Micro RNA (miR-155) regulation and their effect in cancer cells

A Research Internship Report

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Duration : from July 23rd till September 27th, 2010

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Introduction

I am an 18 year high school student and cancer survivor. I was diagnosed with leukemia in 2009 and am still under maintenance treatment, though fully cured. My personal experiences with cancer, while gruelling , painful and exhausting also taught me a valuable lesson : research into this disease and its many forms and complications is crucially important , and is the only thing that can lead to a future world without cancer.

I have been involved in fund raising for cancer research for the past two years with the International Terry Fox Foundation and as a consequence of this involvement, this year I was given an opportunity to work as a research intern in the cancer biology laboratory at the Biosciences department in IIT Madras.

Recently an exciting class of endogenous small RNA molecules called microRNA have been discovered, that have wide ranging influence in the regulation of biological processes in general and in the initiation and progression of cancers in particular. They do this by a previously unsuspected ability to "silence" the expression of genes either by affecting messenger RNA stability or by interfering with the mRNA in their translational work. My research project is aimed at better understanding miR-155, an oncogene influential in the genetic processes that initiate leukemias and lymphomas. The interaction between microRNAs is one of the frontier areas of cancer research, all over the world.

IIT Madras is a premier institute of engineering, research and higher learning and the project introduced me to state of the art laboratory equipment and gave me a hands-on experience with the latest genetic engineering methodologies. This has been an extremely exciting and enriching experience. My personal trial by fire with cancer started me on this journey of discovery and I hope it will be a lifelong one.

Cancer genesis and proliferation, a genetic background.

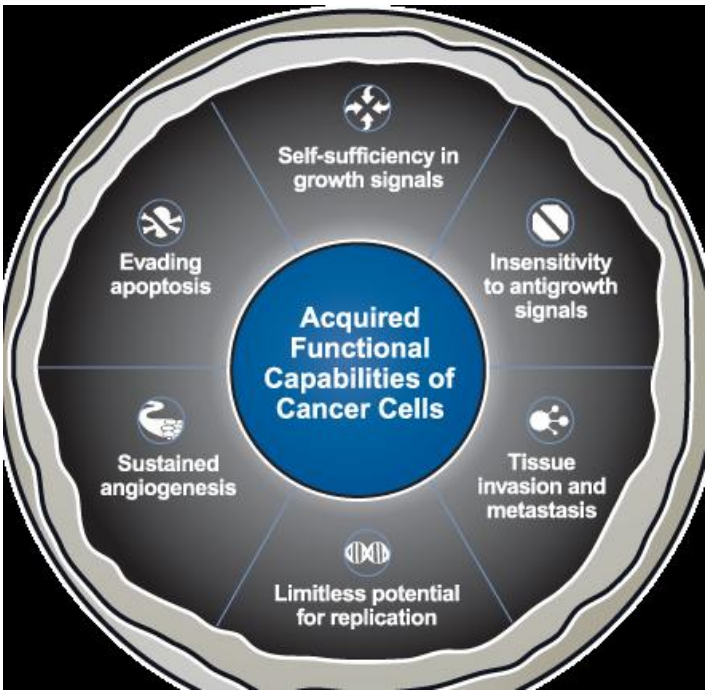
Cancer is the Latin word for crab, the ancients no doubt used it because of the disease's crab like tendency to grab and hold onto any tissue it invades. It is systemic, not a single disease, but rather a group of over 100 different and distinctive diseases. Cancer of the lung, prostrate and breast are now fast replacing heart disease as the number one killer in adult populations. It is a well known fact that there are both genetic susceptibility factors as well as environmental triggers for cancer. Our knowledge and ability to fight this disease is growing daily, with research advances, but each new piece of knowledge also shows how much there is still as yet unknown.

At its most basic level cancer is an uncontrolled, irreversible cell division that causes our own body to turn against itself. Cancers can be localized as tumours or spread all over the body, through either our blood, bones or lymphatic fluids. The disease can either stay in one form or can metastize and change into something different and more dangerous .New variants are being discovered all the time.

There are five major steps for cancer development: initiation, promotion, malignant conversion, progression, and metastasis. Many factors influence the development of cancers: some inhibit tumor development (tumor suppressors), and some promote cancer development (cancer inducers). There is a homeostasis of cells which is disrupted. Scientists have been trying to elucidate the molecular mechanisms that cause cancer initiation and its progression. Although several genes, including oncogenes and tumor suppressor genes , have been identified in human and other model animal genomes, the mechanism of cancer formation in many systems is yet to be identified.

Current theory is based on a two trigger mechanism: a genetic pre disposition that when exposed to a trigger of some sort, probably something in the environment causes cancer cells to be created. A recently identified class of non-protein-coding small RNAs, called microRNAs may provide new and exciting insight into cancer research.

Cancer cells quire certain characteristics that make it very hard to control or reduce their spreading , and the most important mechanisms employed by these rogue cells to survive and perpetuate themselves is summarized in the graphic below :



Approximately 70% of all gene therapy trials initiated since the first approved gene transfer study in humans in 1989 have targeted cancer. This predominance of cancer trials reflects both the promise of genetic technologies and the pressing need to improve treatment options and outcomes for advanced and difficult to treat cancer phenotypes.

Focus areas in cancer research

Our biological system is a world in itself, made of a wonderful series of checks and balances that regulate life's processes through a beautiful synchronization between our DNA, RNA , proteins and the various environments that our body's cells find themselves in. The body uses cell signaling pathways to maintain this balance, which the cancer cells are somehow able to circumvent to survive and proliferate. Research into the disease therefore focuses on the genetic processes in normal cells as against cancer cells. To be able to design weapons for our cancer arsenal, we have to first understand the enemy's weaknesses.

Areas of research that are the current focus all over the world are :

1. Genetic markers specific to the various types of cancer and how these may be used for diagnosis and prognosis.
2. Innovative and easy to use diagnostic assays for the clinical community.
3. Imaging, radiation physics, to improve tumour detection.
4. Statistical analysis of prognosis and post treatment quality of life.
5. Cell signaling pathways and how cancer cells cause them to be altered.
6. Mechanisms of Immuno suppression in cancer.
7. Reducing or alleviating symptoms caused by the intense treatment protocols.
8. Metastasis pathways and triggers.
9. Cell apoptosis or cell suicide, this is the holy grail of cancer research. If cancerous cells could be persuaded to eliminate themselves, this has the potential to avoid chemo and radiation.

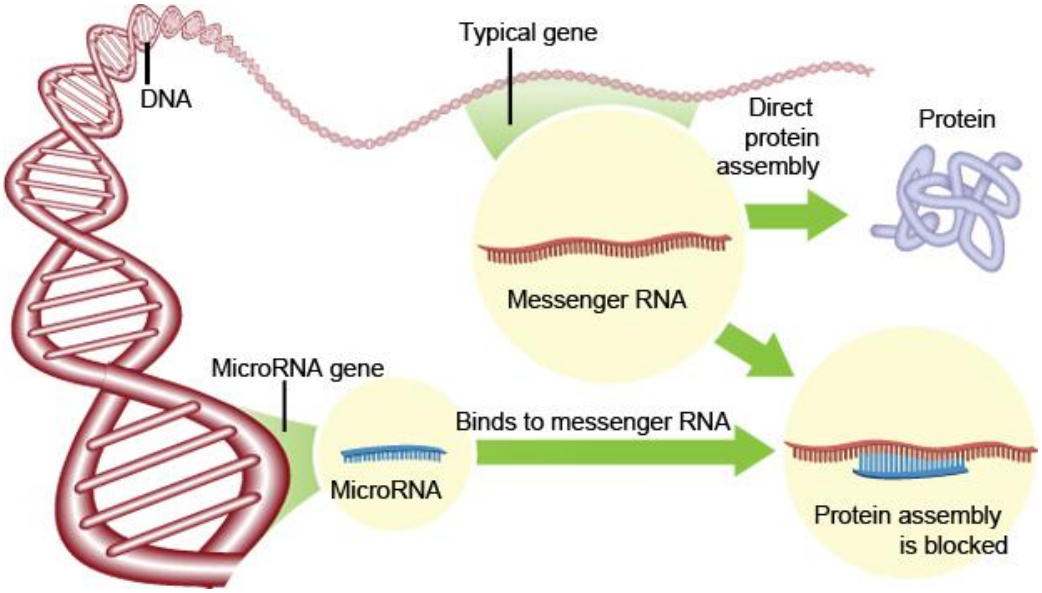
Discovery of micro RNA, a brief history

Micro RNA's are a class of small RNAs (Ribo Nucleic Acid chains) that have revealed a new level of gene regulation in the cell. They are now well known to be involved in the normal functioning of eukaryotic cells, and their dys-regulation in turn has been well associated with disease. Some have been shown[(1)(2)(3)] to be associated with cardio myopathies MiRNAs also seem to regulate the nervous system, and some studies have shown altered expression of micro RNA in schizophrenia [(4)(5)] . However a majority of the studies which we will go into in detail later in this report, deal with the influence miRNAs have in cancer.

miRNA molecules are produced from larger gene transcripts that form hairpin precursors. Two RNase III endonucleases, Drosha and Dicer, cut both strands of the hairpin and generate the miRNA duplex, which is usually about 22 base pairs long.In animal cells, single-stranded miRNAs associated with RISC (RNA-induced silencing complex) bind to target messenger RNAs. The bound messenger RNA is then degraded or remains un-translated, resulting in decreased levels of production of the protein encoded for. A single miRNA can bind to multiple genes and have a possible profound effect on cell physiology.

A new study now shows that microRNAs don't just control the activity of genes within a given cell, they also can move from one cell to another to send signals that influence gene expression on a broader scale. Researchers at the Duke Institute for Genome Sciences & Policy (IGSP), in collaboration with groups at the Universities of Helsinki and Uppsala and the Boyce Thompson Institute for Plant Research at Cornell University, made the discovery while working out the intricate details of plant root development in *Arabidopsis*, a highly-studied mustard plant.

