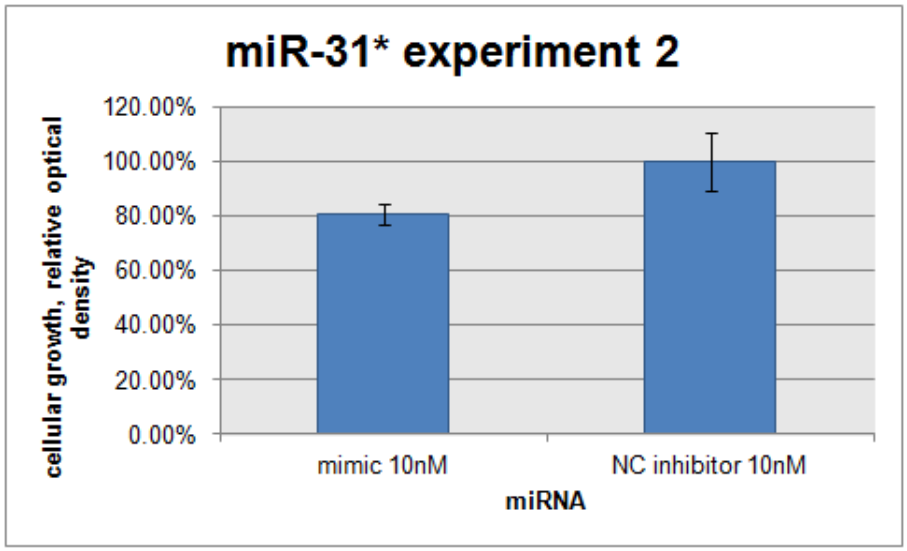
Figure 10. Relative changes in miRNA expression after transfection with miR-148a, miR-196b and miR-215 mimics. The miR-148a mimic administered at 5nM demonstrated the highest miRNA expression as determined by RT-PCR (A). Introduction of miR-196b (B) and miR-215 (C) increased relative miRNA expression with the most significant changes occurring at 10nM of introduced construct.

3.3 Effects of miRNA overexpression or inhibition on cellular growth in HCT-116 cells

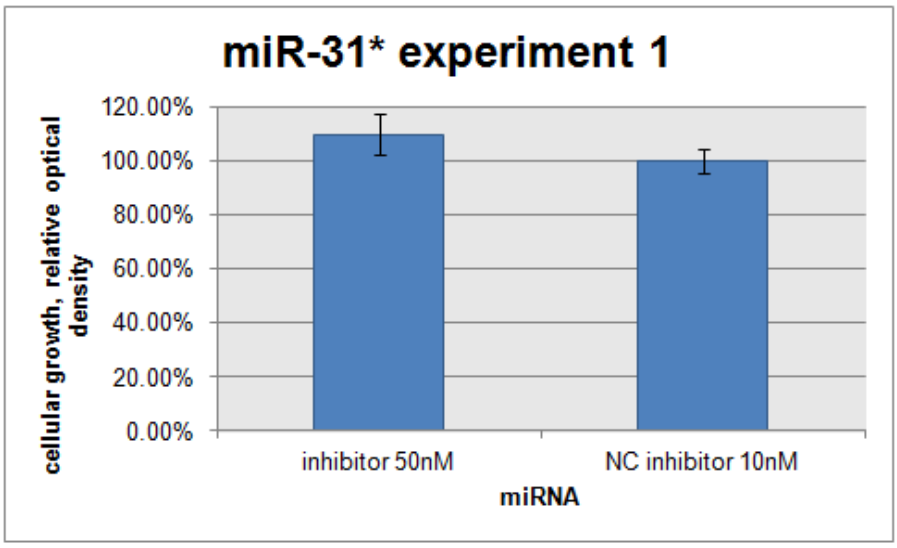
After we established the protocol and determined the optimal concentrations of miRNA mimetics/inhibitors to manipulate the expression of the respective miRNAs in the HCT-116 cell line model system, we studied the impact of these miRNA expression manipulations on fundamental biological functions. To determine the impact of the five selected miRNAs on CRC cell growth, a cellular growth assay was performed using HCT-116 cells transiently transfected with the miRNA mimics and inhibitors. The WST-1 colorimetric assay, which was used for these experiments, indirectly determines the number of viable cells by assessing the cleavage of tetrazolium salts added to the culture medium of adherent cells. Although this assay is an indirect measurement of cell proliferation and viability, its principle is based on the conversion of the tetrazolium salt WST-1 by mitochondrial dehydrogenase enzymes present in the cells. After a given time period, the reaction will result in a color change that is directly proportional to the amount of dehydrogenase enzyme in the tested culture, reflecting the overall cellular metabolic activity and in turn, gives an indirect measurement the number of viable cells.


miR-31* experiment 1
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
mimic 10nM
NC inhibitor 10nM
miRNA

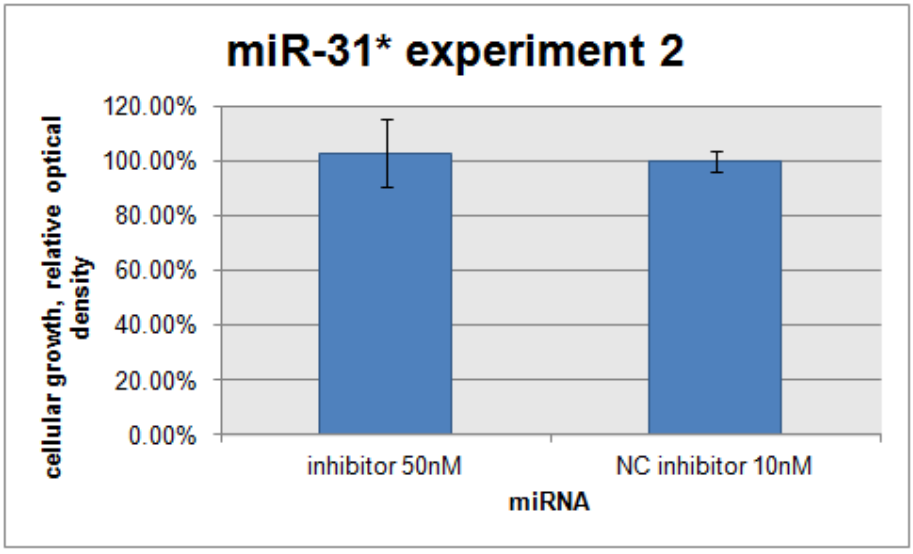
A


miR-31* experiment 2
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
mimic 10nM
NC inhibitor 10nM
miRNA

B


miR-31* experiment 1
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
inhibitor 50nM
NC inhibitor 10nM
miRNA

C



D

Figure 11. Overexpression of miR-31* inhibits cellular growth. Significance was determined using the student's t-test ($p<0.05$). 2 biological experimental repeats were performed and assay measurements were taken at a 3-hour time point. If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Measured absorbance in miRNA mimic experiments 1 and 2 (A, B) indicated that overexpression of the 10nM mimic led to a significant decrease in cellular growth. In the first inhibitor experiment (C), inhibition of miR-31* led to a slight increase of growth in comparison to the negative control, which is consistent with the overexpression data displayed in (A) and (B). However, the second experiment regarding miR-31* inhibition (D) did not demonstrate a significant difference from the negative control.

WST1 assays were performed in 2 biological repeats. If data in both repeats demonstrated the same trend and were significant ($p<0.05$), then the results for this particular miRNA were deemed significant, as the trend was independently verified in two different assays. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. That being said, significant results of each of the 2 individual experiments of the WST-1 proliferation assay showed overall that overexpression of 3 of the 5 miRNAs led to decreased cellular growth of HCT-116 CRC cells (**Figures 11-15**). More specifically, overexpression of miR-148a using the miRNA mimic led to significant results in both repeated experiments (**Figure 12**). This particular miRNA also induced the highest amount of change in absorbance in comparison to the negative control. In the first experiment (**Figure 12A**), overexpression of miR-148a demonstrated a 1.6-fold lower relative optical density than the negative control and in turn indicates that the number of viable cells with metabolic activity was much lower in this group. In the second experiment (**Figure 12B**), the expression was 1.43-fold lower than the negative

control. Inhibition of miR-148a did not yield any significant changes detected by the WST1 assay (**Figures 12C and 12D**).

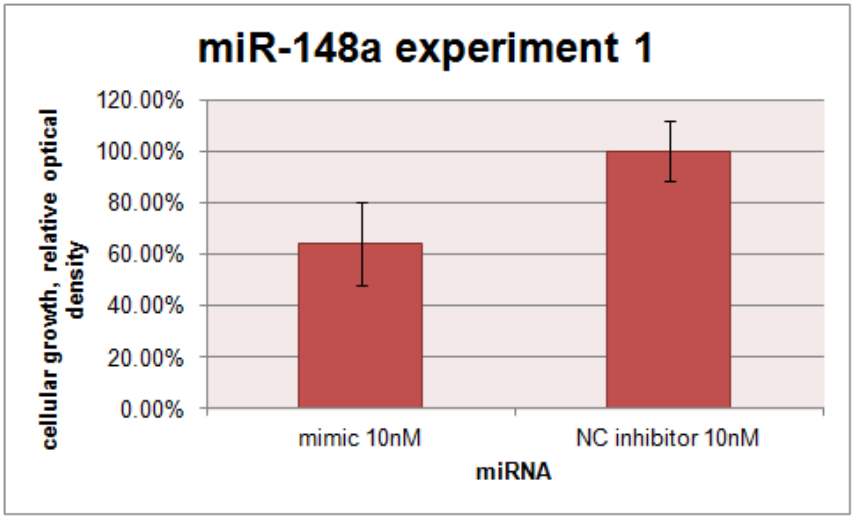
Data pertaining to miR-31* (**Figure 11**) demonstrated the next most drastic alteration in optical density when compared to the negative control. Much like inhibition of miR-148a, data corresponding to the miR-31* inhibitor proved to be insignificant. Although inhibition of miR-31* in the first experiment (**Figure 11C**) led to a significant ($p=0.023$) increase in relative optical density of the cells, the second experiment was unable to independently confirm this increase (**Figure 11D**). HCT-116 cells overexpressing the miR-31* mimic in experiments 1 and 2 demonstrated a 1.2-fold and 1.24-fold decrease in optical density, respectively, when compared to the negative control (**Figures 11A and 11B**).

WST-1 assay results were somewhat contradictory in the case of miR-196b. In the first and second experiments (**Figures 14A and 14B**), overexpression of miR-196b using the miRNA mimic induced a 1.13-fold ($p=0.014$) and 1.2-fold ($p=0.025$) decrease in optical density, respectively, after comparison with the negative control. However, both experiments regarding inhibition of miR-196b illustrated in **Figures 14C** and **14D** showed that inhibition of miR-196b also was able to induce a reduction in optical density in comparison to the negative control, suggesting that it has the same effect on HCT-116 cellular growth as the miR-196b mimic. Inhibition data, however, was only significant in the second of the two experiments ($p=0.004$) and therefore the entire assay for the miR-196b inhibitor was considered insignificant. These contradictory data suggest that it cannot be assumed that overexpression of miR-196b, although significant in our case, inhibits cellular growth. Because both mimic and inhibitor demonstrated similar trends, that is, when introduced into the cells, they decreased the relative optical density as measured in the WST1 assay, these results must be seen as a coincidence rather than as an implication for a biological effect.

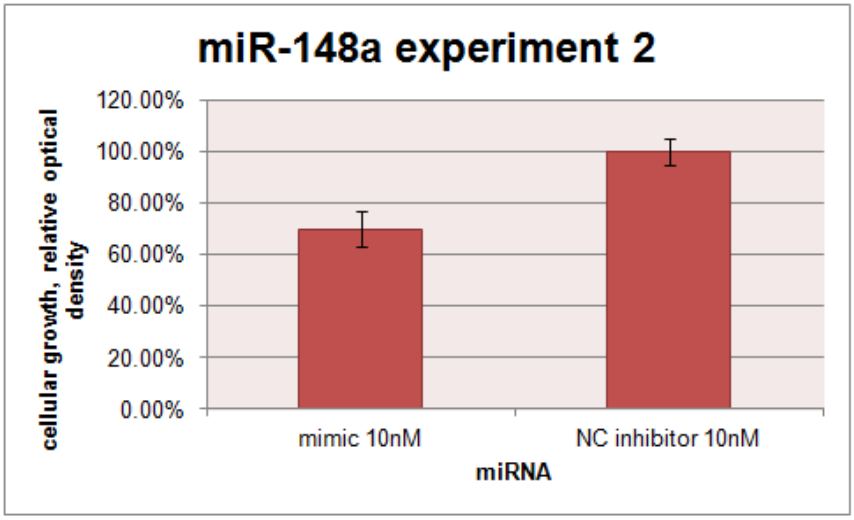
Although overexpression of the miR-342 mimic had a significant anti-growth effect on HCT-116 cells in both of the independently performed assays, results were not as drastic as with the other miRNAs. Cells overexpressing miR-342 demonstrated a 1.04-fold and 1.14-fold increase in optical density and therefore cellular growth in the first and second experiments, respectively (**Figures 15A and 15B**). Neither biological repeat using the miR-342 inhibitor produced a significant change in cellular growth in comparison to the negative control (**Figures 15C and 15D**) with p values of 0.53 and 0.19 for the first and second experiments, respectively.

Finally, the miR-215 mimic was only able to produce a significant change in 1 of the 2 experiments when overexpressed in our model cell line (**Figure 13A**), leading to a 1.1-fold increase of relative optical density in comparison to the negative control. However, this trend

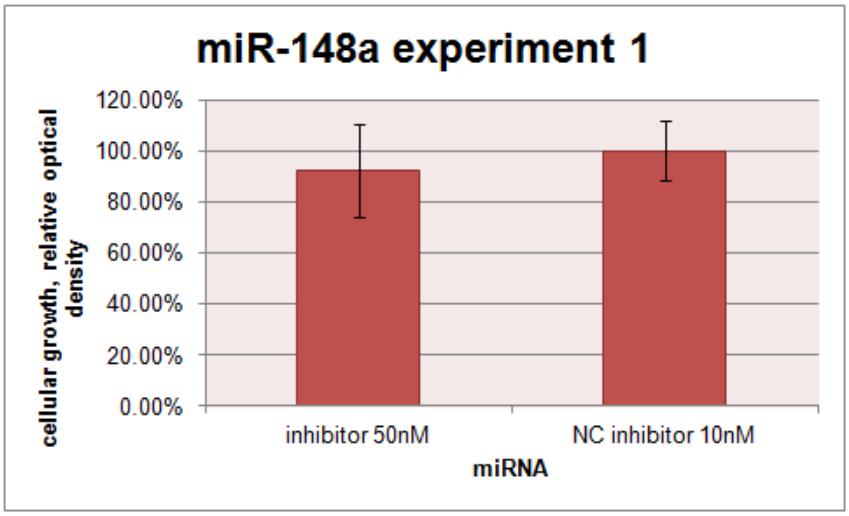
was ruled insignificant as the second biological repeat (**Figure 13B**) failed to demonstrate the same significant increase



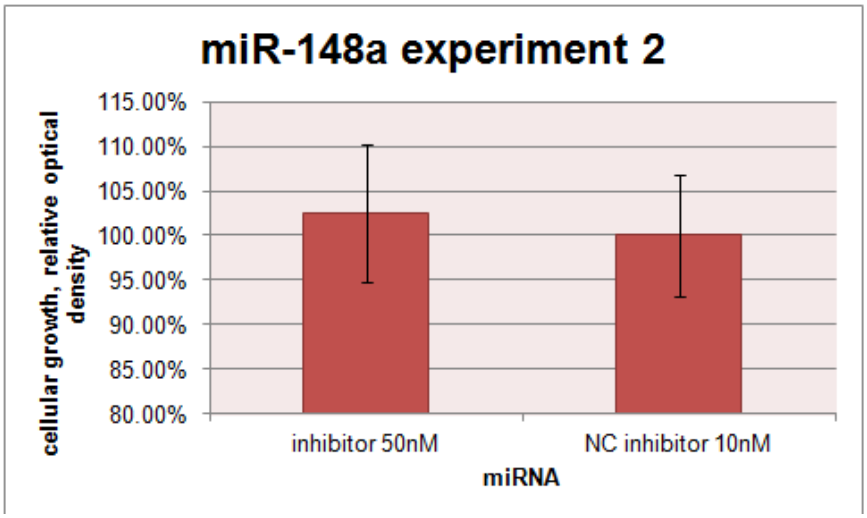
A



B

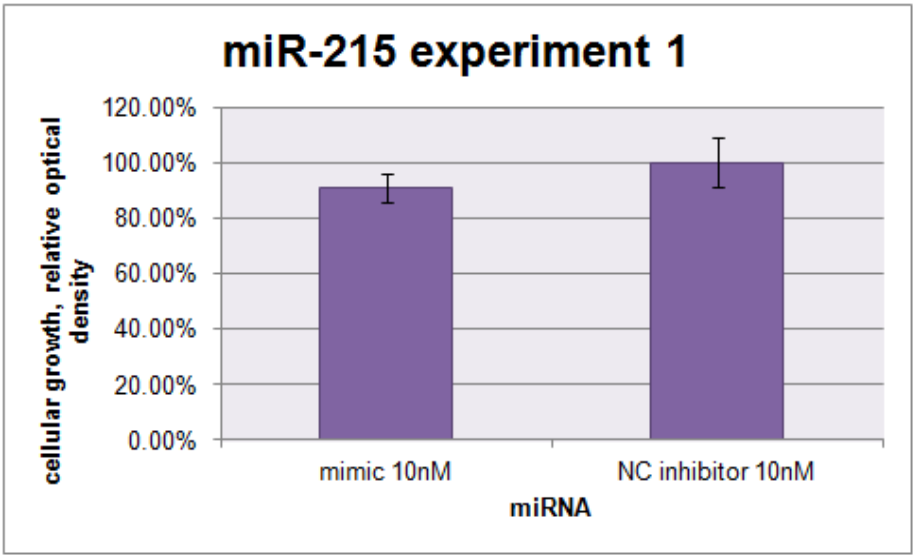


C

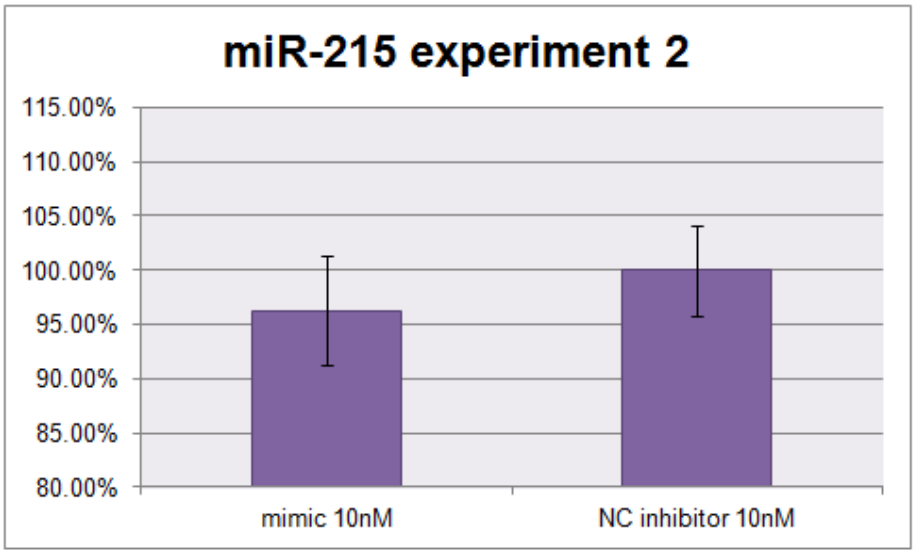


D

Figure 12. Overexpression of miR-148a inhibits cellular growth. Significance was determined using the student's t-test ($p<0.05$). 2 biological experimental repeats were performed and assay measurements were taken at a 3-hour time point. If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Measured absorbance in miRNA mimic experiments 1 and 2 (A, B) indicated that overexpression of the 10nM mimic led to a significant decrease in cellular growth. Results indicate that increasing expression of miR-148a restricts cellular growth in comparison to the negative control. Inhibition of miR-148a expression (C, D) did not lead to significant assay results.

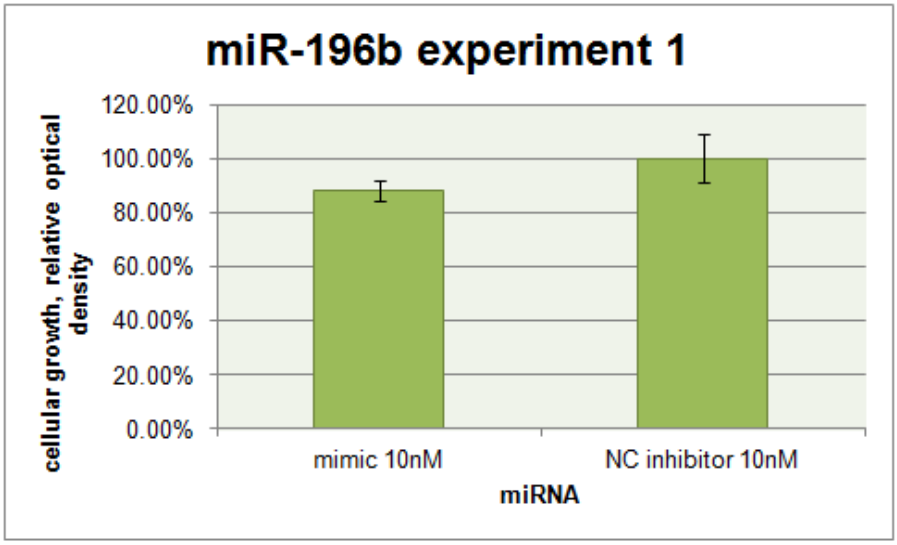


A

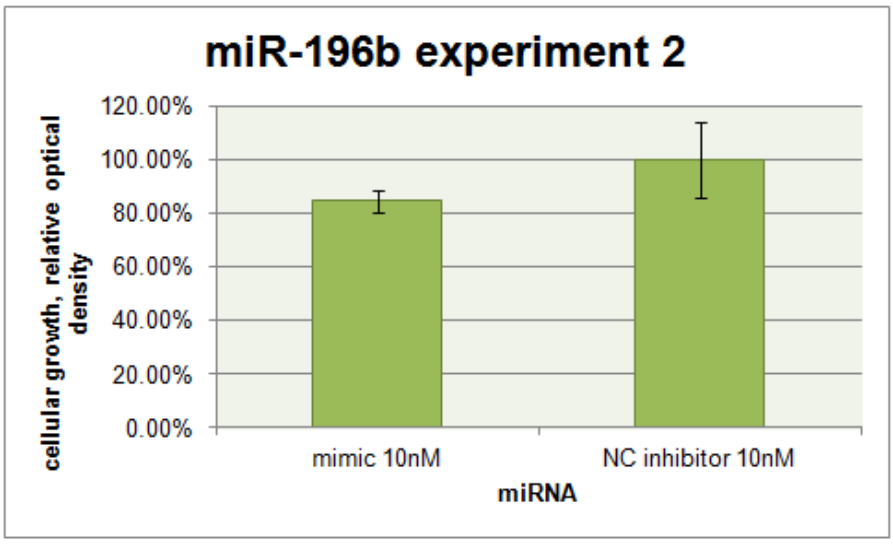


B

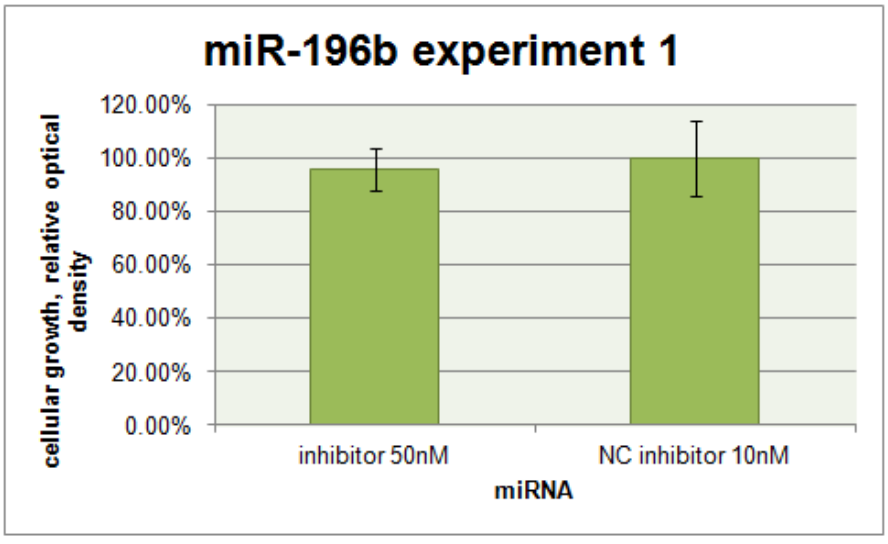
Figure 13. miR-215 overexpression has no effect on cellular growth. Significance was determined using the student's t-test ($p<0.05$). 2 biological experimental repeats were performed and assay measurements were taken at a 3-hour time point. If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Measurement of relative optical density in response to overexpression of miR-215 (A and B) at the 3-hour time point did not demonstrate an observable decrease in cellular growth in comparison to the negative control.


miR-196b experiment 1
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
mimic 10nM
NC inhibitor 10nM
miRNA

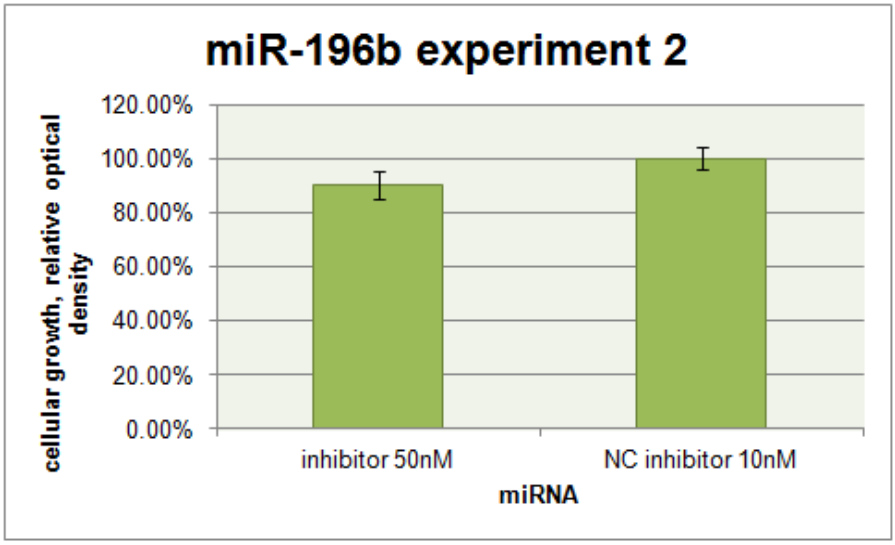
A


miR-196b experiment 2
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
mimic 10nM
NC inhibitor 10nM
miRNA

B

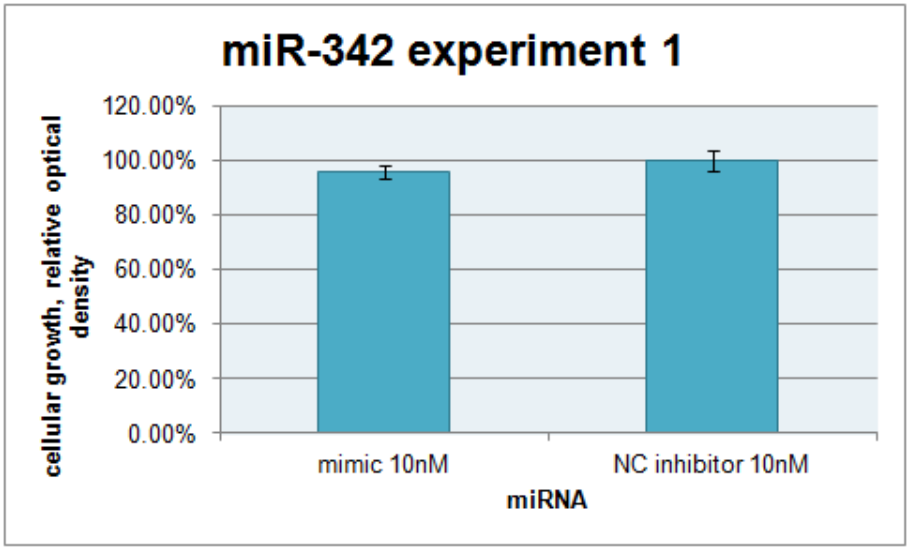

miR-196b experiment 1
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
inhibitor 50nM
NC inhibitor 10nM
miRNA

C

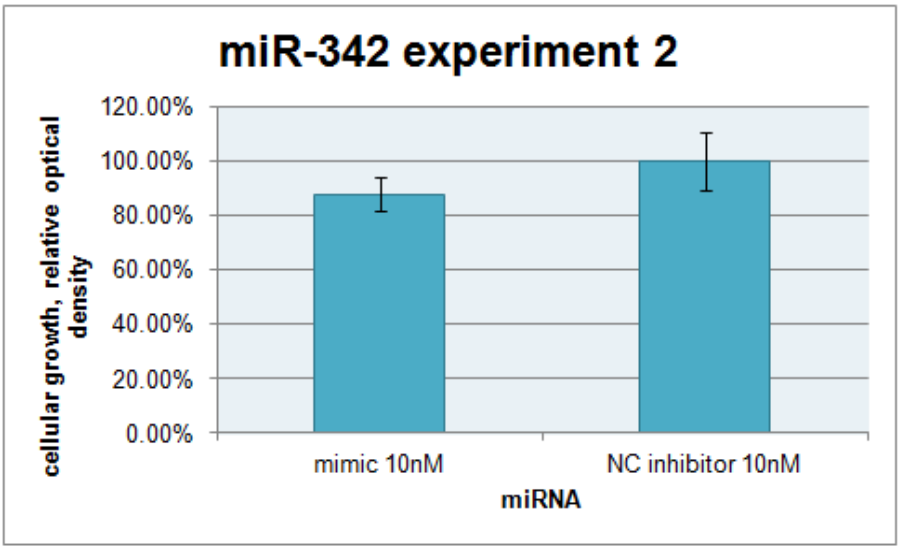


D

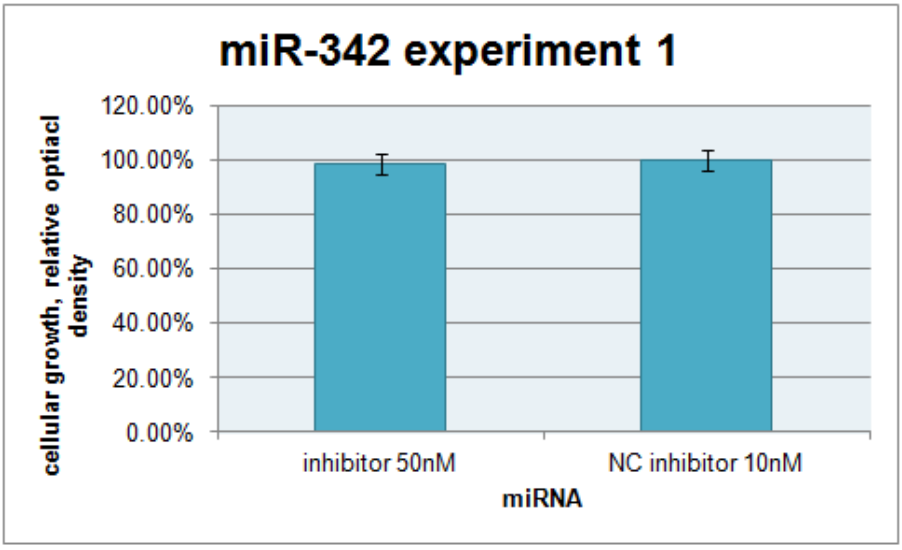
Figure 14. Overexpression or inhibition of miR-196b has no effect on cellular growth. Significance was determined using the student's t-test ($p<0.05$). 2 biological experimental repeats were performed and assay measurements were taken at a 3-hour time point. If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Measured absorbance in miRNA mimic experiments 1 and 2 (A, B) indicated that overexpression of the 10nM mimic led to a significant decrease in cellular growth. Results indicate that increasing expression of miR-196b restricts cellular growth in comparison to the negative control. Inhibition of miR-196b expression (C, D) led to a significant assay result in the second experiment but not in the first. Because trends in cellular growth measurements were the same for both the mimic and the inhibitor, it was concluded that these assay results could not be seen to have biological significance, as opposite trends for mimic and inhibitor were expected.