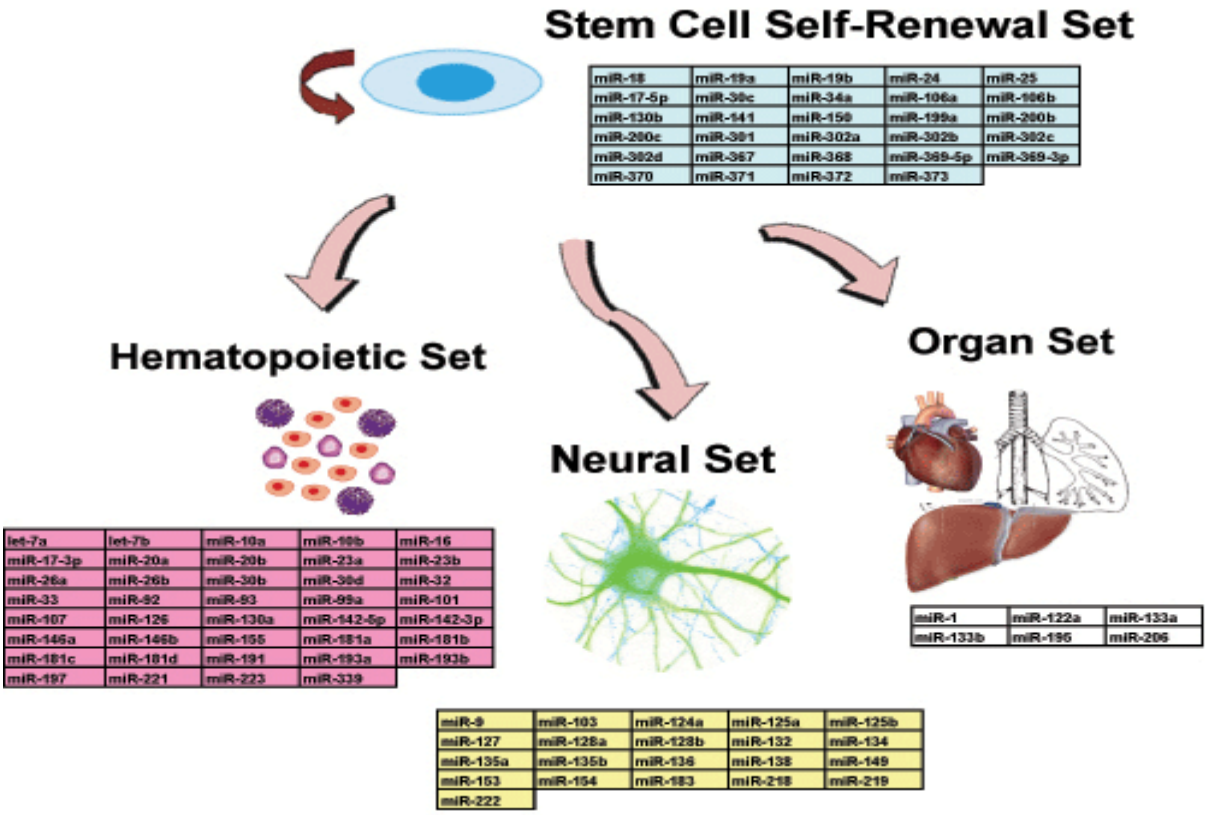
Fig 1. Graphic depiction of micro RNA mode of operation.



MicroRNAs (miRNAs) were discovered in 1993 by Victor Ambros, Rosalind Lee and Rhonda Feinbaum during a study of the gene *lin-14* in *C. elegans* development[6] C. elegans is a eukaryotic model much studied due to its high level (35 %) of homologues with human genes. They found that a protein called LIN-14 was regulated by a short RNA product encoded by the *lin-4* gene. A 61 nucleotide precursor from the *lin-4* gene produced a 22 nucleotide RNA containing sequences partially complementary to multiple sequences in the 3' UTR (Un Translated Region) of the messenger RNA (mRNA) whose job it was to design and produce the protein. This RNA fragment was able to inhibit the translation of *lin-14* mRNA into LIN-14 protein. This was the first miRNA to be identified, though at the time, it was thought to be an idiosyncrasy. It was seven years before the second miRNA was characterized: let-7, which repressed *lin-41*, *lin-14*, *lin-28*, *lin-42*, and *daf-12* expression during developmental stage transitions in *C. elegans*. let-7 was soon found to be conserved in many species,[7][8] indicating the existence of a wider phenomenon.

In the last decade, over 650 miRNAs have been identified, classified and investigated in detail, and the ones active in human cell systems are thought at the present time to be around 230 in number. Research is ongoing at a furious pace with new and exciting discoveries being made almost on a daily basis.

Fig 2 : miRNA classified into groups depending on the type of cells they affect.



Micro RNA form and function as we now understand it.

Most miRNA genes are found either in intergenic regions or in an anti-sense orientation to the genes[9] .They contain their own miRNA gene promoter and regulatory units[9][10][11][12] . As much as 40% of miRNA genes may lie in the introns of protein and non-protein coding genes or even in exons.[13] miRNA genes are usually transcribed by RNA polymerase II (Pol II).[10][14]

Animal miRNAs are usually complementary to a site in the 3' UTR (Un Translated Region) whereas plant miRNAs are usually complementary to coding regions of mRNAs. [15] Perfect or near perfect base pairing with the target RNA promotes cleavage of the RNA .[16] In what has proven to be the primary mode of operation of plant miRNAs.[12] In animals cells however, microRNAs more often only partially base pair and inhibit protein translation of the target mRNA.[17]This style of functioning does also exist in plants but is less common [18]. The differences must be a consequence of the basic divergence in evolutionary paths between plant and animal cells.

MiRNAs that are partially complementary to the target can also speed up the process of deadenylation (removal of the adenylate group) thereby causing mRNAs to be degraded sooner. [19] For partially complementary miRNA to recognise their targets, its enough if nucleotides 2–7 of the miRNA (also known as the " seed region') are perfectly complementary.[20] miRNAs occasionally can also causes histone modification and DNA methylation of promoter sites , affecting the expression of targeted genes.[21][22]

The system of nomenclature of miRNA is complicated and can be a little difficult to understand. Names are assigned to experimentally confirmed miRNAs before publication of their discovery.[23][24] The prefix "mir" is followed by a dash and a number based on the order of discovery. For example, mir-123 was named and likely discovered prior to mir-456. The uncapitalized "mir-" refers to the pre-miRNA, while a capitalized "miR-" refers to the mature form. miRNAs with nearly identical sequences with just one or two nucleotide differences are annotated with an additional lower case letter.

For example, miR-123a would be closely related to miR-123b. miRNAs that are 100% identical but are encoded at different places in the genome are indicated with additional dash-number suffix: miR-123-1 and miR-123-2 are identical but are produced from different pre-miRNAs. Species of origin is designated with a three-letter prefix, e.g., hsa-miR-123 would be from human (*Homo sapiens*) and oar-miR-123 would be a sheep (*Ovis aries*) miRNA. Other common prefixes include 'v' for viral (miRNA encoded by a viral genome) and 'd' for *Drosophila* miRNA (a fruit fly commonly studied in genetic research). microRNAs originating from the 3’ or 5’ end of a pre-miRNA are denoted with a -3p or -5p suffix. (In the past, this distinction was also made with 's' (sense) and 'as' (antisense)). When relative expression levels are known, an asterisk following the name indicates an miRNA expressed at low levels relative to the miRNA in the opposite arm of a hairpin. For example, miR-123 and miR-123* would share a pre-miRNA hairpin, but relatively more miR-123 would be found in the cell.

Fig 3. A detailed schematic description of the various processes involved in miRNA production and its effect in gene silencing is shown in the figure below :