miR-342 experiment 1
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
mimic 10nM
NC inhibitor 10nM
miRNA

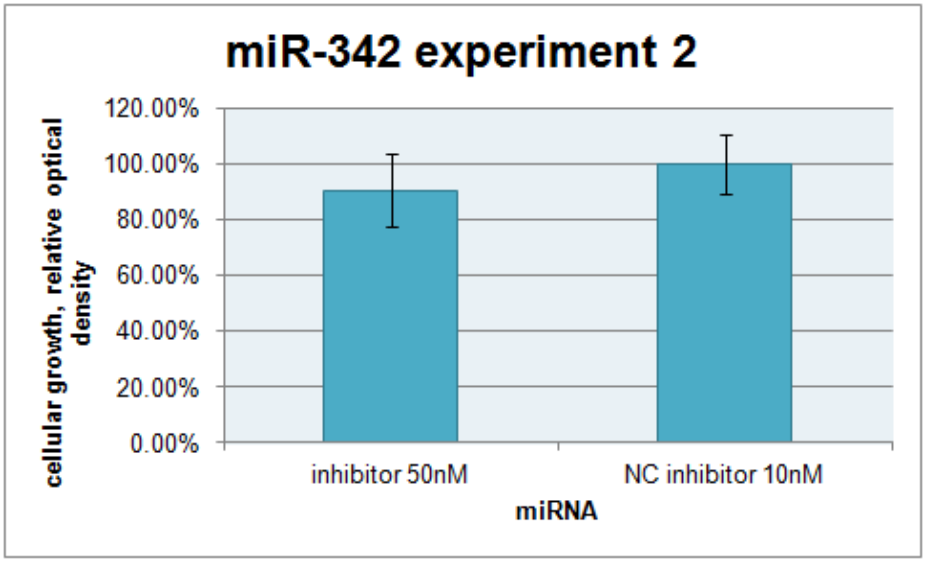
A


miR-342 experiment 2
cellular growth, relative optical density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
mimic 10nM
NC inhibitor 10nM
miRNA

B


miR-342 experiment 1
cellular growth, relative optiacl density
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
inhibitor 50nM
NC inhibitor 10nM
miRNA

C



D

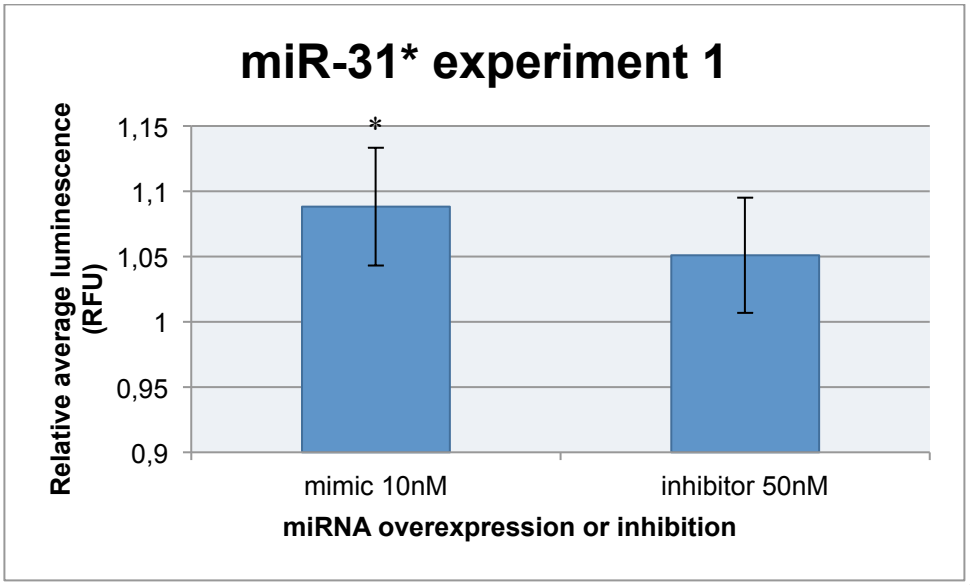
Figure 15. Overexpression of miR-342 has an inhibitory effect on HCT-116 growth. Significance was determined using the student's t-test ($p<0.05$). 2 biological experimental repeats were performed and assay measurements were taken at a 3-hour time point. If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Measured absorbance in miRNA mimic experiments 1 and 2 (A, B) indicated that overexpression of the 10nM mimic led to a significant decrease in cellular growth. Results indicate that increasing expression of miR-342 restricts cellular growth in comparison to the negative control. Inhibition of miR-342 expression (C, D) did not lead to significant assay results.

3.4 Effects of miRNA overexpression or inhibition on apoptosis activity in HCT-116 cells

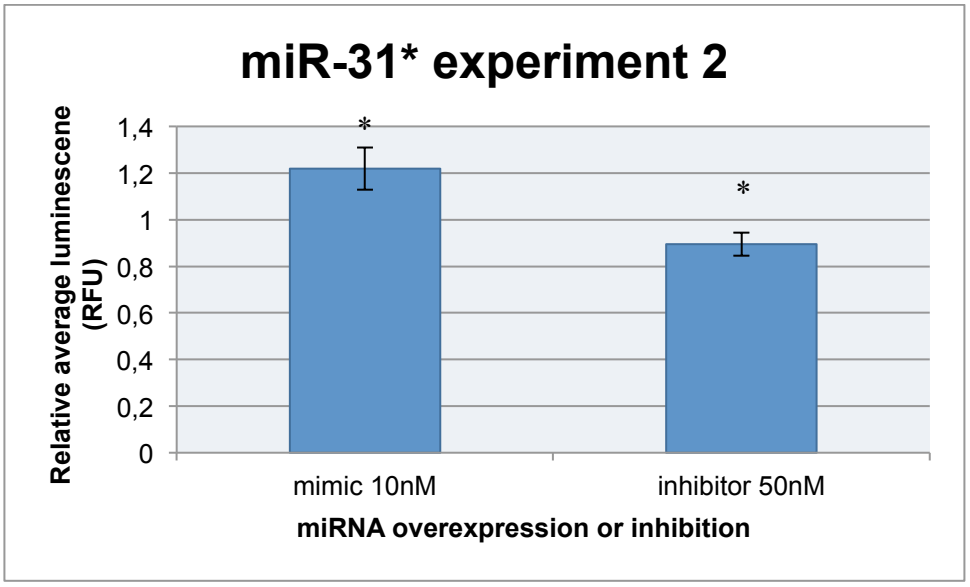
Apoptosis is one cellular feature that can influence the cellular growth rate of cells. An increased apoptosis rate induced by a particular miRNA would result in reduced cellular growth (viable cells) rates in the WST-1 assay. In addition, we were interested if a respective miRNA could induce apoptosis, as this effect can be useful for pharmaceutical targeting of this miRNA. Based on the results of the WST-1 assay in combination with a literature search, the effect of miR-31*, miR-148a and miR-215 on apoptotic activity in HCT-116 cells was assessed using the Caspase-Glo 3/7 assay, since apoptosis is ultimately mediated by caspases 3 and 7.[112] miR-196b and miR-342 were not included in this set of experiments. The principle of this luminescent assay is based on providing a luminogenic substrate containing the DEVD sequence, which can be cleaved by caspases, in turn leading to the release of a luciferase substrate, aminoluciferin.

A luciferase reaction ensues and the subsequent production of light can be measured on a luminometer. Because luminescence is proportional to caspase activity, the light measurements can be used to determine apoptotic activity within the cells, or more specifically, caspase 3 and caspase 7 activity. The AllStars Hs Cell Death Control siRNA was used as a positive control and is composed of siRNAs that target ubiquitously expressed human genes that are required for cell survival. Knockdown of these genes therefore initiates a high level of cell death.

As with the WST1 assays, all caspase assays were performed in 2 biological repeats. Once again, if data in both repeats demonstrated the same trend and were significant ($p<0.05$) when compared to the negative control, then the results for this particular miRNA were deemed significant, as the trend was independently verified in two different assays. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Because raw luminescence values varied so drastically among technical triplicates, individual data points were normalized to the negative control to account for any discrepancy as a result of the luminescence reader. Results from the WST-1 assay suggested that overexpression of miR-31* mediated the inhibition of HCT-116 cell growth; it would therefore be expected that overexpression of miR-31* might increase caspase-3/7 activity and induce apoptosis, thereby leading to reduced cellular growth rates in the WST-1 assay. The Caspase-Glo 3/7 assay demonstrated that this most likely was the case in both experiments, with the miR-31* mimic construct exhibiting a significant ($p=0.028$) observable increase in caspase activity in comparison to the corresponding miR-31* inhibitor (**Figure 16A**) and negative control (data not shown). These data suggest that miR-31* could induce apoptosis by increasing the activities of caspase-3/7 in vitro and the trend in the second experiment (**Figure 16B**) was replicated and significant ($p=0.013$). Data corresponding to the cells inhibiting miR-31* were unable to pass the significance parameter, as only 1 of the 2 independent experiments was significant ($p=0.02$) when compared to the negative control.



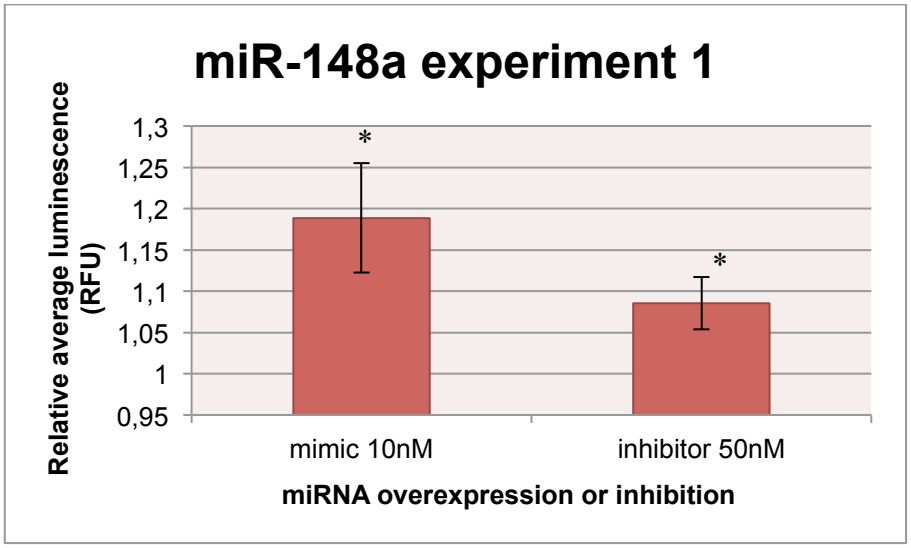
A



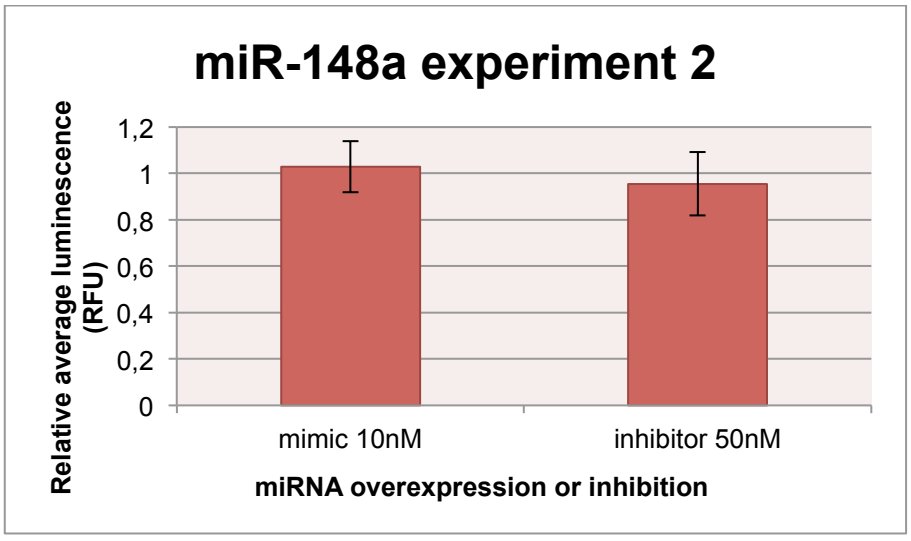
B

Figure 16. miRNA-31* has no consistent effect on caspase-3/7 activities in HCT-116 cells. 2 independent experiments were performed and assays measurements were taken at a 48-hour time point. Significance was determined using the student's t-test and significant data ($p<0.05$) are shown and denoted by an asterisk (*). If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Averaged relative luminescence values depict significant increased light production in cells expressing the miR-31* mimic in the first (A) and second (B) experiment. A cell death control (CD) was used as a positive control to ensure that both transfections and assay reagents functioned properly. In the first experiment, the miR-31* mimic led to a 1.22-fold and 1.2-fold increase in caspase activity in comparison to the negative control and miR-31* inhibitor, respectively. Cells inhibiting miR-31* did not produce significant values when compared to the negative control.

Much like miR-31*, overexpression of miR-148a using the corresponding mimic also exhibited the tendency to inhibit cell growth in HCT-116 cells as measured by the WST-1 assay. It was thus expected that the Caspase-Glo 3/7 assay would detect heightened caspase activity in cells overexpressing miR-148a and, inversely, lower luminescence signals in cells inhibiting miR-148a. These data were very similar to those from the miR-31* assay, but the miR-148a mimic was only able to induce a significant increase of caspase activity in 1 of the 2 independent assays, with a p-value of 0.012 and 0.29, respectively (**Figures 17A** and **17B**). Although the trend remained consistent, no conclusion about a biological effect of miR-148a in this assay could be reached since the significance of the result could not be replicated. Similarly, cells inhibiting miR-148a produced a significant decrease in caspase activity (**Figure 17A**) when compared to the negative control, but this effect could not be replicated in the second experiment (**Figure 17B**).