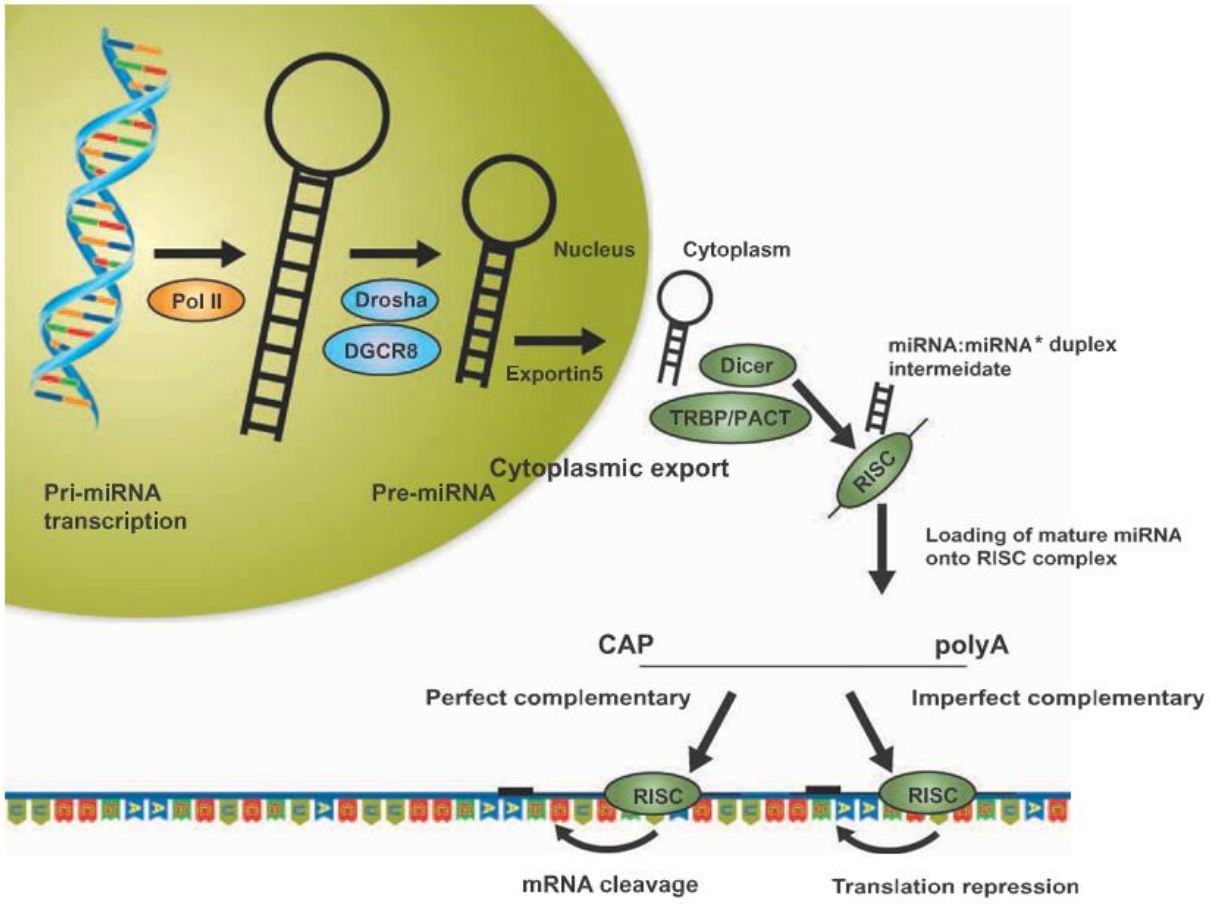


Figure 2. miRNA processing. RNA polymerase II transcribes a primary miRNA sequence, which is then cleaved by a complex including Drosha and the double-stranded RNA-binding domain protein DGCR8 (DiGeorge syndrome critical region gene 8) resulting in a precursor miRNA. Exportin-5 exports the precursor miRNA from the nucleus to the cytoplasm where it is cleaved by Dicer in a complex with TAR RNA-binding protein and protein kinase R-activating protein leaving a miRNA:miRNA* complex intermediate. The miRNA strand of the complex is incorporated into a RISC and guides the complex to the 3¦UTR of the target mRNA by way of a 7- to 8-nucleotide seed sequence. This binding will result in either translational repression or mRNA degradation depending on several factors including the extent of complementarity between the miRNA seed sequence and the 3¦UTR and the Ago protein in the RISC.

Micro RNA and Cancer

Several miRNAs have been found to have links with types of cancer [25][26] and in recent years are making a major impact in our understanding of cancer biology. They can be involved in metastasis, invasion, proliferation, cell life cycle or even apoptosis. A recent study demonstrated that more than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile regions. [27] The most exciting discovery is that they can function either as tumour suppressors or as oncogenes. These totally opposite functionalities for the same class of compounds have led to miRNA's becoming a priority area in cancer research worldwide.

A study of mice altered to produce excess c-Myc — a protein with mutated forms implicated in several cancers — shows that miRNA has an effect on the development of cancer. Mice that were engineered to produce a surplus of specific miRNA found to be upregulated in lymphoma cells developed the disease within 50 days and died two weeks later. In contrast, mice without the surplus miRNA lived over 100 days.[28] Leukemia could be caused by the insertion of a virus next to the miR 17-92 cluster leading to increased expression of this microRNA.[29] Another study found that two miRNA inhibit the E2F1 protein, which regulates cell proliferation [30] . Transgenic mice that over-express or lack specific miRNAs have provided insight into the role of small RNAs in various malignancies. [31]

By measuring activity among 217 genes encoding miRNA, patterns of gene activity that can distinguish types of cancers can be discerned and miRNA signatures may enable classification of cancer. This will allow doctors to determine the original tissue type which spawned a cancer and to be able to target a treatment course based on the original tissue type. miRNA profiling has already been able to determine whether patients with chronic lymphocytic leukemia had slow growing or aggressive forms of the cancer .[32]

Studies on the effect of various miRNAs and the type of cancer they act upon, are just too many and varied for the scope of this report. I have tried therefore to list only some of the more salient results in this section. The initial evidence for the involvement of miRNAs in cancer came from a molecular study characterizing the 13q14 deletion in chronic lymphoblastic leukemia [33] It was observed that two miRNAs miR-15a and miR-16a are located in 13q14, a region deleted in more than half of B cell CLL cases. A recent study [34] has shown BCL 2 is one of the targets of these miRNAs suggesting they could possibly be used therapeutically to cure tumours over expressing the BCL 2 gene.

Lung cancer is one of the most common cancers in adults and a leading cause of cancer related deaths in economically advanced countries. Emerging evidence suggests that mi RNA let-7 may control lung cancer development. Expression levels of let-7 were found to be frequently reduced in both in vivo and in vitro studies of lung cancer [35] and was found to be associated with shortened post operative survival, independent of disease stage. In contrast to miRNA let-7, the expression of miRNA cluster miR-17-92 is increased in lung cancer, particularly in the most aggressive form: small cell lung cancer. [36]

Breast cancer is one of the most prevalent cancers in adult females. Studies in 2005 have shown miR-125b, miR-145 , miR-21 and miR-155 to be significantly reduced in breast cancer tissues. [37] In 2010 however contradictory results have been reported [38] with miR-155 shown to be over expressed and targeting a tumor suppressor gene named "suppressor of cytokine signaling 1" or (socs1) in breast cancer cells. mir-155 expression has been found to be inversely correlated with socs1 expression in breast cancer cell lines as well as in a subset of primary breast tumors.

Colon cancer is also associated with alterations in miRNA expressions. 28 different miRNAs have been identified so far in colonic adenocarcinomas and normal mucosa , and it has been found that both miR-143 and miR-145 are consistently reduced [39]

It has been reported [40] that as breast cancer cells metastize, expression of miR-126 and miR-355 is lost, while over expression of these miRNAs in cancer cells decreases lung and bone metastasis in vivo. Conversely miR-373 and miR-520c enhance tumour invasion and metastasis both in breast cancer cells and in vivo, which suggests they may target genes required to suppress cell growth and metastasis. [41]

miRNAs are also involved in human brain cancer. Glioblastoma multiforme (GBM) is the most frequent occurrence and malignant form of primary brain tumors. These are highly invasive, very aggressive, and one of the most incurable cancers in humans. However, an understanding of the molecular mechanisms involved with this tumor is still very poor. Recently, a study [42] employed microRNA microarry analysis to examine the global expression levels of 245 miRNAs in GBM. They observed that *miR-221* was strongly upregulated in glioblastoma samples from patients. They also found that *miR-181a*, *miR-181b*, and *miR-181c* were down-regulated in glioblastoma compare to normal brain controls.

Papillary thyroid carcinoma (PTC) is the most common malignancy in thyroid tissue; about 80% of incident thyroid cancers are PTC. Although PTC is usually associated with alterations in the RET/PTC-RAS-BRAF signaling pathway[43][44] the detailed molecular mechanism is unclear. Recently,it has been demonstrated [45] that numerous miRNAs are overexpressed in PTC tumors compared with normal thyroid tissues. Of these miRNAs, *miR-221*, *miR-222*, and *miR-146* were strongly overexpressed (11- to 19-fold) in thyroid tumors compared with unaffected thyroid tissues.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the liver. Studies investigated miRNA expression profiles of HCC and adjacent non tumorous tissue and found that *miR-18* and *miR-224* were significantly over expressed in HCC compared with non tumor tissues. In contrast, *miR-199a**, *miR-195*, *miR-199a*, *miR-200a*, and *miR-125a* were under expressed in HCC tissues. [46]

Last but certainly not the least in importance **: p53** (also known as tumor protein 53), is a protein that in humans is encoded by the *TP53* gene.[47][48][49] P53 is extremely important in multicellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor that is involved in preventing cancer. P53 is so important it has been named "the guardian angel gene." In its anti-cancer role, p53 works through several mechanisms:

- It can activate DNA repair proteins when DNA has sustained damage.
- It can induce growth arrest by holding the cell cycle at the G_1/S regulation point on DNA damage recognition (if it holds the cell here for long enough, the DNA repair proteins will have time to fix the damage and the cell will be allowed to continue the cell cycle).
- It can initiate apoptosis, the programmed cell death, if DNA damage proves to be irreparable.

A team of investigators from The Cancer Institute of New Jersey and the Institute for Advanced Study -- which includes a researcher who co-discovered p53 some 30 years ago have this year reported that miR-504 is directly shown to reduce expression of the P53 gene, removing a natural defense and leaving us vulnerable to a cancer onslaught.

Simply put, cancer genetics has been revolutionized today by micro RNA studies. The questions that need answering are these :

1. What regulates the expression of miRNAs?
2. What are the targets of each miRNA?
3. Do miRNAs act mainly to 'fine-tune' gene expression or more often as binary on/off switches?

Given that each miRNA may regulate numerous targets, it is possible that thousands of protein-coding genes could be regulated by a just few hundred miRNAs. All we can say for sure is that a great deal of experimental work remains to be done in validation.

My research hypothesis, in perspective.

Micro RNA have in recent years generated a lot of interest not just because of their unique gene silencing abilities but also because it is theoretically conceivable to engineer modifications to biological systems using their interactions with each other and with other chemicals that exist in the cytoplasm. A study conducted in MIT's Centre for Cancer Research in 2007 [(50)] opened up exciting new possibilities with the development of what have been named " miRNA sponges ". This is a revolutionary method to inhibit the action of miRNA by genetically altering the cell. By incorporating a complementary gene into the cell's DNA, the cell is given the ability to produce miRNA " sponges " that are capable of destructively binding to the miRNA and rendering them inactive before they can reach their targets, the messenger RNAs . Earlier attempts had been made to reduce miRNA activity using artificially synthesized oligonucleotides [(51)] but the MIT study found it to be a much more effective hindrance when the cells produced the sponges themselves.

Micro RNA that are found to be more expressed in cancerous systems and seem to be involved in the processes that initiate or spread cancer are classified as "oncogenes ". Other micro RNA equally clearly fall into the category of tumour suppressors. The research work that I am to be a part of, is to try to understand the effect of some known oncogenes and tumor suppressors and to further investigate the effect of "mir sponges" on their ability to interact with each other and manipulate cell regulation. I selected for my work miR-155 which is known to be up-regulated in a wide range of cancers.