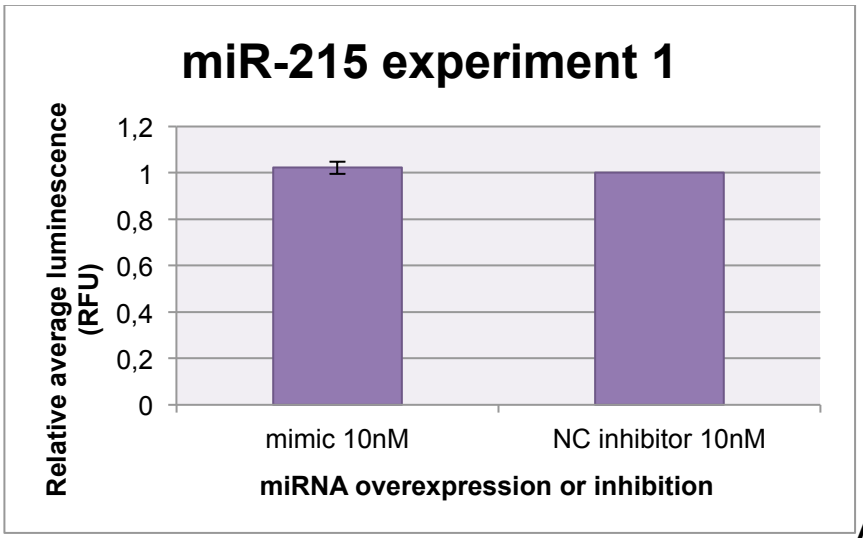
A



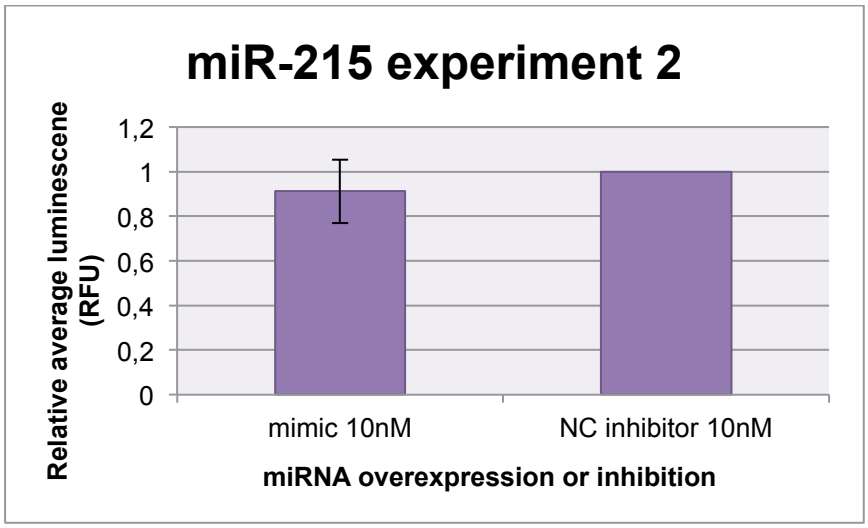
B

Figure 17. miRNA-148a has no consistent effect on caspase-3/7 activities in HCT-116 cells. 2 independent experiments were performed and assays measurements were taken at a 48 hour time point. Significance was determined using the student's t-test and significant data ($p<0.05$) are shown and denoted by an asterisk (*). If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. Averaged relative luminescence values depict significant increased light production in cells expressing the miR-148a mimic in the first experiment (A) but not in the second (B), although the trend is similar. A cell death control (CD) was used as a positive control to ensure that both transfections and assay reagents functioned properly. Cells inhibiting miR-148a significantly decreased luminescence readings when compared to the negative control in the first experiment (A) but not in the second (B).

None of the results of the apoptosis assay for miR-215 were ruled significant in either of the 2 experiments, as depicted in **Figures 18A** and **18B**, which were compared to the negative control in this case as the miR-215 inhibitor was not used.



A



B

Figure 18. Caspase activities remain unaffected after miR-215 overexpression. 2 independent experiments were performed and assays measurements were taken at a 48-hour time point. Significance was determined using the student's t-test and significant data ($p<0.05$) are shown and denoted by an asterisk (*). If data in both repeats were significant ($p<0.05$), then the entire assay for this particular miRNA was deemed significant. Results with $p<0.05$ in only 1 biological repeat were not considered to be significant. A cell death control (CD) was used as a positive control to ensure that both transfections and assay reagents functioned properly. The mimetic for miR-215 was unable to induce significant caspase activities when compared to the negative control.

3.5 Influence of miRNA on PARP expression levels and cleavage

To further investigate the therapeutic potential of our selected miRNAs regarding apoptosis, HCT-116 cells overexpressing miR-148a, miR-31* or miR-215 and inhibiting miR-148a or miR-31* were screened for PARP expression in its cleaved and uncleaved form via a western blot. PARP is a target of apoptotic caspases, which leads to cleavage and the creation of proteolytic fragments, one with a molecular weight of 89kDa.[113] Analysis of PARP cleavage could thus provide evidence of apoptosis occurring in cells as a result of cellular manipulation via miRNAs. 25µg of isolated protein was used per lane and the membrane was stripped after probing for PARP to detect the true quantity of protein loaded by detecting beta actin, which is ubiquitously expressed in cells. PARP in its uncleaved form has a molecular weight of 116kDa and was clearly detected in this western blot, as depicted by the thick bands for each sample in Figure 26. Cells expressing the miR-31* inhibitor and miR-215 mimic proved to have the highest protein concentration based on band thickness alone whereas cells overexpressing miR-148a and miR-31* demonstrated the least thickness. Cleaved PARP, on the other hand, should have a molecular weight around 89kDa and is only seen very subtly in several samples in **Figure 19**. Although the bands representing cleaved PARP were very faint across all miRNA samples, the miR-148a mimic was actually the only sample to have no band whatsoever.

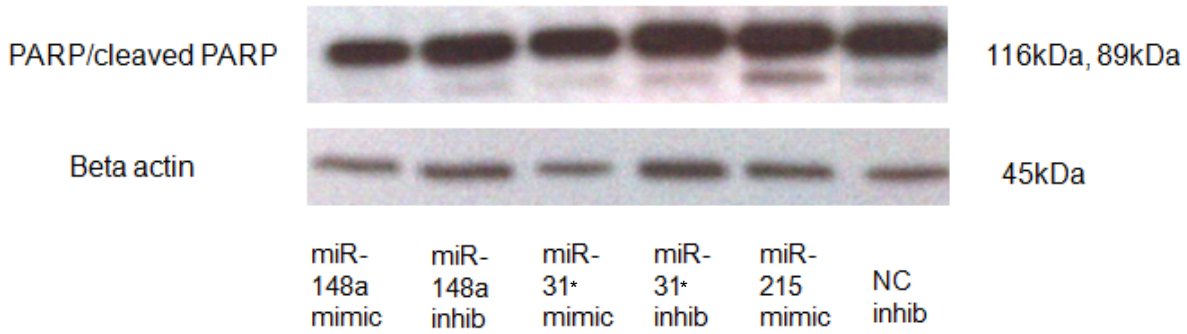


Figure 19. Western blot for detection of PARP protein in uncleaved and cleaved form in HCT-116 cells manipulated with miRNA constructs. HCT-116 cells previously individually transfected with either mimic or inhibitor constructs of miR-148a, miR-31*, miR-215 or a negative inhibitor control (NC inhib) were lysed to obtain protein 48 hours post-transfection. 25µg of isolated protein were used per well of the pre-cast gel. PARP in its uncleaved form has a molecular weight of 116kDa whereas its cleaved form can be detected at 89kDa. Beta actin (45kDa) was used as a loading control and was probed for after the membrane was stripped of PARP antibody. The quantity of protein loaded into each well appeared to be consistent with the exception of the miR-31* and miR-148a inhibitors, which both had a slightly thicker band then the rest of the samples.

Examining the blot more closely, it appears that the cells overexpressing miR-215 induced the most clear cases of apoptotic PARP cleavage in comparison to cells overexpressing miR-31* or miR-148a. While the bands for uncleaved PARP are comparable for all cells overexpressing the miRNA mimics, there is a clear distinction between cleaved PARP at 89kDa. Cells overexpressing miR-215 produced a much denser, darker band for PARP in its cleaved form in comparison to the cells overexpressing miR-31* or miR-148a, which had protein bands with less than half of the intensity of the miR-215 sample. Figure 14 shows that cells inhibiting miR-31* and miR-148a exhibited a stronger cleaved PARP protein concentration in comparison to their mimic counterparts. However, the cells expressing or inhibiting miR-31* and miR-148a constructs did not produce band intensities stronger than that of the negative control at 89kDa. The beta actin bands at 45kDa in the lower half of the image seem to represent consistent band intensities and therefore consistent protein loading with the exception of cells expressing the miR-31* and miR-148a inhibitors, which both had a band thickness slightly greater than the others. With the exception of these samples, it is assumed that roughly 25µg of protein was equally loaded into each lane while performing the western blot.

3.6 Gene expression of apoptosis-related markers as a result of miRNA overexpression or inhibition

To further verify the results obtained from the WST-1 and caspase 3/7 functional cellular assays, RT-PCR was performed to detect expression levels of two apoptosis-related markers, BAX and Bcl-2, after cellular manipulation with miRNA mimics and inhibitors. We were interested in whether the gene expression pattern reflects and confirms the functional cellular assays. BAX is a member of a protein family which shares homology with Bcl-2 proteins that are known for their involvement in the regulation of apoptosis.[114] Overexpression of Bax should promote cell death. Overexpression of Bcl-2 from this Bcl-2 family, on the other hand, enhances cell survival by protecting the cells from apoptosis-inducing stimuli.[114] Fold changes were calculated after normalizing to the GAPDH housekeeping gene and negative control via the ddCt method. Fold changes above or equal to 1.2 were considered significant and upregulated whereas fold changes equal to or below 0.8 were considered significant and downregulated after performing a student's t-test.

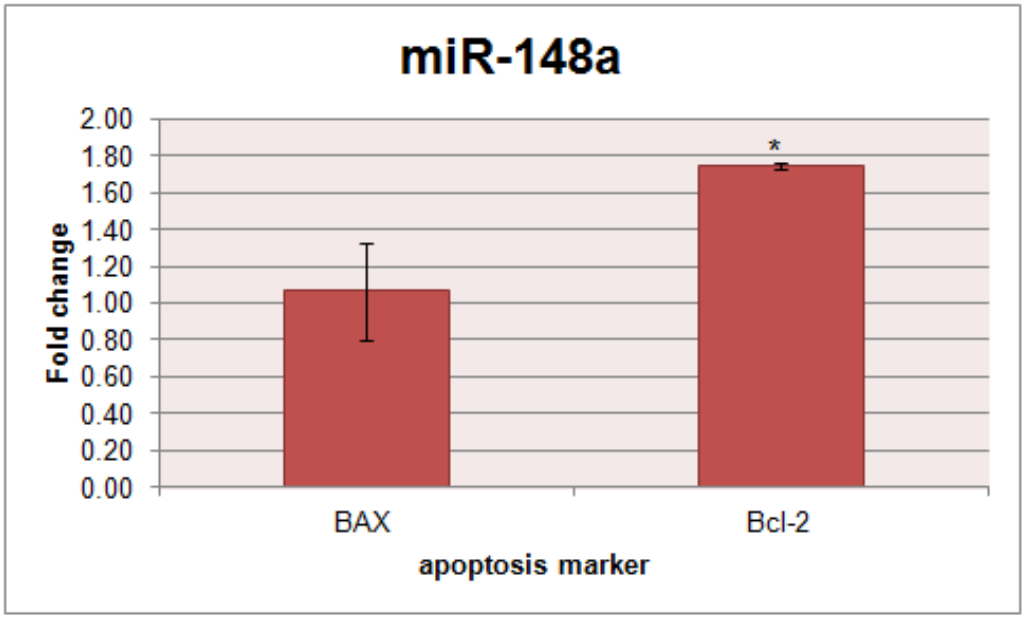


Figure 20. Overexpression of miR-148a significantly increases Bcl-2 expression. Significance was determined using the student's t-test. Here, the miR-148a mimic led to a 1.75-fold increase in Bcl-2, the anti-apoptotic marker. BAX expression remained relatively unchanged after overexpression of miR-148a. The asterisk (*) indicates that the value has a significant fold change and t-test result ($p<0.05$) for this particular marker.

Overexpression of miR-148a in HCT-116 cells demonstrated a 1.75-fold increase in Bcl-2 expression as seen in **Figure 18**. The pro-apoptotic marker BAX did not yield significant results, as the fold change remained 1.06, indicating no difference in expression when compared to the negative control. No RT-PCR data is available for these genes regarding inhibition of miR-148a, as it was previously determined that endogenous expression of miR-148a in the HCT-116 cell line is much too low to yield significant results detectable by RT-PCR upon inhibition.

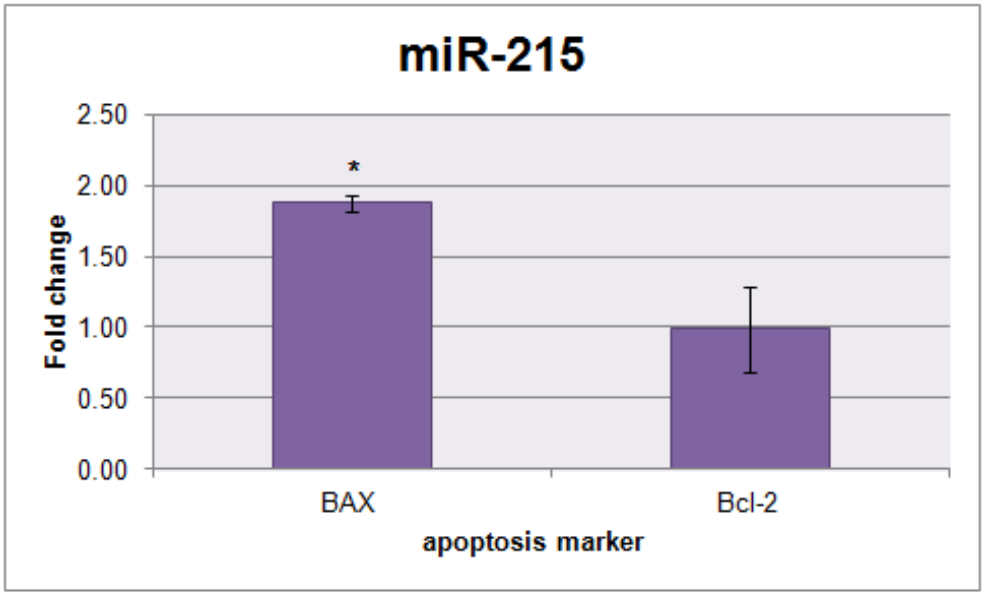


Figure 21. Overexpression of miR-215 significantly increases BAX expression. Significance was determined using the student's t-test. Overexpression of miR-215 led to a significant increase in expression (about 1.87-fold) in the pro-apoptotic gene marker BAX. Bcl-2 expression remained unaffected. The asterisk (*) indicates that the value has a significant fold change and t-test result ($p<0.05$) for this particular marker.

miR-215 overexpression was able to demonstrate a 1.87-fold increase for BAX expression in HCT-116 cells as displayed in **Figure 19**, whereas Bcl-2 expression remained unchanged (fold change (FC)=0.986). For similar reasons as with the miR-196b data to follow, there is no inhibitor data for miR-215 regarding these two apoptosis markers.

miR-196b was the only miRNA mimic which did not yield any change in either BAX or Bcl-2 detection levels after overexpression in HCT-116 cells. In fact, **Figure 22** shows nearly identical expression levels for both markers, just under the 1.2-fold change cutoff level.

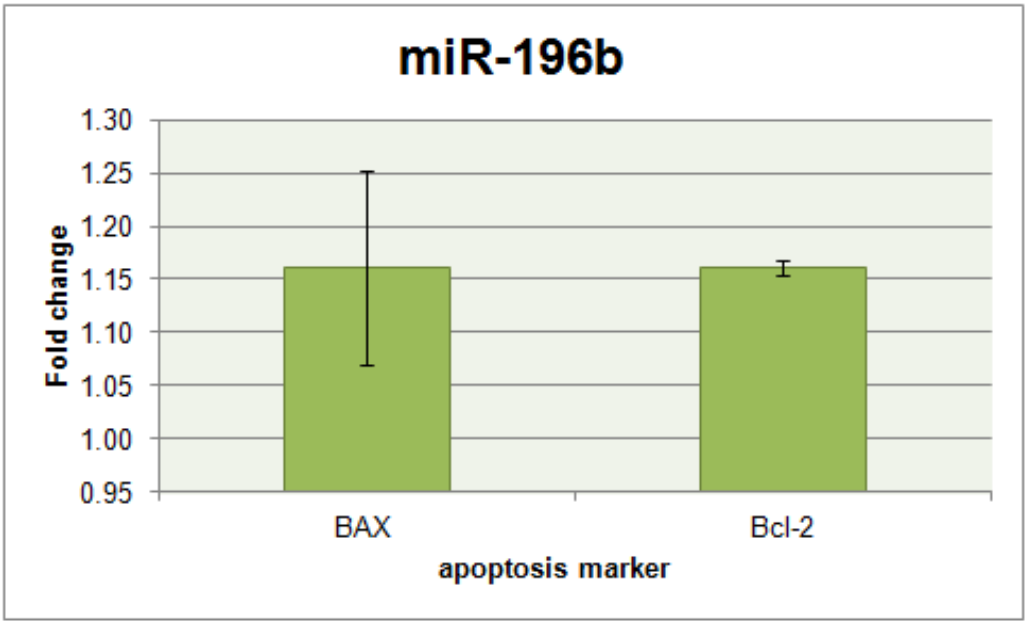


Figure 22. miR-196b overexpression does not lead to change in apoptosis marker expression. Significance was determined using the student's t-test. miR-196b overexpression did not produce significant changes in BAX or Bcl-2 expression, as neither of these values reached the 1.2-fold change cutoff. The asterisk (*) indicates that the value has a significant fold change and t-test result ($p<0.05$) for this particular marker.

Unlike the previous miRNAs, miR-31* and miR-342 inhibitors were administered to HCT-116 cells in parallel to their mimic forms. The main reason for using both mimetics and inhibitors only for these two miRNAs was that the expression values of these miRNAs were high enough that we could expect effects by inhibitors with a higher probability (see also **Figure 7**). Interestingly, overexpression of the miR-31* mimic led to a 1.43-fold increase in BAX expression as well as a 0.707-fold decrease in Bcl-2 expression, as depicted by the dark columns in **Figure 23**. Although the data corresponding to Bcl-2 expression after miR-31* overexpression was not significant after conducting a t-test, overexpression of this miRNA still proved to have the opposite effect on Bcl-2 as it did on BAX expression. Data corresponding to the miR-31* inhibitor was not as clear and neither BAX nor Bcl-2 values passed the significance threshold. Similarly, overexpression of miR-342 upregulated BAX expression 1.711-fold but seemed to have no impact on Bcl-2 levels in HCT-116 cells, as seen in Figure 19. Results corresponding to the miR-342 inhibitor, on the other hand, were not as expected. Inhibition of miR-342 (**Figure 24**, light blue columns) did not quite lead to downregulation of BAX expression but instead led to a slight downregulation in Bcl-2, the anti-apoptotic gene marker. Although inhibition of miR-342 decreased BAX expression in comparison to miR-342 overexpression, data did not pass the t-test regarding significance nor did it reach the 0.8-fold cutoff level.

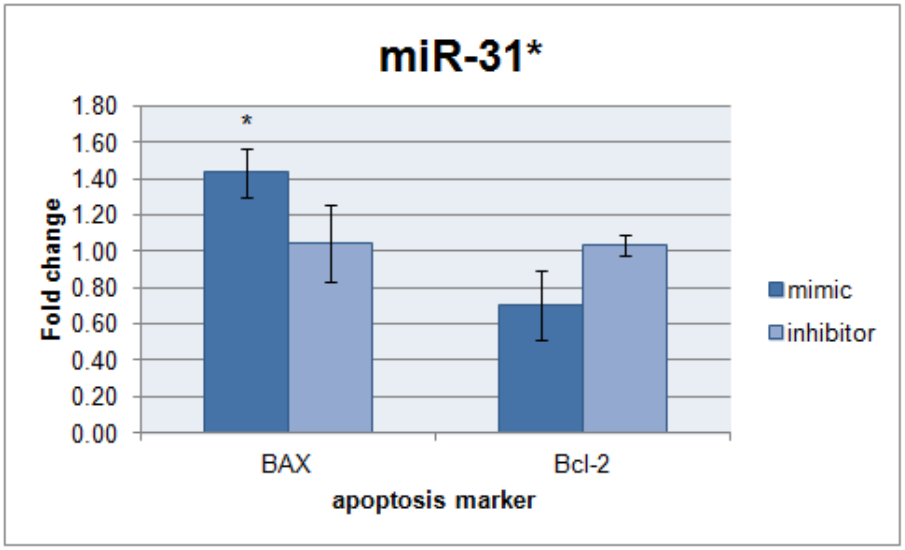


Figure 23. Overexpression of miR-31* increases BAX expression and lowers expression of Bcl-2. Significance was determined using the student's t-test. Overexpression of miR-31* led to a 1.43-fold increase in BAX expression as well as a 0.707-fold decrease in Bcl-2 expression (dark columns). Inhibition of miR-31* did not seem to significantly alter either BAX or Bcl-2 expression, as represented by the light gray columns. The asterisk (*) indicates that the value has a significant fold change and t-test result for this particular marker.

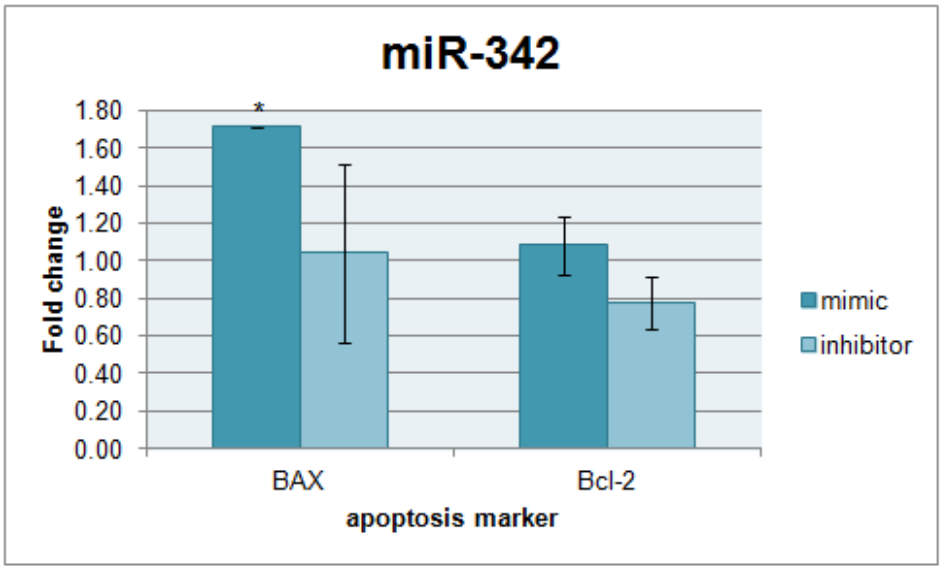


Figure 24. Overexpression of miR-342 leads to an increase in BAX expression. Significance was determined using the student's t-test. Overexpression achieved by introduction of a miR-342 mimic caused significant upregulation of BAX expression but had no significant impact on Bcl-2 expression. Conversely, suppression of miR-342 expression via the miR-342 inhibitor led to downregulation in Bcl-2 but the change was insignificant. The asterisk (*) indicates that the value has a significant fold change and t-test result for this particular marker.

3.7 Gene expression of proliferation and markers for epithelial-mesenchymal transition as a result of miRNA overexpression or inhibition

In order to further characterize the selected miRNAs for their therapeutic potential, RT-PCR was performed to assess the expression levels of several proliferation and epithelial-mesenchymal transition (EMT) markers after HCT-116 cellular manipulation. The genes profiled were cyclin D1 (CCND1), E-cadherin (CDH1), vimentin (VIM) and cyclin-dependent kinase inhibitor 1B (CDKN1B). Overexpression of these selected genes could represent alterations in cell cycle progression, attachment, proliferation and invasion, ultimately leading to tumorigenesis. Once again, fold changes were calculated after normalizing to the GAPDH housekeeping gene and negative control via the ddCt method. Fold changes above or equal to 1.2 were considered significant and upregulated whereas fold changes equal to or below 0.8 were considered significant and downregulated after performing a student's t-test.

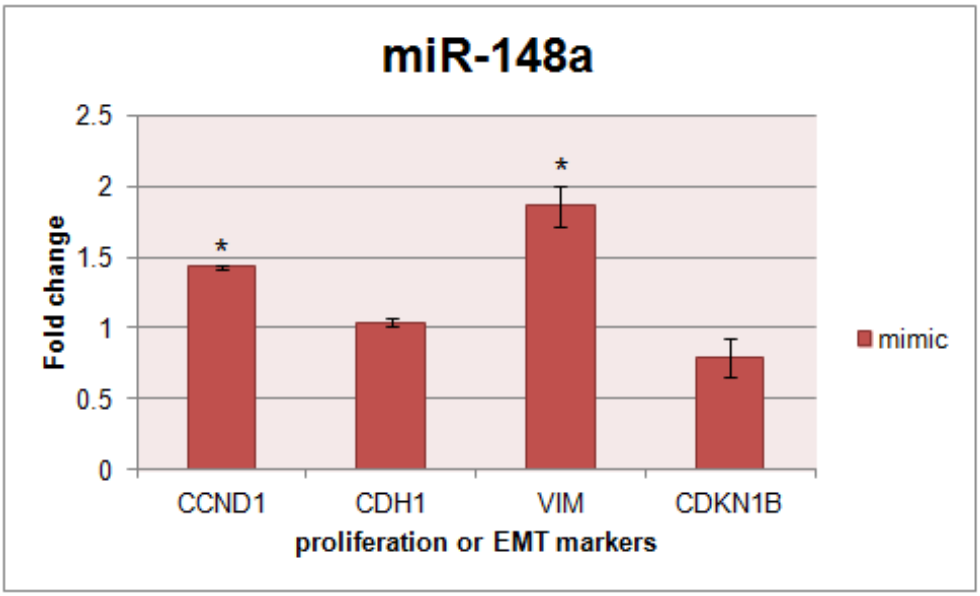


Figure 25. The miR-148a mimic alters expression in CCND1 and VIM. Overexpression of miR-148a led to significant gene upregulation in CCND1 and VIM. CCND1 increased 1.43-fold in comparison to the negative control whereas VIM exhibited a 1.86-fold change in expression. CDKN1B was slightly downregulated in HCT-116 cells overexpressing miR-148a. The asterisk (*) indicates that the value is significant for this particular marker after conducting a t-test ($p<0.05$).

Overexpression of the miR-148a mimic exhibited influence on the expression of the several genes in the selected gene panel, as depicted in **Figure 25**. CCND1 and VIM were upregulated 1.43-fold and 1.86-fold, respectively, in the manipulated HCT-116 cell line.

Overexpression of miR-148a led to a small yet insignificant decrease in CDKN1B expression with a fold change of 0.793. The miR-215 mimic in **Figure 26** shows significant alterations in expression of CCND1, CDH1 and CDKN1B. Overexpression of miR-215 slightly upregulated CCND1 and CDH1, with a 1.2-fold and 1.22-fold change in gene expression, respectively. A dramatic decrease was seen in CDKN1B expression with a fold change of 0.27, suggesting downregulation of the gene. A nearly 2-fold increase in expression of vimentin was the only significant change in cells overexpressing miR-196b as seen in **Figure 27**.

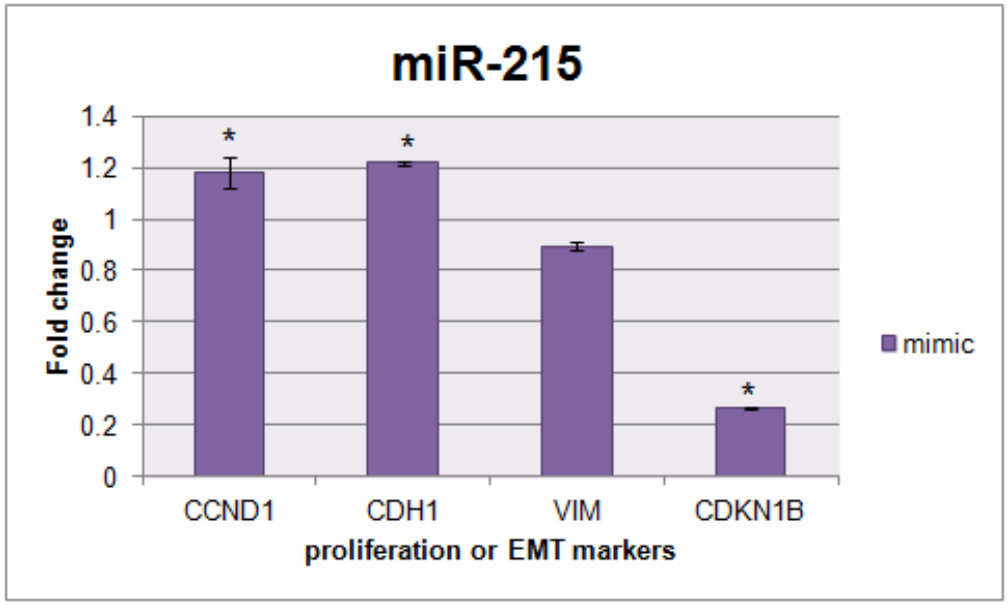


Figure 26. The miR-215 mimic alters expression in CCND1, CDH1 and CDKN1B. Overexpression of miR-215 led to significant upregulation of CCND1 and CDH1 in comparison to the negative control. CDKN1B was very significantly downregulated with a 0.27-fold decrease in gene expression. The asterisk (*) indicates that the value is significant for this particular marker after conducting a t-test ($p<0.05$).

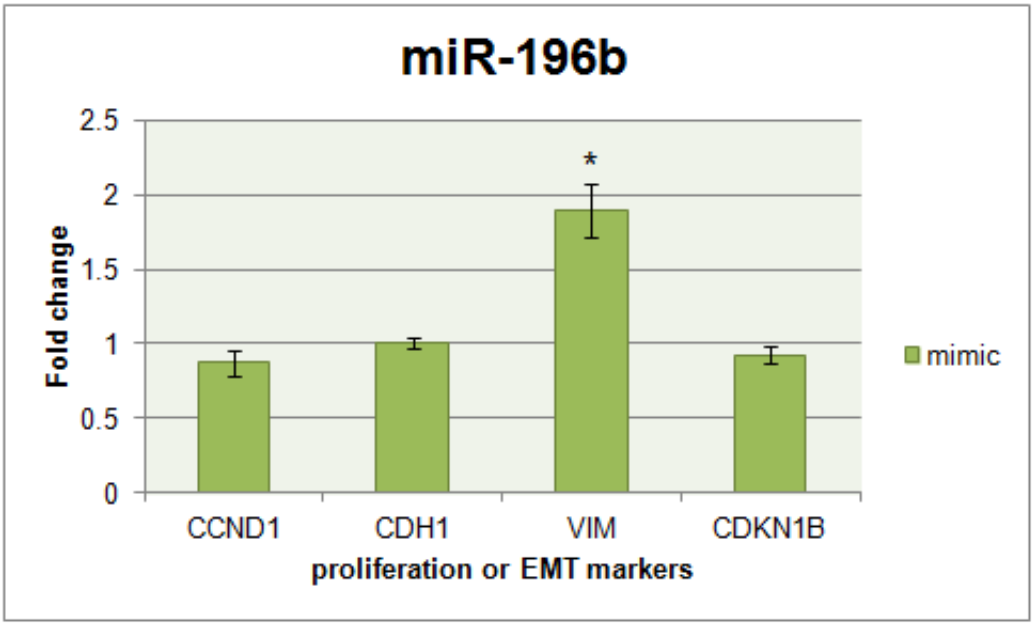


Figure 27. The miR-196b mimic upregulates expression of VIM. miR-196b overexpression only affected one gene in the panel, upregulating vimentin about 1.9-fold when compared to the negative control. The asterisk (*) indicates that the value is significant for this particular marker after conducting a t-test ($p<0.05$).

miR-31* and miR-342 were the only two of the five miRNAs to be tested with their inhibitors in this RT-PCR gene panel. Surprisingly, the miR-31* mimic appeared to have little effect on most of the selected markers, as inhibition of miR-31* had no significant effect. **Figure 28** shows that overexpression of miR-31* using the mimic only had an effect on one gene, leading to a 1.3-fold increased in the expression of CDKN1B. Overexpression of miR-342, on the other hand, led to a 0.743-fold downregulation of CCND1 as seen in **Figure 29** whereas inhibition of miR-342 caused vimentin expression to increase 1.3-fold in HCT-116 cells.