The presence of cancer initiation signals by the micro RNA can be measured by adding a sensor construct into the plasmid vector. When the micro RNA is successfully transcribed, this sensor construct is also activated and the effect can be measured using a luciferase assay. Luciferase is a bioluminescent molecule originally derived from fire flies. Heightened activity due to the micro RNA's expression will result in more light emission which can be measured and compared against control cells without the plasmid in them, using a luminometer. The light measurement data can therefore be taken as a direct measurement of the carcinogenic capabilities of the miR-155.

My research hypothesis : A successful plasmid vector with the miR-155 gene ligated into it, when incorporated into normal HEK (Human Embryonic Kidney) cells should be able to promote cancer initiation signals in the healthy cells.

Micro RNA -155

The oncogene I have elected to investigate is miR-155 and I would like in this section of my report to give an analysis of the research undertaken on this molecule till date. miR-155 is influential in the regulation of hematopoietic cells, which are cells that later mature and become different red blood cells. For this reason therefore, in several types of lymphomas, including Burkitt's lymphoma , the expression of miR-155 is increased when compared to normal cells . [53] [54] In addition, it is well known that the BIC gene is related to several such cancers, and its activation accelerates the pathogenesis of both lymphomas and leukemias. However the molecular basis of BIC related cancers is still unknown.

A recent study [55] has indicated that miR-155 is located in the only phylogenetically conserved region of the BIC gene, on chromosome 21 , suggesting that miR-155 may be responsible for the oncogenic activity of the BIC gene [53] . This study has also found that miR-155 is over expressed in a wide range of lymphomas derived from B cells of different developmental stages, specially in aggressive B cell neoplasms (growths) such as diffuse large B cell lymphoma (DLBCL). An elevation of 10 to 60 fold was found in miR-155 levels in these lymphomas. The study also found significantly higher levels of miR-155 in cells with the ABC phenotype, when compared to cells with the GC phenotype, suggesting that miR-155 may be useful diagnostically for curing the ABC type lymphomas. A diagnostic assay for B cell lymphomas using measured levels of mir-155 in the B cells has already been approved by the US patent office

One possible mechanism for miR-155 involvement in this type of cancer may be that miR-155 may down-regulate the expression of the transcription factor PU-1 which is necessary for later differentiation of the B cells. miR-155 levels change dynamically during both hematopoietic lineage differentiation and the course of the immune response.

Different mouse models developed recently indicate that miR-155 plays a critical role during hematopoiesis and regulates lymphocyte homeostasis and tolerance. A moderate increase of miR-155 levels is observed in many types of malignancies of B cell or myeloid origin, and transgenic over-expression of miR-155 in mice results in cancer.

Another consequence of miR-155's gene positioning in the BIC region of chromosome 21 is its possible influence on the incidence of Down's Syndrome. Individuals with Down's Syndrome have an extra copy of chromosome 21 so it is logical to assume that miRNA located here may be a causal factor. A 2009 study [(56)] at Ohio State University investigated the levels of a protein called methyl-CpG-binding-protein 2 (MeCP2) . A mutation in this protein leads to a cognitive disorder called Rett's syndrome and they were actually able to show that MeCP2 which seems to be involved in normal brain development is indeed a target of miR-155. Further investigation is now ongoing.

miR-155 while known primarily for its effect on lymphomas, is also known to be influential in breast cancer tissues, and the contradiction described in the last section between results obtained in 2005 and 2010 is just a sign of how our methods of analysis and experimental precision are improving in leaps and bounds. The contradiction will have to be reconciled as is usual in science by collecting evidence to support the winning hypothesis over a period of time.

Head and neck/oral cancer (HNOC) is a devastating disease. Despite advances in diagnosis and treatment, mortality rates have not improved significantly over the past three decades. Improvement in patient survival requires a better understanding of the disease progression so that HNOC can be detected early in the disease process and targeted therapeutic interventions can be deployed. MicroRNA alterations that consistently identified in HNOC are the up regulation of miR-21, miR-31, miR-155, and down regulation of miR-26b, miR-107, miR-133b, miR-138, and miR-139. [(57)]

New research in 2010 also shows miR-155 can silence genes that protect the genome from cancer-causing mutations. The study, led by researchers at the Ohio State University Comprehensive Cancer Center-Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, shows that microRNA-155 (miR-155) can inhibit the activity of genes that normally correct the damage when the wrong bases are paired in DNA. The loss or silencing of these genes, which are called mismatch repair genes, causes inherited cancer-susceptibility syndromes and contributes to the progression of colorectal, uterine, ovarian and other cancers.

The above study was published recently in the Proceedings of the National Academy of Sciences and clearly shows the following:

1. Over expression of miR-155 reduced the expression of the human mismatch repair genes MLH1, MSH2 and MSH6 by 72 percent, 42 percent and 69 percent, respectively, in a colorectal cancer cell line.

2. High expression of miR-155 in human colorectal tumors correlates with low expression of the genes MLH1 and MSH2.

3. Human tumors that feature unexplained mismatch repair inactivation showed miR-155 over expression.

In general therefore, it seems safe to conclude that over expression of miR-155 is a common feature in both the initiation as well as the metastasis of many cancers. If it were possible to somehow engineer a mechanism to down regulate this part of chromosome 21 , it could conceivably lead to some dramatic therapeutic results. A word of caution however: studies with mice in which the mir-155 gene have been " knocked out " have shown wide ranging problems with the immune system and the development of auto-immune disorders.[58] They developed lung disease and were less able to resist bacterial infections such as salmonella. Gene therapy obviously still has a long way to go !

Fig 4a. Nucleotide sequence and chromosomal location of miR-155.

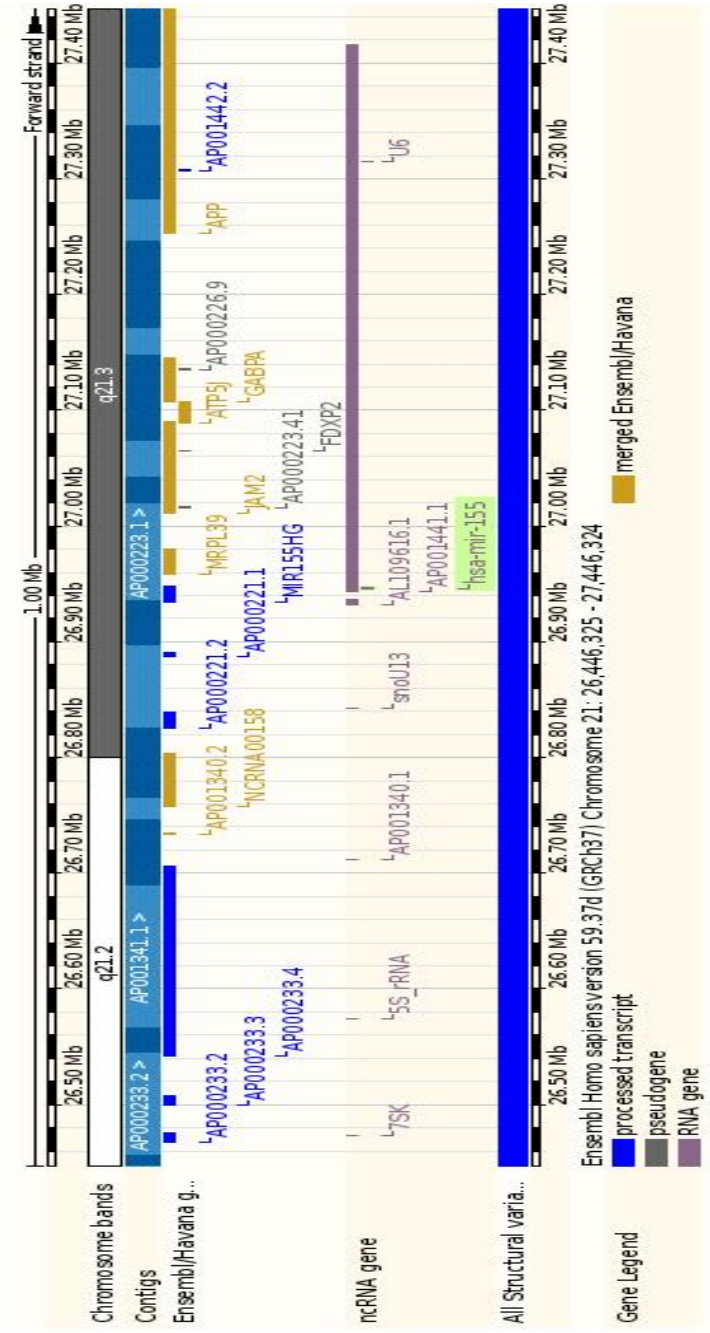
The pre mir-155 loop structure is :

```
     c   a        -u   c
cuguuaaugcuaau gug uagggguu  uug c
|||||||||||||| ||| ||||||||  |||
gacaauuacgauua uac auccucag  aac u
              -   -        uc   c
```

The mature form of mir-155 is :

uuaaugcuaaucgugauaggggu

Fig 4b. The gene for miR-155 is located on the non coding BIC region of chromosome 21



Equipment and Protocols used.

Objective : I would like to state my two main objectives in undertaking this research internship at this stage . (1) To learn and understand the experimental techniques and protocols used in cutting edge cancer biology experiments. (2) To use this new found knowledge to push forward our understanding of miRNA manipulation and their expression in cancer cells with special reference to miR-155 and miR-22.

The first steps I took in achieving my objectives was to spend time in the cancer biology laboratories getting trained by my supervisors in the lab techniques I would need to master. I have briefly described these below. I have split the description into two sections , one small section detailing the equipment I learnt to use and the other section with the experimental protocols that had to be followed.