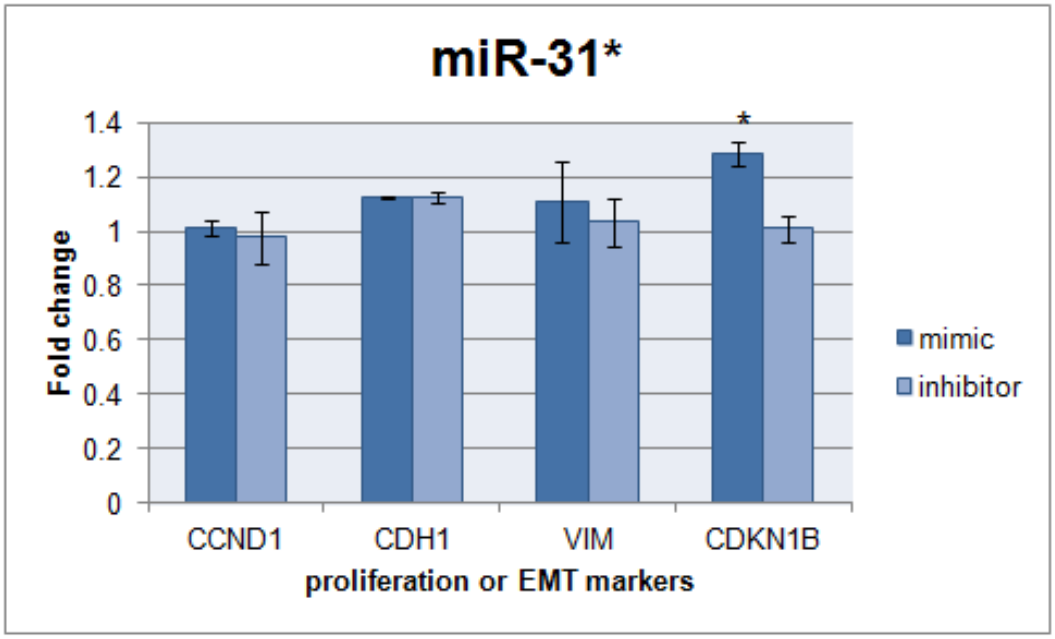


Figure 28. The miR-31* mimic upregulates CDKN1B expression. miR-31* overexpression led to a 1.3-fold increase in CDKN1B expression in HCT-116 cells. Inhibition of miR-31* seemed to have no effect on the rest of the proliferation and EMT markers in HCT-116 cells under the given conditions. The asterisk (*) indicates that the value is significant for this particular marker after conducting a t-test ($p<0.05$).

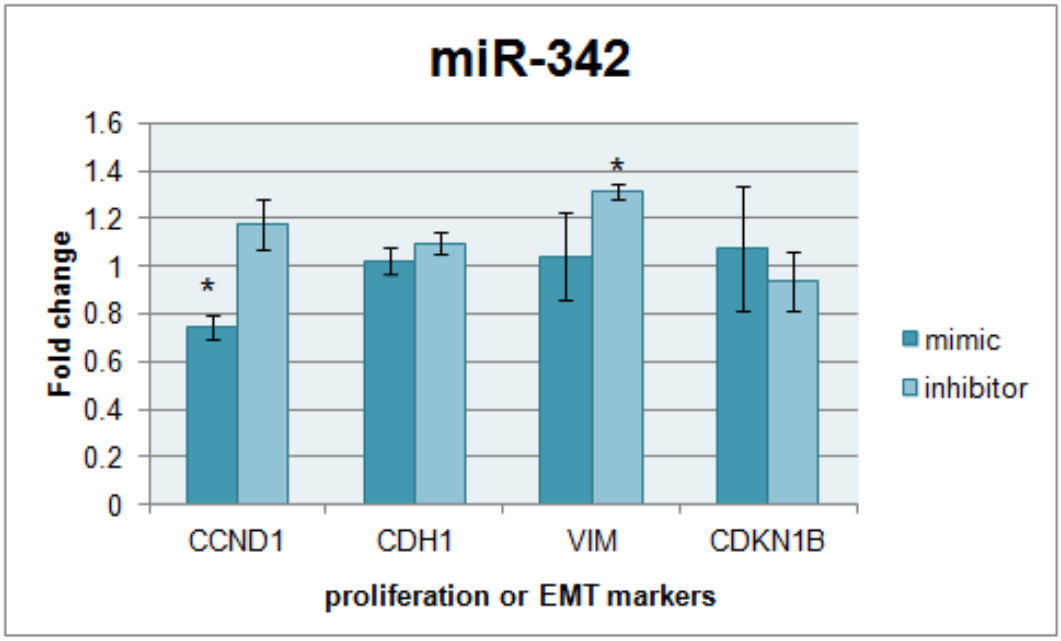


Figure 29. Effects of miR-342 overexpression and inhibition on proliferation and EMT markers. miR-342 overexpression caused a 0.743-fold decreased in CCND1 expression. Inhibition of miR-342 demonstrated a 1.31-fold upregulation of vimentin. The asterisk (*) indicates that the value is significant for this particular marker after conducting a t-test ($p<0.05$).

3.8 miRNA expression in adherent cells compared to tumor spheres

Total RNA from tumor spheres derived from the CRC cell lines HCT-116, HT-29 and Caco-2 (kindly provided by Dr. Martin Pichler) was used for further investigation. All of our 5 miRNAs of interest were measured via RT-PCR in these individual cell lines to determine any link between the miRNAs and potential stem cell-like characteristics. miRNA expression was also measured in adherent cells to serve as a reference. Fold changes above our equal to 1.2 and below or equal to 0.8 were considered to be meaningful after performing a t-test.

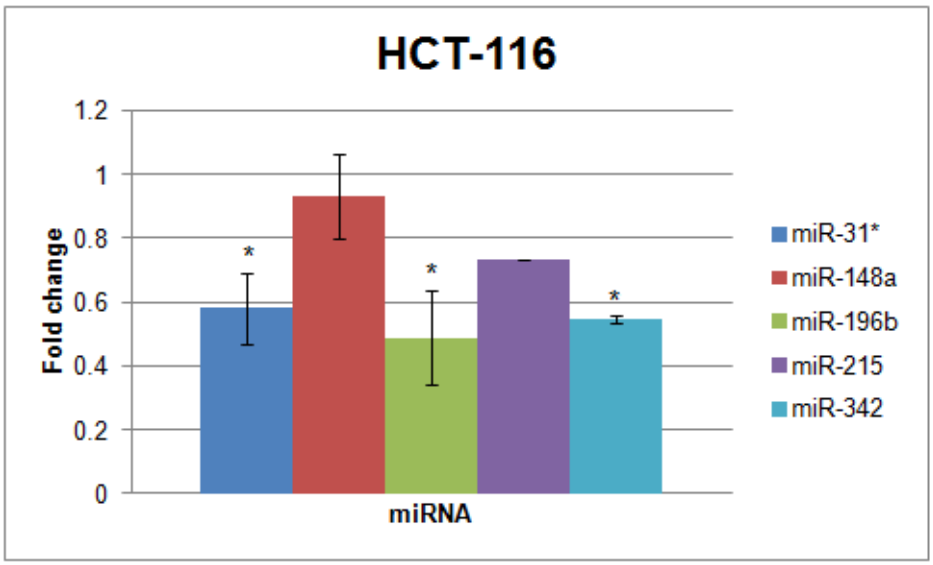


Figure 30. miRNA expression in HCT-116 colorectal carcinoma tumor spheres. 3 of the 5 measured miRNAs were significantly downregulated in HCT-116 tumor spheres compared to the adherent growing parental cells. miR-148a and miR-215 overexpression did not result in a significant fold change when compared to reference cells. The asterisk (*) indicates that the value is significant ($p<0.05$) for this particular miRNA.

miRNA expression levels in HCT-116 tumor spheres were significantly lower than in their adherent cell counterparts as pictured in **Figure 30**. miR-196b demonstrated the lowest fold change of 0.488, whereas as miR-31*, miR-215 and miR-342 had fold changes of 0.581, 0.733 and 0.546, respectively. miR-148a did not exhibit any observable change in expression when compared to adherent cells. Results for miRNA expression in HT-29 tumor spheres are depicted in **Figure 31**, showing that 4 of the 5 miRNAs had significantly different expression levels when compared to adherent cells. miR-215 was the only example of upregulation, exhibiting a fold change of 2.92. miR-31*, miR-196b and miR-342 expression was downregulated with fold changes of 0.576, 0.705 and 0.730, respectively. Caco-2 tumor sphere miRNA expression

shown in **Figure 32** clearly depicts the most dramatic changes in RT-PCR detection levels. miR-31*, miR-148a and miR-215 were all strongly upregulated with fold changes of 10.63, 23.73 and 56.62, respectively.

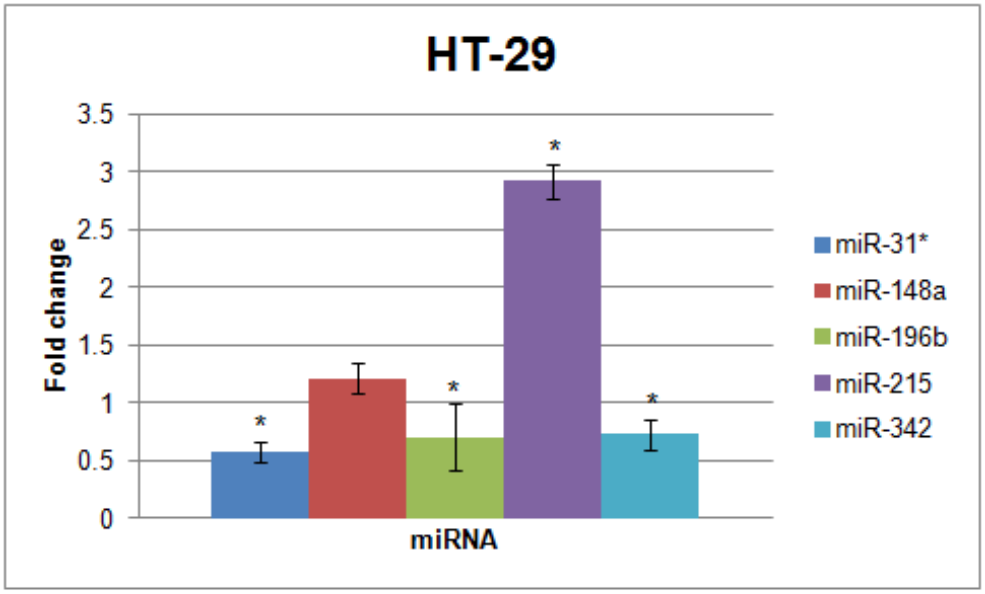


Figure 31. miRNA expression in HT-29 colorectal adenocarcinoma tumor spheres. 4 of the 5 miRNAs had significant fold changes in HT-29 tumor sphere cells. miR-148a was slightly upregulated yet insignificant whereas miR-215 demonstrated an almost 3-fold increase in expression when compared to adherent cells. miR-31*, miR-196b and miR-342 all had decreased expression in comparison to the reference. The asterisk (*) indicates that the value is significant for this particular miRNA.

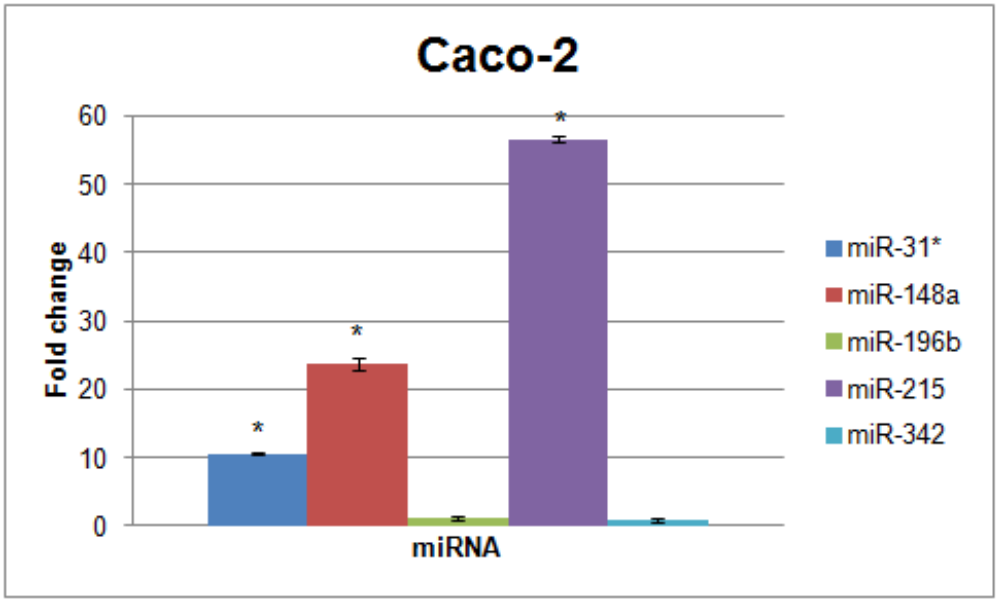


Figure 32. miRNA expression in Caco-2 colorectal adenocarcinoma tumor spheres. 3 of the 5 miRNAs demonstrated significant upregulation in Caco-2 tumor sphere cells. miR-215 exhibited nearly a 60-fold increase in expression when compared to the adherent control cells. miR-196b and miR-342 did not have significant changes in expression in comparison to their references. The asterisk (*) indicates that the value is significant for this particular miRNA.

3.9 Influence of miRNA on expression of stem cell markers

Encouraged by our findings that some miRNAs are significantly differentially expressed in the tumor spheres (i.e. putative cancer stem cell enriched model), we aimed to explore the influence of miRNA manipulations on stem cell marker expression. Several cancer stem cell markers were chosen for Western blot/protein expression analysis in HCT-116 cells to assess the potential link of cancer stem cells and miRNA expression.

Once again, 25µg of protein was loaded per lane and beta actin was used to verify that equal amounts of protein were used per sample to prevent misinterpretation of the results. Because Nanog, Oct4 and beta actin have similar molecular weights, the membrane needed to be stripped and re-probed several times in order to avoid overlapping of the bands.

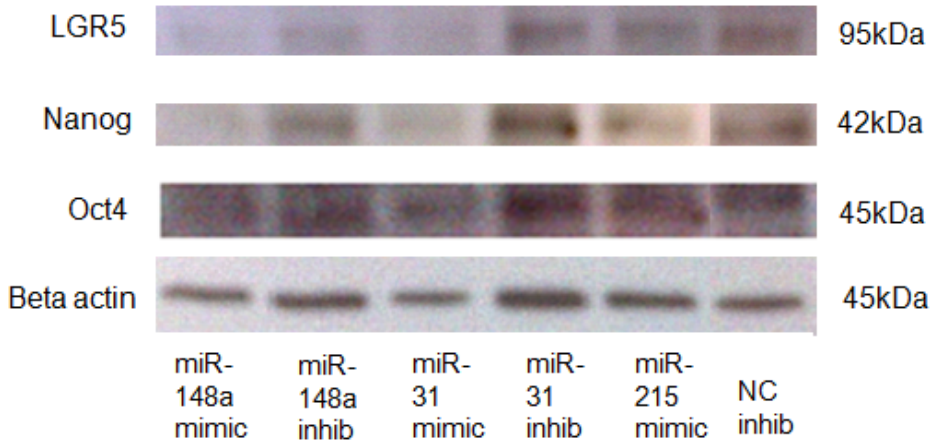


Figure 33. Western blot for detection of stem cell markers in HCT-116 cells manipulated with miRNA constructs. HCT-116 cells previously individually transfected with either mimic or inhibitor constructs of miR-148a, miR-31*, miR-215 or a negative inhibitor control (NC inhib) were lysed to obtain protein 48 hours post-transfection. 25µg of isolated protein were used per well of the pre-cast gel. Stem cell markers LGR5, Nanog and Oct4 were probed for and detected at 95kDa, 42kDa and 45kDa, respectively. Beta actin (45kDa) was used as a loading control and was probed for after the membrane was stripped of other previous antibodies. The quantity of protein loaded into each well appeared to be consistent with the exception of the miR-31* and miR-148a inhibitors, which had a slightly stronger band then the rest of the constructs.

Western blotting results of this panel of cancer stem cell markers were not quite as clear as the results for PARP protein expression. **Figure 33** displays the protein bands indicating expression levels for LGR5, Nanog and Oct4 at 95kDa, 42kDa and 45kDa, respectively. Beginning with the 95kDa protein LGR5 in the first row of the image panel, it appears that across all three miRNAs that inhibition of the respective miRNA led to an increase in LGR5 protein expression when compared to the samples pertaining to the miRNA mimic construct. Results for the miR-31* sample seem to be most significant when compared to the negative control. LGR5 expression for cells inhibiting miR-31* shows a more concentrated band than the negative control and is distinctly more intense than the neighboring band representing the miR-31*-overexpressing cells. The band representing LGR5 protein expression after overexpression of miR-215 resembles that of the negative control. One aspect of this interpretation which proves problematic is that the beta-actin loading control band is actually stronger in the miRNA mimetic group, making it very difficult to reach a conclusion.

Nanog expression at 42kDa had a similar pattern as that of LGR5, with inhibition of the selected miRNAs leading to higher protein expression than seen in constructs overexpressing the respective miRNA. Cells inhibiting miR-31* demonstrated a similar protein band thickness was observably stronger than that of the negative control. Cells overexpressing miR-31* produced a protein expression less than half of the protein expression corresponding to the miR-31* inhibitor.

Results pertaining to the Oct4 antibody are the most difficult to interpret, as the bands at 45kDa are not tight and focused as in the LGR5 and Nanog scenarios. Despite this fact, it still appears as if the expression pattern remains consistent with the two previous cases in it that inhibition of the selected miRNAs leads to an increase in Oct4 protein expression. Although the bands are blurry, it even appears as if Oct4 expression is slightly higher in the miR-148a inhibitor construct than in the miR-148a mimic construct or in the negative control sample. The distinction between Oct4 protein expression in the miR-31* mimic and miR-31* inhibitor samples is the strongest amongst the three miRNAs. Inhibition of miR-31* in cells led to roughly a 2-fold increase in Oct4 band thickness in comparison to cells overexpression miR-31*. There is only a very subtle difference, if at all, between the miR-215 mimic and negative control samples of miR-215. The sample overexpressing miR-215 could potentially have a slightly darker protein band than the negative control to its right in **Figure 33**, but the results are not clear enough to draw an accurate conclusion.

Again, for all these Western blot bands, one limitation is the unequal distribution of the loading control, which makes an interpretation in some cases difficult.

4 DISCUSSION

Treatment decisions for CRC are still largely based on the anatomical location of the tumor in combination with the stage of disease. Current limitations of these selected therapies have brought about a demand to discover new technologies to add to the arsenal of therapies for managing this disease. Emerging evidence from studies involving manipulation of miRNAs provides arguments for harnessing the therapeutic potential of these molecules for the treatment of cancer. For this reason, current research from both academic and industrial fronts has focused on bringing the use of miRNAs from "benchtop to bedside". miRNAs belong to the class of RNAi-based therapeutics and are able to modulate multiple targets simultaneously, unlike protein inhibitors, which only intercept individual targets. Modulation of these molecules has the potential of altering hundreds of transcripts, which could eventually lead to the controlled regulation of entire deregulated pathways or gene programs. In addition, specific features of these molecules, such as stability, ease of control, simple detection and tissue specificity, can help personalize cancer treatment.[115] The two current technologies for investigating miRNA therapeutic approaches are downregulation of oncogenic miRNAs via the use of miRNA antagonists and the upregulation of tumor suppressor miRNAs via the use of miRNA mimics. Ultimately, manipulation of miRNA should ideally restore a normal (nonpathogenic) miRNA profile to eradicate the disease state.[100,116,117]

In this thesis, we hoped to manipulate targeted miRNA expression levels in the HCT-116 CRC cell line to ascertain which of these molecules could serve as a potential candidate or target for miRNA-based cancer therapy. We sought out to evaluate the pharmacological outcomes via overexpression and inhibition assays of the following miRNAs: miR-31*, miR-342, miR-215, miR-196b and miR-148a. In general, the miRNA mimics provided more significant results in comparison to their inhibitor counterparts, ultimately making a conclusive interpretation of the data more difficult, as the mimic and inhibitor should induce opposite effects upon introduction into the cells in order to confirm the consistency of the observed phenotype. Overall, the data in this thesis suggested that these miRNA candidates functioned as pro-apoptotic molecules in this particular *in vitro* model, with perhaps the exception of miR-148a. Because the main goal of this thesis was to deduce which miRNAs could serve as anti-cancer molecules, the following discussion is divided so that results corresponding to each miRNA could be expanded upon individually.

4.1 miR-31

Of the five investigated miRNAs, effects of the miR-31* mimic offered the most promising data. Results of the WST-1 cellular growth assay demonstrated the decrease in cell viability of HCT-116 cells after overexpression of miR-31*. Although for our purposes we decided that two independent assays needed to confirm the biological observation for it to be deemed significant, it is worth noting that in one of the two independent WST-1 experiments, inhibition of miR-31* led to a statistically significant ($p=0.023$) increase in cell viability and therefore indicated an increase in cellular growth capability. To further support this evidence that overexpression of miR-31* can lead to inhibition of cellular growth, we tested the effect of overexpression and inhibition of miR-31* on caspase activity. In both independent biological experiments, we were able to demonstrate a significant increase in activity of the caspase cascade as measured via the selected caspase assay upon introduction of the miR-31* mimic into HCT-116 cells. These data correlate with the effect observed from the cellular growth assay; that is, overexpression of miR-31* led to statistically significant decreased growth in addition to increased caspase activity. Much like the data from the WST-1 assay, evaluation of caspase activity after inhibition of miR-31* only demonstrated statistical significance ($p=0.02$) in one of the two biological repeats. Once again, we were unable to reproduce this effect in the second experiment, although the trend of decreased caspase activity in the first inhibition experiment would seem to correlate with the increased caspase activity observed after overexpression of miR-31* However, this effect along with other questionable insignificant results for the other 4 miRNAs could be simply due to coincidence or a technical error; to truly assess the effects of inhibition, multiple experiments in various CRC cell lines need to be performed.

Given the implications discussed above, we hoped to observe a consistent trend in regards to the effects of miR-31* expression when analyzing the RT-PCR data for the selected proliferation, EMT and apoptosis markers. Inhibition of miR-31* did not produce any change in expression of any of the proliferation (CCND1, CDH1, VIM, CDKN1B) or apoptosis (BAX, Bcl-2) markers in comparison to the negative control. However, we were able to demonstrate further that overexpression of miR-31* correlates with anti-cancer properties in HCT-116 cells. There was a statistically significant increase in the gene expression of CDKN1B when miR-31* expression was high. This data is consistent with results of both WST-1 and caspase assays, as CDKN1B is a known cell cycle inhibitor protein which functions to slow down or even stop the division of cells.[118] This increase in CDKN1B expression suggests that expression of

extracellular growth factors responsible for promoting cell division should in turn be decreased when miR-31* is highly expressed in cells.[119] Moreover, expression of the apoptotic markers BAX and Bcl-2 inversely correlated with one another as a result of miR-31* overexpression, as RT-PCR demonstrated an increase and decrease in each of these markers, respectively. This observation is consistent with the fact that BAX promotes apoptosis and, reversely, that Bcl-2 inhibits apoptosis.[120,121] Because the caspase assay and apoptosis marker results are in agreement with the data obtained from the WST-1 assay, that is, miRNAs which suppressed cellular proliferation in one assay demonstrated the activation of caspases-3/7 to induce apoptosis in the other assay, it can be concluded that miR-31* acts as a cellular growth suppressor under these given conditions.

To further investigate the pro-apoptotic capabilities of miR-31* overexpression, western blot analysis was performed to evaluate the levels of protein expression of PARP in its whole and cleaved form. In our HCT-116 experiments, it was expected for expression levels of cleaved PARP to be higher in cells harboring the miR-31* mimic when compared to the cells harboring the miR-31* inhibitor. This expectation stems from the fact that PARP is cleaved by caspase-3 during apoptosis, resulting in an 89kDa fragment.[122] Therefore, given our observations discussed above, we expected to detect little to no cleaved PARP expression in the cells in which miR-31* expression was inhibited, as we have thus far shown that miR-31* has served as a pro-apoptotic molecule in HCT-116. This, however, was not the case, in the western blot result. In fact, expression of cleaved PARP was slightly higher in the cells harboring the miR-31* inhibitor in comparison to the cells expressing the mimic. This, however, can most likely be attributed to the discrepancy between beta actin expression levels seen in the lower panel, as the loading control for the miR-31* inhibitor cells is clearly much thicker than that of the miR-31* mimic cells. This limits the interpretation of the result and we could therefore not deduce if the observation had actual biological relevance.

To further explore the anti-cancer potential of our microRNAs, we examined the effects of miRNA manipulation on the expression of select cancer-related stem cell markers via western blot analysis in the hopes of linking cancer stem cells to miRNA expression. The cancer stem cell hypothesis suggests that tumors can develop from a subset of cancer-initiating cells which possess the ability of self-renewal and differentiation.[123] The three selected markers were LGR5, Nanog and Oct4. Leucine-rich repeat-containing G-protein-couple receptor 5, or LGR5, is a membrane protein closely related to tumorigenesis and is active in colorectal cancer.[124] Nanog, which is an embryonic stem cell-specific transcription factor, regulates cell proliferation, epithelial-mesenchymal transition and self-renewal among other cancer stem cell features.[125,126]

Much like Nanog, Oct4 is a transcription factor and cancer stem cell marker and potentially linked to tumorigenesis.[127] High expression levels of these markers would suggest potential links to increased cell proliferation, invasion and metastases.

At first glance of the results of the stem cell marker protein expression data, it would appear that overexpression of miR-31* led to a dramatic decrease in LGR5, Nanog and Oct4 expression when compared to both miR-31* inhibitor and the negative control. Unfortunately, this data cannot be quite accurately interpreted due to the major discrepancy between the loading control expression of beta actin for the miR-31* mimic and inhibitor. Beta actin expression was approximately twice as high in the sample inhibiting miR-31* in comparison to the sample in which miR-31* was overexpressed. That being noted, however, we could then roughly estimate the protein panel corresponding to the miR-31* mimic to be about half of what it technically should be. Based on this estimation, we could still deduce that miR-31* overexpression in HCT-116 cells caused a reduction in protein expression of our selected cancer stem cell markers, with Nanog expression demonstrating the most drastic effect. More detailed analysis using quantitative software such as ImageJ might help to better estimate the true volume and intensity differences between the beta actin expression of miR-31* mimic and inhibitor. However, because the western blot itself is only a semi-quantitative technique, it should also be noted that this analytical method is limited in terms of fully interpreting the data.

Adherent growing monolayer tumor cells can form three-dimensional tumor spheres under specific growth conditions ("ultra-low attachment conditions"). These tumor spheres are considered as enriched by cells with CSC features.[128] As miRNAs regulate cancer stem cell formation and these cancer stem cells themselves represent attractive therapeutic targets, we aimed to further characterize the possible differential expression of our miRNA panel in tumor spheres. Investigation of miRNA expression in tumor spheres compared to adherent cells, however, proved to be quite complex. None of the cell line tumor spheres exhibited identical miRNA expressional patterns across the miRNA panel. Levels of miRNA expression were most similar between HT-29 and HCT-116 tumor spheres. The trend of miR-31* downregulation was consistent in both cell lines. This downregulated expression of miR-31* in tumor spheres supports the possibility of miR-31* acting as a tumor suppressor, as is consistent with our other findings described throughout this paper. Caco-2 results were exceptional in it that fold changes were tremendously higher than in the other two cell lines. In this particular cell line model, expression of miR-31* was upregulated in the tumor spheres in comparison to the adherent monolayer tumor cells, which is the exact opposite of the phenomenon observed in both HCT-116 and HT-29 cell line models. Interestingly, a study by Wu et al. elaborated on this exact

observation that the Caco-2 colorectal cancer cell line is an ineffective model for use in sphere formation assays, as they were unable to detect colorectal CSCs in the derived colon spheres.[129]

Interestingly, there is overwhelming evidence in the literature that proposes the exact opposite case of what our results here propose regarding the effects of miR-31* in colorectal cancer. However, it needs to be clarified that our study involved the investigation of miR-31*, otherwise known as miR-31-3p, which is the passenger strand of the miR-31 molecule, rather than the miR-31-5p strand, which is referred to simply as miR-31 without the asterisk. In certain cases during miRNA biogenesis, the stem-loop pre-miRNA can give rise to two different mature miRNAs, termed the "5p" and "3p" strands, which possess different sequences and have varying mRNA targets.[130,131] There is little information regarding the targets and effects of miR-31-3p let alone its effects in CRC, as most of the literature pertains miR-31-5p. For this reason, studies investigating miR-31-5p will primarily be discussed.

It has been demonstrated that miRNAs in general can be multi-functional and miR-31 is a classical example thereof.[132-134] Typically in breast cancer and ovarian cancer, miR-31 has been shown to inhibit proliferation, migration and invasion of cells, essentially acting as a tumor suppressor.[133,135] This effect, however, is dependent on the cell context, as miR-31 has been demonstrated to act as an oncogene in epithelial cancers such as that of the lung or colon, in which case cells tend to proliferate and lead to tumorigenesis.[136,137] In fact, when Wang et al. analyzed miRNA expression levels in 98 cases of CRC, 91% had an overexpressed level of miR-31, of which 63% had expression levels greater than 10 times than that of the normal mucosa and 17% had an expression level over 100 times greater than normal tissue.[138] Our results do not agree with previous findings regarding the pro-cancer and metastatic function of miR-31 in colon cancer cell lines.[133,136,138,139] One study regarding miR-31* demonstrated similar implications as does miR-31 in different CRC. Manceau et al. established a link between miR-31-3p expression and increased progression in metastatic CRC patients being treated with anti-EGFR therapy.[140] However, given that miRNAs act on multiple targets, it could very well be that there is a deeper complexity as to the function and role of miR-31* in not only colorectal cancer models, but human biology as well. A greater understanding and better knowledge regarding miR-31* targets is required in order to propose a more solid explanation as to why our results varied so strongly from previous studies.

4.2 miR-215

Overall assessment of the effects of miR-215 proved to be more difficult than was the case for miR-31*. Our findings for this particular miRNA remain mostly inconclusive due to several insignificant assay results and incomplete data regarding the effects of the miR-215 inhibitor. First and foremost, we were unable to detect any significant changes in cellular growth after manipulation of miRNA expression in either of the biological repeats of the WST-1 assay. Further limiting our ability to determine the basic functionality of this miRNA in our HCT-116 model cell line, caspase activities appeared to remain unaffected after overexpression of miR-215 as determined by the caspase activity assay. Given this lack of significant evidence regarding changes in growth capability or induction of cell death made it difficult to suggest a potential role of miR-215 at this point.