1. **Centrifuge :**

   This is a piece of equipment, driven by an electric motor that puts an object in rotation around a fixed axis, applying a force perpendicular to the axis. The centrifuge works using the sedimentation principle, where the centripetal acceleration causes more dense substances to separate out along the radial direction (the bottom of the tube). Lighter objects will tend to move to the top.

2. **Gel electrophoresis**

   This is a technique used for the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein molecules using an electric field applied to a gel matrix. The gel is a crosslinked polymer whose composition and porosity is chosen based on the specific weight and composition of the target to be analyzed. When separating proteins or small nucleic acids (DNA, RNA, or oligonucleotides) the gel is usually composed of different concentrations of acrylamide and a cross-linker, producing different sized mesh networks of polyacrylamide. When separating larger nucleic acids (greater than a few hundred bases), the preferred matrix is purified

agarose. An electromotive force (EMF) is used to move the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an electric field, the molecules will move through the matrix at different rates, determined largely by their mass when the charge to mass ratio (Z) of all species is uniform, toward the anode if negatively charged or toward the cathode if positively charged.

3. Flow cytometry

This is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in both research and clinical practice. A beam of light (usually laser light) of a **single wavelength** is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle.

4. PCR

The **polymerase chain reaction** (**PCR**) is a scientific technique in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using heat resistant TAQ polymerase. . Primers (short DNA fragments) containing sequences complementary to the target region along with the enzyme are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

5. Real time PCR

Real-time polymerase chain reaction, also called *quantitative real time polymerase chain reaction* (Q-PCR/qPCR/qrt-PCR) or *kinetic polymerase chain reaction* (KPCR), is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample.

The real PCR procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in *real time*, a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

Frequently, real-time PCR is combined with reverse transcription is used to quantify messenger RNA and Non-coding RNA in cells or tissues.

Experimental protocols

1. **Plasmid Transformation :**

    a. Take competent cells out of -80 degree storage and keep on ice to thaw.
    b. Add plasmids to one of the tubes containing the component cells, using 25 mg of plasmid : 50 micro litres of competent cells. Mark other tubes as –ve control.
    c. Flick to mix.
    d. Keep on ice for 15 minutes
    e. Provide heat shock in the water bath at 42 degrees C for 90 seconds.
    f. Return back to ice for 5 – 10 minutes.
    g. Add 800 micro litre medium to tubes.
    h. Store tubes for 45 minutes to one hour at 37 degrees C in constant motion.
    i. Spin down in centrifuge and remove supernatants.
    j. Re suspend pellet.
    k. Plate the suspension onto an LB agar plate, with appropriate antibiotic.
    l. Invert the plates and store for 16 hours at 37 degrees C in the incubator.
    m. When the 16 hours are over, seal the plates and store at 4 degrees C.

    Points to remember :

    1. Return all pipettes to max value, as written.
    2. Keep other half of re-suspended solution at 4 degrees C till colonies are seen.
    3. Plates have to be sealed after 16 hours, or satellite colonies will form and contaminate samples.
    4. The ampicillin on the LB agar plates ensures that only cells with the survival factor imparted by the plasmid can survive.
    5. The heat shock is to seal the pores of the bacteria.

2. **Making of the agar plates :**

    a. Microwave the agar solution till it is fully liquid.
    b. Add ampicillin when the beaker can be touched with the bare skin, but before the agar solidifies.
    c. Mix thoroughly.
    d. Pour plates, seal with paraffin and store.

3. **Plating :**

    a. After resuspension of the pellet, take half of the solution in the pipette and spread evenly on the agar gel.
    b. Fire sterilize glass rod and first test on the gel before using it to spread the cells. Keep spreading till the solution is solid (it will feel rough).

4. **Inoculation of cells after transformation :**

    a. **Preparing LB (Laria Bertoni) broth :**

        (i) Take 150 ml of triple distilled water.
        (ii) Mix with 3.75 gms of LB broth powder.
        (iii) Shake thoroughly and didvide among three beakers(50 ml in each)
        (iv) Seal beakers with aluminium foil, paper and a rubber band.
        (v) Send for autoclaving.

b. Procedures :

For weighing out powder put a piece of paper on the weighing machine and tare it. Using the spoon, measure out the needed amount. Fold the paper and pour into the beaker. Wash spoon and discard the paper.

When measuring ml, take the reading always from the lower meniscus.

Use autoclave tape on the beakers.

After preparing broth add the Ampicillin as per the ratio

Ampicillin : Broth = 1 micro L : 1 ml

Pick a colony with the tip of the beaker, and drop tip into the beaker.

Store at 37 degrees C, in a shaking incubator for 16 hours.

5. Plasmid Isolation :

a. Spin down the bacterial pellet from the broth.
b. Remove supernatant.
c. Resuspend the bacterial pellet (Solution I) in cold alkaline lysis solution.
d. Resuspend the pellet but just with the tip.
e. Ratio to use of Solution I : LB broth = 2 ml : 50 ml.
f. Add 4 ml of Solution II, roll mix and store on ice for 3 – 5 minutes.
g. Solution II is to be 1 % SDS in 0.2 % normal NaOH.

h. Add 3 ml of Solution III and gently invert mix. Solution III is also a lysis solution used in conjunction with the other two.
i. Centrifuge at 12,000 rpm for 5 minutes. This will lyse the bacterial cells, releasing the contents of the cytoplasm. The rest of the steps are to separate the Vector.
j. Transfer the supernatant to a new tube.
k. Add 20 ml of RN-ase.
l. Incubate at 37 degrees C for 2 hours. This step is to remove the RNA.
m. Add equal amount of chloroform as there is liquid in the container.
n. Spin down at 8000 rpm for 5 minutes.
o. Remove the transparent top layer.

6. Gel Electrophoresis protocol :

a. Use 35 ml of 1 X TAE
b. Measure out 0.35 gms of gel powder.
c. The powder will dissolve when its microwaved.
d. Keep constantly swirling beaker till it can be held on the bare skin.
e. Add 10 micro L or 1 mg / ml.
f. Pour gel into the cast.
g. Mix the samples to be run.
h. Add dye in the ratio 1 : 5 of the total solution.
i. Add sample and buffer.
j. Load wells, one well with the reference DNA ladder.

7. Restriction Enzyme reaction :

a. First decide which enzymes to use, depending on availability.
b. Check cut zones on the vector.
c. Choose one zone in backbone and one in MCS(multiple cloning site)
d. Size should be big enough to be visible.
e. Find the solution at which enzyme reaction is compatible.
f. Add plasmid.
g. Add water.
h. Add buffer.
i. Add enzyme.
j. Top mix.
k. Spin down (small centrifuge).
l. Store at 37 degrees C for 2 hours.
m. Run gel and check size of fragments.

8. Elution (removal of DNA from agarose gel)

a. After restriction enzyme digestion of 2- 3 hours, run the product through a gel. The gel must be thicker than a check gel ,70 ml, 0.7 g and 20 micro L Etbr.
b. Cut out plasmid band from gel.
c. Add 500 micro L of chaotropic salt solution. The amount of salt solution used should be three times the volume of the gel.
d. Store at 55 degrees C till the gel dissolves completely.
e. Add glass powder, for the first 2 micro gm of DNA use 6 micro L and 2 micro Litre for every micro gm after that.
f. Spin down and remove supernatant.
g. Wash thrice with 500 micro L ethanol and buffer.

h. Add 20 ml water.

i. Keep at 55 degrees C in a water bath and tap mix every 5 minutes.

9. PCR volumes (with PFU Turbo Polymerase)

Ingredient	Total volume 10 micro L	Total volume 100 micro L	Total volume 200 micro L
H_2O	7 micro L	70 micro L	140 micro L
10 X buffer	1 "	10 "	20 "
d NTP	0.8 "	8 "	16 "
Genomic DNA	0.4 "	4 "	8 "
F Primer	0.3 "	3 "	6 "
R Primer	0.3 "	3 "	6 "
PFU Polymerase	0.2 "	2 "	4 "

10. Restriction Reaction volumes for miR – 155, with plasmid PSDNA3.1

Water	2 micro L
Elute	40 micro L
Buffer	5 micro L
Bam H1	1 micro L
Hind 3	2 micro L

11.Restriction Reaction volumes for miR-155, with plasmid pSRP

Water	4 micro L
miR-155 elute	20 micro L
Buffer 3	3 micro L
Hind 3	1 micro L
Bgl2	2 micro L

12. Gel check for pSRP

Water	7.1 micro L
Plasmid	0.5 micro L
Tango Buffer	2 micro L
Xho 1	0.2 micro L
Eco R1	0.2 micro L

13. Ligation volumes with Quick-Ligase (control with 12 micro L water)

Plasmid	2 micro L
Amplicon	10 micro L
Water	2 micro L
Buffer	5 micro L
Ligase	1 micro L

14. Luciferase Assay protocol

a. Inoculation of the plasmid with the microRNA into bacterial culture, for the normal 16 hour time period.
b. Plasmid isolation
c. Preparation of the HEK cells, involving rejuvenation by passaging and seeding, a procedure of about 8 hours.
d. Co Transfection of the human cells with the plasmids and the luciferase reporter contruct (another plasmid) and incubation for a 24 hour period.
e. Induction of micro RNA expression reactions, by addition of Lithium chloride for a 12 hour period.
f. Run sample through the luminometer and compare bioluminescence against control sample of normal HEK cells.

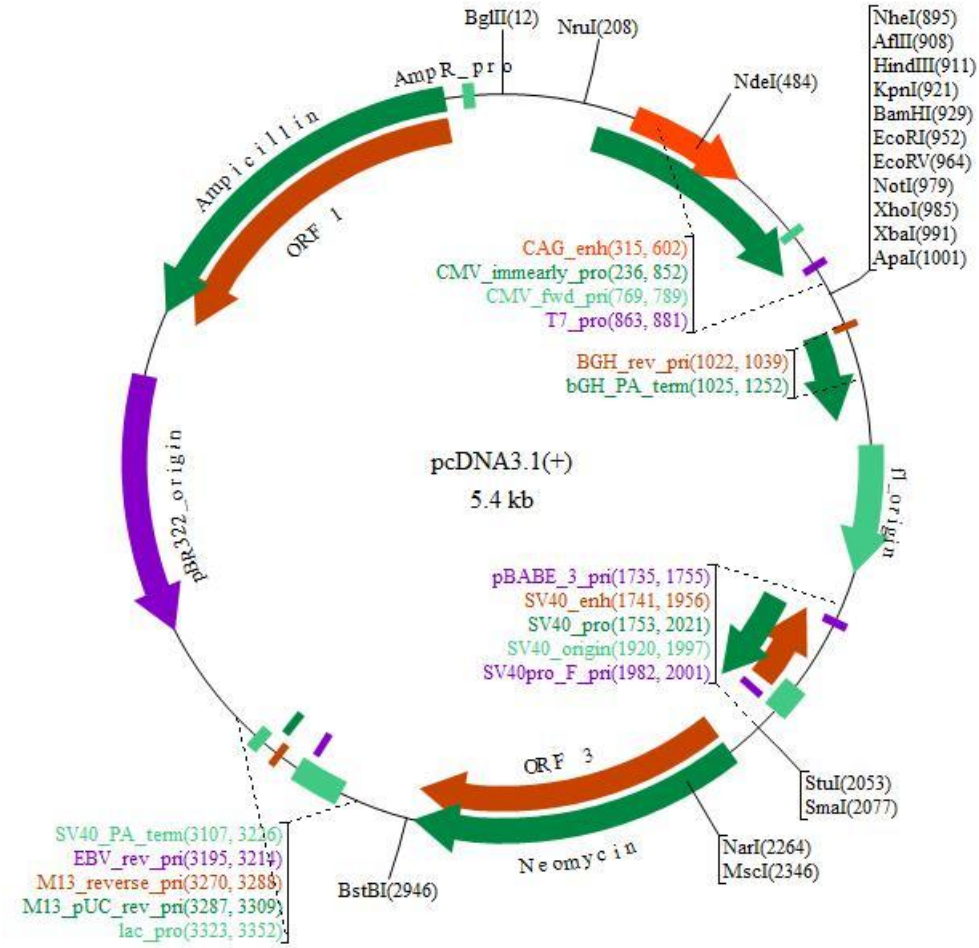
Experimental journal, results and conclusions.

My supervisor Dr Rao gave me a series of introductory lectures on the basics of genetics and the genetic foundations for cancer. As a first step we analysed the karyotype and fish study reports of my cancer so I could familiarize myself with the way chromosomal detaisl are analysed and reported. I was then introduced to the concept of a plasmid: covalently closed circular, bacterial DNA into which the gene for the miRNA to be tested has to be incorporated. This would be done using special enzymes called restriction endo nucleases. Using two distinct and different endo nucleases would create a spot or gap that only the DNA we wish to incorporate would fit into ,with its sticky ends in the right orientation. In my case this would be the gene that codes for miR-155 and for this appropriate primers (customized and produced to order) would need to be used. The vector or carrier, which is the plasmid would be treated with a special chemical that removes the 5 prime end phosphates , thereby reducing chances of re-ligation. We would be using E Coli in our cloning experiments, as this is a very common, easy to use bacteria, that replicates every 20 minutes. The most important, indeed crucial stage would be that of plasmid isolation, when the genetically engineered DNA is removed from the bacterial colonies. The plasmids used produce a substance called D-luciferin, a bioluminescent chemical originally from fire flies. This can be measured qualitatively with a scanner and hence makes a useful marker. My project will be to incorporate miR-155's gene into a plasmid vector and test the effect of its expression in a human cancer cell line , on proteins and subsequent proliferation abilities of the cell. I would spend the first two to three weeks of my internship assisting the Ph D students in the laboratory and learning the techniques by daily usage.

On August 10th I got my first opportunity to prepare for my own research , a dry run using a left over sample of plasmid ligated with miR-196. I had to introduce my vector with the DNA amplicon spliced into it, into bacteria, which then had to be plated and incubated overnight. I carried out all the various steps in the protocol carefully and slowly under the watchful eye of my supervisor but the experiment did not succeed and when we ran the eletrophoresis gel, I didnt get the correct length DNA out. Dr Rao explained that a possible reason for this could be because the initial ligation had not been done accurately. My bacteria did grow successfully, but the DNA could not be used.

I was able to start preparing the construct, the specific plasmid (PCDNA3.1) that I was to use, on August 12th. The plasmid was available but not in enough quantity, so I started my work by transforming bacteria : introducing the empty vector into them for amplification. I left the bacteria to incubate overnight as before , the cloning was a success and I was able to harvest a good number of bacterial colonies with the plasmid imbued survival factor to ampicillin in them. I stored the construct away in readiness to wait for the next stage. Primers had been ordered for miR-155 and once they arrive I will start the next, crucial ligation process. I was able to successfully separate my plasmids from the bacteria, mix with dye and run through the gel and ensure they were indeed the right size DNA, showing that my cloning had been successfully accomplished.

Fig 6. Plasmid PCDNA3.1 V5 His topo (modified)



.The next important stage of my work, which is to make the plasmid linear and check conclusively that it is the right size could be started only on August 24th, as I had to wait for fresh stock of the restriction enzymes. The plasmid had to be digested with two separate restriction enzymes PCDNA3 (Bam H1 and Hind3) and a purity check run using a spectrophotometer.

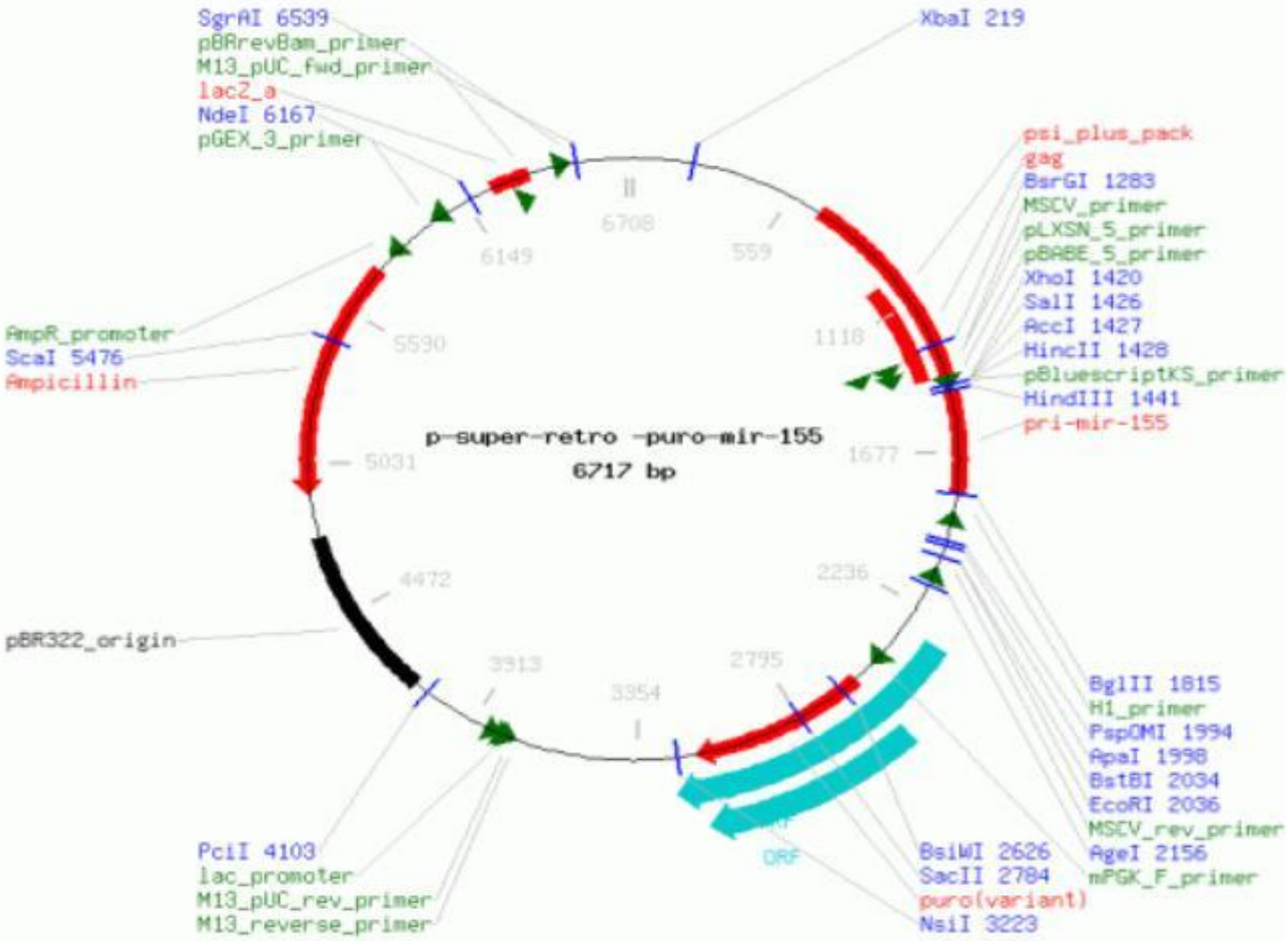
There is only a specific amount of light DNA can absorb, so that this makes a sensitive measure. We can check purity and estimate DNA by measuring the absorbance at 260 nm and 280 nm. The PCDNA3 restriction was contaminated with a lot of protiens from the cell after lysis and needed a second clarification with the centrifuge.

When I finally ran my restricted DNA fragments through the gel, it was possible to see that two reactions had worked, producing DNA fragments of the right size, but one sample had somehow failed. Dr Rao explained that sometimes factors come into play in these living systems that we cannot always account for.