The expression of proliferation and EMT markers after overexpression of miR-215 offered significant yet conflicting data. In two cases, evidence suggested that miR-215 has an anti-apoptotic function and one case pointed to a pro-apoptotic function. The altered expression of CCND1, CDH1 and CDKN1B after miR-215 overexpression was statistically significant. CCND1 and CDH1 expression increased whereas high expression of miR-215 led to a decrease in CDKN1B expression. The changes observed in CCND1 and CDKN1B support the case for miR-215 anti-apoptotic activity. It is known that CCND1 plays a role in cell cycle progression and is often overexpressed in tumors, thus suggesting its potential role in tumorigenesis.[141] CDKN1B, on the other hand, is a cell cycle inhibitor protein, and our observation of its decreased expression after miR-215 overexpression suggests that miR-215 might be able to interfere with the ability of CDKN1B of properly regulating the cell cycle process.[118] An increase in CDH1 expression, however, is suggestive of miR-215 pro-apoptotic activity. Downregulation of this gene causes the cells to lose their adhesion capability, which ultimately results in increased cellular motility. It has been established that CDH1 is a tumor suppressor and loss of its function has implications in the progression of cancer and metastasis.[142,143] For these reasons, our data imply that an increase in miR-215 leads to an increase in this tumor suppressor function, which is of course a goal for an anti-cancer therapy.

Further supporting the concept of miR-215 as a pro-apoptotic miRNA, overexpression of miR-215 led to an increase in the expression of the known promoter of apoptosis, BAX. Any changes in Bcl-2 expression were not statistically significant, thus making an interpretation of these proliferation, EMT and apoptosis markers rather questionable. Detection of PARP in its cleaved form proved to be more informative for miR-215 than for miR-31*, as expression of the

beta actin loading control was consistent between samples overexpressing miR-215 and the negative control, making the case for a more solid interpretation of the data. Levels of PARP expression at 116kDa (uncleaved form) appeared to be similar if not identical for the miR-215 mimic sample and the negative control, whereas the level of protein expression for cleaved PARP at 89kDa in the miR-215 mimic sample was roughly double in comparison to the negative control. This would suggest that overexpression of miR-215 leads to an increase in caspase-3 activity and thus apoptosis, as PARP is cleaved by caspase-3 when the cells undergo programmed cell death. Further evidence pointing towards miR-215 tumor suppressor function, cancer stem cell marker expression was lower in cells overexpressing miR-215. When compared to the negative control, LGR5 and Nanog appeared to demonstrate decreased protein expression levels, suggesting that miR-215 might potentially play a role in the inactivation or degradation of these protein transcripts which have a known role in the EMT process, cell proliferation and tumorigenesis. Although Oct4 expression remained unaffected, lower protein levels of LGR5 and Nanog were at least consistent in terms of miR-215 functioning as a tumor suppressor. Data pertaining to the expression of miR-215 in tumor spheres was perhaps contradictory to the evidence provided thus far. Expression of miR-215 in tumors spheres in comparison to their adherent cells was not statistically significant in the HCT-116 cell line model. In HT-29 cells, however, miR-215 demonstrated an almost 3-fold increase in expression when compared to the adherent cells. This trend contradicts the notion that miR-215 functions as a tumor suppressor, as higher expression of miR-215 should have been observed in adherent cells in comparison to the spheres. It would of course be necessary to repeat a sphere formation assay followed by RT-PCR measurement of miR-215 expression in order to confirm these results. Much like the data for miR-31*, Caco-2 spheres demonstrated an incredible increase in expression of the measured miR-215 when compared to Caco-2 adherent cells. As mentioned before with the referenced publication, Caco-2 cells are not actually good candidates for producing reliable sphere formation assay results; this established fact might best explain the nearly 60-fold higher levels of miR-215 expression in the spheres of Caco-2 than in the adherent cells.

Overall, our data regarding overexpression effects of miR-215 are consistent with previous studies which suggest that miR-215 acts as a tumor suppressor. Boni et al. showed that an increase in miR-215 expression was able to reduce cell proliferation and had an impact on several key cell cycle genes in several colorectal cancer cell lines.[144] Braun et al. and others were also able to confirm that miR-215 was highly expressed in normal tissue but drastically reduced in the analyzed colon cancer samples.[145,146] However, one study demonstrated an

interesting contradiction to the theory that miR-215 can be defined as a tumor suppressor. Although miR-215 expression was dramatically reduced in the colon cancer samples, prognostic results demonstrated that patients expressing lower levels of miR-215 had a better survival rate. The study also confirmed that although high levels of miR-215 are able to suppress proliferation of tumor cells and induce cell cycle arrest but also that this high expression led to a decreased chemosensitivity in the cells.[146] This somewhat contradictory evidence seems to have similarities with our results regarding both a tumor suppressor and oncogene function for this miRNA. We also were able to confirm that overexpression of miR-215 led to an increase in the pro-apoptotic gene BAX, which correlates with other studies which ascertained that higher levels of miR-215 expression led to a significant increase in the number of measured apoptotic cells in the HCT-116 cell line.[147]

4.3 miR-148a

Assessment of the therapeutic potential of miR148a was also not an easy task, as many of the results obtained were not statistically significant. However, unlike the other two previous miRNAs discussed, much of the evidence from our experiments suggests more of an anti-apoptotic role for miR-148a in our main HCT-116 cell line model. However, we were able to independently confirm in both WST-1 assay repeats that overexpression of miR-148a leads to a decrease in cellular growth, indicative that miR-148a might function as a tumor suppressor as well. Inhibition of miR-148a in HCT-116 cells did not produce a statistically significant effect. It should be noted that none of the miRNA inhibitors used in these experiments were able to induce a significant effect upon introduction into the cells for the WST-1 assay, which could have been the result of a variety of factors. In the case of miR-148a (as well as miR-215 and miR-196b), endogenous expression in untreated HCT-116 cells was extremely low, of which detection came at a very late threshold cycle as measured by RT-PCR. It could very well be the case that introduction of an inhibitor into the cells would have minimal to no effect considering that the HCT-116 cells expressed very little of the target to begin with. Results of the caspase assay offered no further insight into the role of miR-148a in colorectal cancer since the data obtained did not pass the significance parameters or our experiments.

Measurement of the proliferation and EMT markers after overexpression of miR-148a demonstrated the trend of an anti-apoptotic role. Expressional upregulation of CCND1 and VIM were significant, with 1.43-fold and 1.86-fold changes in comparison to the negative control, respectively. As discussed earlier, overexpression of CCND1 is known to alter cell cycle

progression, which would suggest here that overexpression of miR-148a does not lead to anti-cancer activity. Furthermore, the upregulation of VIM indicates a potential role of miR-148a in the EMT process. Because VIM is a major component of mesenchymal cells, it is often used to identify cells which might be undergoing EMT and could potentially be used as a marker of metastatic progression.[148,149] Given these implications, we could perhaps assume that, under these conditions, miR-148a functions in an oncogene-like manner. To further substantiate this claim, we would then likewise expect for our selected apoptosis markers, BAX and Bcl-2, to be underexpressed and overexpressed in HCT-116 cells, respectively, upon overexpression of miR-148a. This was the case for Bcl-2; as Bcl-2 is an anti-apoptotic marker, its overexpression, which we assume is directly correlated with the high levels of miR-148a in the cell, we might interpret that miR-148a inhibits apoptosis. No significant changes were observed in BAX, for which we expected detect high levels of expression.

Interestingly, we made a similar observation that high levels of miR-148a might inversely correlate with apoptosis activity. When examining the sample in which cells overexpressed miR-148a, we were unable to detect PARP protein in its cleaved form, whereas the negative control demonstrated a slight yet clearly distinct protein band at 89kDa. This would suggest that overexpression of miR-148a leads to undetectable caspase-3 activity in this particular assay when compared to non-treated cells. If this miRNA truly functions in such a way that it interferes with or inhibits apoptosis, we would expect to see more cleavage of PARP at 89kDa upon inhibition of miR-148a. We were indeed able to observe this trend, although it could most likely be attributed to the fact that slightly more protein was loaded for cells inhibiting miR-148a than protein loaded for cells overexpressing miR-148a, albeit a slight discrepancy. However, once again, as western blot analysis is only a semi-quantitative method and given the fact that we did not use any quantification software for our purposes, we should be careful not to overinterpret these results. Data pertaining to expression measurements of our selected stem cell markers contradicted the tumor suppressor trend of miR-148a. As was the case for miR-31* and miR-215, both LGR5 and Nanog protein levels were decreased after overexpression of the target miRNA when compared to the negative control. After observing this contradictory effect, we hoped to turn to our tumor sphere data to help make a more decisive claim about the role of miR-148a in colorectal cancer cells, as the tumor spheres are known to be enriched with cells harboring CSC-like features. We were unable, however, to reach any conclusions that might help support any of the previous evidence due to the fact that no significant change was detected in either HCT-116 or HT-29 tumor sphere cell models and that Caco-2 once again produced unreliable results, for reasons mentioned earlier.

Although our results have some clear correlations with previous studies, there are also some very interesting incongruities. With the WST-1 assay, we were able to observe a significant tendency for HCT-116 cells to cease proliferation, which is consistent with other studies that suggested that miR-148a could act as a repressor of tumor cell proliferation by targeting TGIF2 and DNMT1.[150,151] However, in our study we also demonstrated that overexpression of miR-148a leads to an increase in expression of cell growth or EMT markers as well as the increase of expression in Bcl-2, suggesting an anti-apoptotic role. A study by Zhang et al. revealed the exact opposite of our findings. They constructed a miR-148a expression vector which harbored the human miR-148a genomic sequence and ectopically expressed the vector in several colorectal cancer cell lines. They were able to deduce that introduction of miR-148a into the cells promoted apoptosis in both RKO and Lovo cells and also that expression of an antisense miR-148a inhibitor was able to partially reverse this increased apoptosis.[152] Moreover, they confirmed that Bcl-2 is a functional target site of miR-148a in the 3'UTR after designing a vector which coded for the seed sequence of miR-148a found in the Bcl-2 gene.[152] This clash of evidence with our study should not be overinterpreted. We were unable to obtain any significant caspase assay data which would help clarify the overall role of miR-148a in colorectal cancer cells. An individual RT-PCR analysis is by no means a method of exhaustive validation; for this reason, we might even be able to attribute the increase in Bcl-2 expression we observed after miR-148a expression to mere coincidence. It is also worth mentioning once more that miRNAs have multiple targets; more studies need to be conducted in order to elucidate further potential roles of miR-148a in colorectal cancer.

4.4 miR-342

The last two miRNAs to be discussed, among them miR-342, were the most inconclusive due to their exclusion from the protein expression analysis experiments and caspase activity measurements in the interest of time as well as financial reasons and due to discrepancies in the initial growth assay analysis. However, despite these limitations to a final interpretation, the data which was obtained did not demonstrate any conflicting trends in terms of labeling miR-342 a tumor suppressor miRNA. In the WST-1 assay, we were able to independently identify a significant inhibitory effect on HCT-116 cellular growth after overexpression of miR-342 in the two biological repeats. Furthermore, assessment of the proliferation and EMT markers demonstrated that overexpression of miR-342 correlated with a decrease in CCND1 expression and that VIM expression was significantly increased after

inhibition of miR-342. The former suggests that miR-342 can potentially inhibit unrestricted cell cycle progression while the latter indicates that inactivation of miR-342 expression leads to an increase in cell motility and thus invasion, both points which give evidence to a tumor suppressor role of miR-342. In terms of a role in apoptosis, overexpression of miR-342 was able to increase BAX expression in HCT-116 cells, which would indicate a correlation with increased cell death. Interestingly, miR-342 expression proved to be significantly downregulated in both HCT-116 and HT-29 tumor sphere models when compared to adherent cells, but this time not in Caco-2 cells. These data might suggest that miR-342 is not associated with these CSC-like features.

Although our data points were few, our analysis remains consistent with previous reports regarding the tumor suppressor function of miR-342. Wang et al. were able to inhibit CRC cell proliferation by 44% as well as metastasis by restoring lost expression of miR-342 both in vitro and in vivo. In their in vivo model, xenograft tumor growth was significantly reduced after being treated with their miR-342 expression vector construct.[153] Similar to our observations regarding cell growth inhibition due to miR-342 overexpression, others have confirmed that high levels of miR-342 could be associated with an increase in apoptosis in CRC cells.[154]

4.5 miR-196b

Obtaining an overview of miR-196b as a potential therapeutic molecule in colorectal cancer cells using our selected analytical methods proved to be the most difficult. As stated before, we did not collect protein expression data or caspase activity data for miR-196b; thus it was not possible to determine an overall classification of miR-196b as an oncogene or tumor suppressor using our cellular model. Results from the WST-1 assay demonstrated that overexpression of miR-196b significantly led to a decrease in cellular growth in both independent biological repeats. However, the experiments in which miR-196b was inhibited in HCT-116 cells were problematic for our interpretation of the true effect of miR-196b. Although only 1 of the 2 biological repeats was of statistic relevance for the miR-196b inhibitor, the trend remained similar to that of miR-196b overexpression. This means that both overexpression and inhibition of miR-196b was able to decrease cellular growth in this assay, which is of course both contradictory and questionable. As mentioned before in the case of miR-148a and miR-215, introducing an inhibitor into the cells would most likely have minimal to no effect due to the low endogenous expression in untreated HCT-116 cells. Nevertheless, this potential scenario does not explain why inhibition of miR-196b in the second biological repeat was statistically

significant and looked almost identical to the experiment in which cells were overexpressing miR-196b. This observation could quite possibly be the result of a series of off-target effects within the cell, suggesting that introduction of the mimic or inhibitor indirectly caused a decrease in cellular growth by affecting other targets other than the intended growth factors related to cellular proliferation. Examining the data obtained from the measurement of proliferation and EMT markers in cells harboring the miR-196b mimic, only expression of VIM appeared to be effected, in which the gene marker was upregulated about 2-fold when compared to the negative control. This would suggest that miR-196b activity could potentially trigger cells to undergo EMT, an indication that miR-196b itself might have an oncogenic role. Nonetheless, this single individual data point is by no means conclusive, nor can it be interpreted solely by itself without the support of the other biological assays.

Reports in the literature have suggested the deregulation of miR-196b in several forms of cancer as well as its role in the regulation of metastasis-related genes which promote cell migration and invasion.[155-157] Mo et al. was able to verify the regulation of the FAS-mediated apoptosis pathway by miR-196b in CRC and furthermore demonstrated that an antagonist inhibiting miR-196b was able to restore FAS expression and therefore apoptosis function in CRC cells. Our results are partially consistent with these studies if the WST-1 data for the miR-196b inhibitor is taken into account along with upregulation of VIM expression after miR-196b overexpression. To truly be able to interpret the function of miR-196b in our study, more biological repeats of the WST-1 assay would need to be performed in order to clarify the contradictory cell growth data. Furthermore, we would need to determine the effects of miR-196b overexpression with a caspase assay.

5 CONCLUSIONS

Although the initial approach taken towards miRNA classification trended towards labeling these molecules as either oncogenes or tumor suppressors, accumulating evidence continues to challenge this school of thought, as it is now understood that an individual miRNA can have multiple functions rather than just one solitary role.[158] The path to discovering novel therapeutic agents for the effective treatment of cancer has been made possible through the miRNA mimic and inhibitor technology now at hand that allow researchers to manipulate targeted expression of miRNAs of interest. Our study has shed some light onto the complexity of these small RNA molecules in terms of screening for the perfect anti-cancer therapy. The expression of any given miRNA can simultaneously affect multiple pathways and their genes involved, both desirable or undesirable, making the simple classification of individual miRNA expression as either “good” (tumor suppressor in nature) or “bad” (oncogenic in nature) rather difficult if not altogether inaccurate. While not the aim or scope of this thesis, the data provided here in this study were unable to overwhelmingly convince us that any of these selected miRNAs are solid candidates for moving onto pre-clinical studies, with the exception of miR-31*, which demonstrated some promising implications. A more exhaustive approach, including in vivo models, must be taken to further confirm or repudiate any of the stated outcomes. Although the path to clinical trials for any drug candidate is a long and complex one, investigations continue to show the true potential of miRNA-based therapy for the treatment of CRC and other cancers.

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