I also started in parallel a second set of reactions using a retro viral vector that I had transformed and extracted in preparation earlier : p SUPER retro puro(pSRP) . These vectors are better able to be transfixed into human cells and my trial would be the first time that this vector is to be tried in Dr Karunagaran's laboratory. If my results were successful , this would be pioneering work for this lab.

pSRP is a plasmid that can be easily incorporated into cellular systems using retroviral vectors. This leads to better gene delivery and works in most cell lines. Upto now our lab had only used PCDNA3 in all the trials to date, but due to the pCDNA3's tendency for self-ligation , it was thought necessary to try a different plasmid. My supervisor was also very eager to see how my experiments would turn out, due to this reason.

Fig 7. The pSRP plasmid construct after the incorporation of miR-155.



I spent some time on the Fermentas website (the company that supplies the restriction enzymes) which has a detailed description of plasmids and their unique cut sites, so as to familiarize myself further with the theoretical basis for restriction enzyme choices that would enable to design the correct restriction sites when I am ready to insert my amplicon.

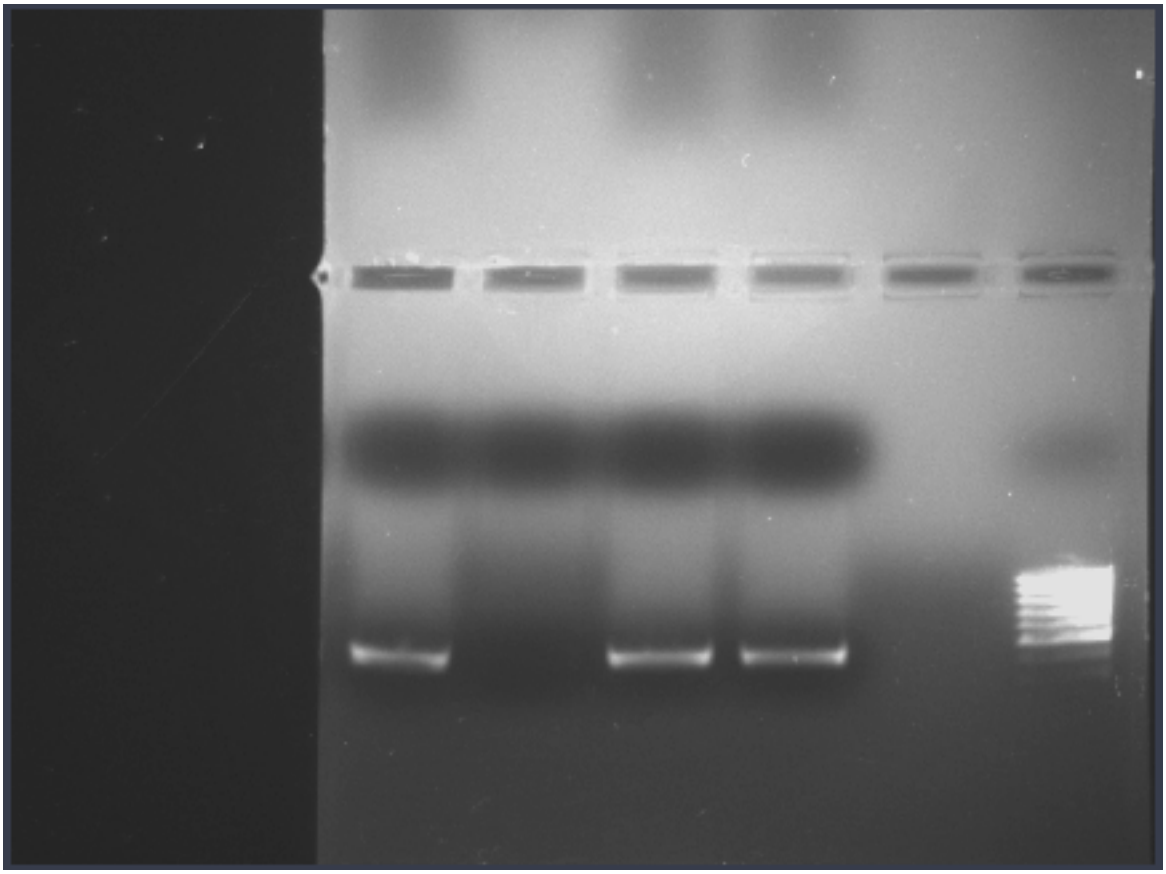
On August 25th., I started the theoretical planning work on designing my primers. In order to do this, we begin with the amplicon sequence, which is typically a few hundred base pairs long, with a flanking region on either side. After the flanking region are the sticky ends, which will attach only to the sites where my plasmid had been cut. I ran my restricted plasmid once again through the gel, to be doubly sure it was the right size. On Friday, August 27th, I was ready to set up the restriction reaction for the two plasmids I had cloned . I planned to use restriction sites BamH1 and Hind3 for the PCDNA3 plasmid. For the other plasmid, PSRP, I planned to use Hind3 and Bgl2 as my restriction sites.

On September 1st., my ordered primers were finally cleared through customs and would arrive in a day, so i was able to start planning for the transformation and subsequent cloning. The first step was to hydrate and dilute the primers to a usable level. I set up a test cycle with the PCR , then set my genomic DNA with the miR-155 gene spliced into it by using the primers. I checked the gel with the UV scanner and was happy to get a crisp, clean band at the right size which was about 500 base pairs. The test reaction on the PCR was with only 10 micro L and the next step will be running the PCR with 200 micro L in order to ensure a good enough quantity of the miR-155 gene , for incorporation into the bacterial cell.

When I ran the 200 micro L PCR result through the gel, I found only 3 of the 4 PCR tubes had worked and there had been no amplification in the 4th tube, for some unknown reason. I had to therefore run another complete set through the PCR in order to ensure I would have enough amplicon available for ligation.

The yield though better than the first run was still not very high. The gel checks showed my construct had succeeded and the combined yield was sufficient for the experiments planned.

Fig 8. miR-155 contruct against the 100 bp ladder, gel screen shot dated Sep 3, 2010.



On September 6th , I set up the ligation experiment with the first plasmid I had cloned , the PSDNA3. Each time, four plates have to be used. One is the actual experimental plate, one is a self ligation control and the other two are to be used for a positive and negative control. Self ligation is when the plasmid re-sticks to itself, without taking in the insert. The ingredients of importance are the plasmid, the amplicon I had designed and engineered and the all important enzyme : ligase.

My bacterial plates had grown well, and the plate which had plasmid plus insert on it had more colonies than the control with only plasmid, which was a hopeful sign.

Fig 9. My colony plate for first trial set, plasmid PCDNA3.1 with miR-155

The next step was plasmid isolation, on 20 colonies picked at random. This is a day-long procedure involving total cell lysis . At the end of the day when I ran the gel however only one of the 20 colonies produced a " pop-out " of the right size so I decided to repeat the isolation step again, after giving the one colony for sequencing and checking. The result was not what I had hoped for, the one colony upon sequencing revealed itself to be random cellular DNA and not my amplicon at all. The repeated 20 colonies after a further day's work , also showed only self ligation.

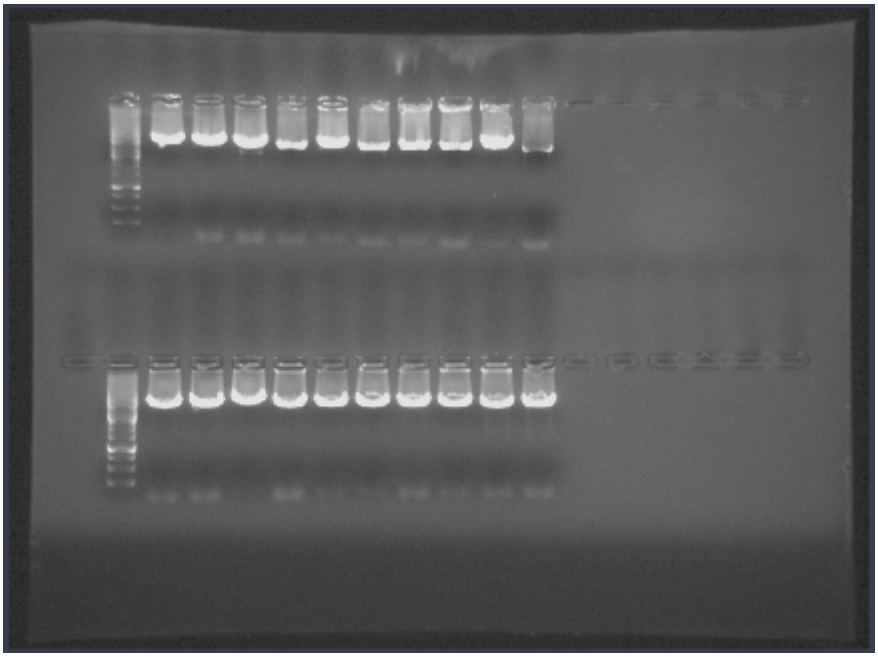
Restriction Enzyme Screening of the PCDNA3.1 plasmid , for 20 colonies

Plasmid	5 micro L	115 micro L
Water	3.7 micro L	85.1 micro L
Bgl2	0.3 micro L	6.9 micro L
Buffer	1.0 micro L	23 micro L
Totals	5 micro L	115 micro L

Restriction Enzyme Screening of the PCDNA3.1 plasmid , second 20 colonies

Plasmid	5 micro L	110 micro L
Water	3.7 micro L	81.4 micro L
Bgl2	0.3 micro L	6.6 micro L
Buffer	1.0 micro L	22 micro L
Totals	5 micro L	110 micro L

Fig 10. PCDNA3.1 gel check showing self ligation (no pop outs) Sep 15th, 2010



On September 15th I started the long process of ligation all over again, with the second plasmid PSRP. After repeating the entire sequence from restriction through transformation to inoculation and plasmid isolation, which took three days, I was deeply disappointed when the gel run showed pop outs of 900 base pairs, which was clearly wrong as I was expecting a pop out of around 400 bps. 900 bp is mathematically impossible with PSRP and the restriction enzymes I had selected and the only possible explanation was that my PSRP plasmid samples had somehow been contaminated with the PCDNA3 plasmid. This must have self ligated as before and with the Hind3 and Bgl2 restriction enzymes had come out with a 900 bp pop out and a 5.5 Kbp reminder.

My supervisor was as disappointed as I was and told me it was possible that the cross contamination could have occurred in the inoculation hood. I decided to repeat the experiment a third time safe guarding as far as possible from contamination and started this set of experiments on Saturday, September 18th.

Restriction Enzyme Screening of the pSRP plasmid , 20 colonies

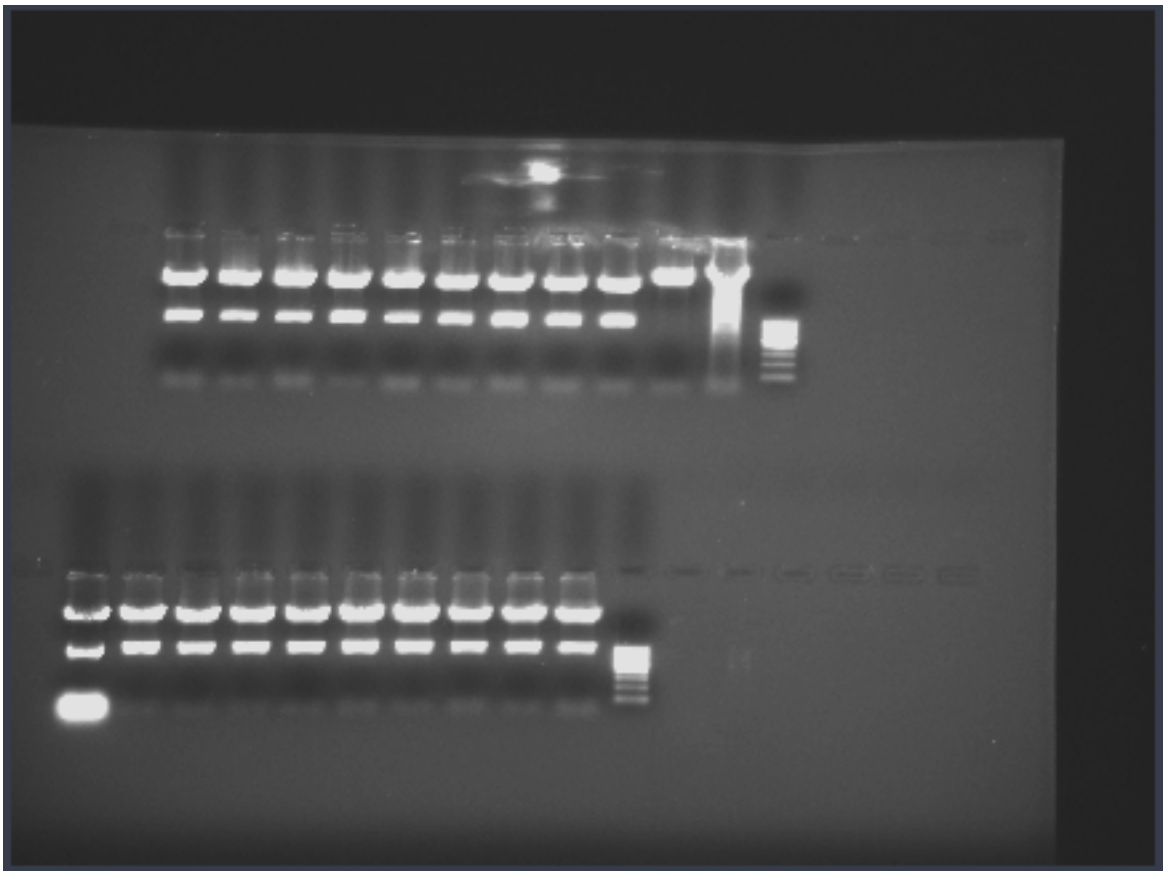
Plasmid	5 micro L	115 micro L
Water	3.1 micro L	71.3 micro L
Bgl2	0.6 micro L	13.8 micro L
Hind 3	0.3 micro L	6.9 micro L
Buffer	1 micro L	23 micro L
Totals	5 micro L	110 micro L

Fig 11. My colony plate for plasmid pSRP with miR-155

Working all weekend I was able to start the plasmid isolation step on Monday, September 20th. My supervisor Dr Rao, spent the three days with me , every step of the way , so I could be sure there was no chance of any handling errors, due to my inexperience. The gel run results however were a little unexpected. Instead of a sharp band at 400 bps, I got instead slightly diffuse broad bands from 400 to 900 bps in 6 tubes.

I would only know for sure whether my mIR-155 gene had ligated in or not, after sequencing the samples. Among the 20 tubes there was one self ligated plasmid and hence a no pop out result, which was confirmation that pSRP had indeed got into the bacterial cell, but of course the more important point was whether my miR-155 had got in as well and only sequencing could answer that all important question.

Fig 12. pSRP gel check showing pop out band



The research work on miR-155 had to be put aside at this stage, due to problems with the sequencing apparatus. I was however able to carry out the luciferase assay on a previously successfully ligated plasmid and complete my learning curve.

An miR-22 "sponge" had been ligated previously into a PCDNA3.0 plasmid and was ready to be used. The plasmid was designed in such a way so as to have 3 regions that will produce RNA strands that are the exact complement of miR-22. The theory is that their expression should theoretically be sufficient in quantity to shut off any naturally occurring miR-22 before it can be effective. If the sponge works then a similar sponge could then also be designed and procured for the other microRNA the lab is working with including miR-155.

I grew the colony overnight and was able to successfully isolate the plasmid on September 22nd. The HEK cells were rejuvenated by overnight passaging and allocated into 24 wells, with 1 lakh cells in each well. The cells had to allowed to grow and become confluent for 24 hours, at which point the cells were rendered permeable and three plasmids (with Beta galactose, topflash and the plasmid with the sponge gene) incorporated over a further 24 hours. Micro RNA gene expression was induced in both the controls and samples for a period of 12 hours at the end of which the luciferase assay was carried out.

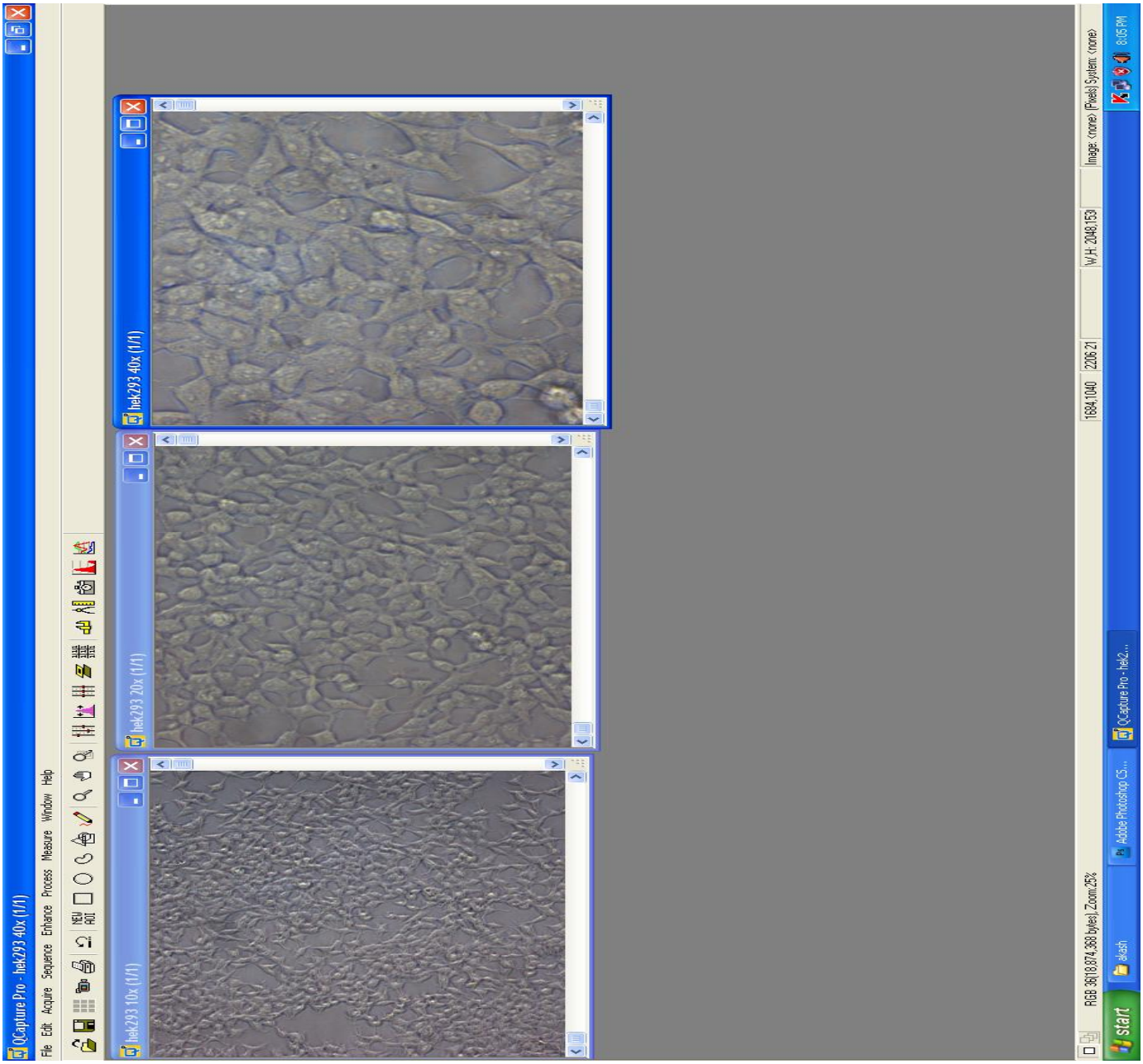


Fig 13. HEK (Human embroyonic Kidney) cells magnified X10, X20 and X 40

The results of the luciferase assay are shown below . Cancer initiation signals are seen to be clearly reduced in all the samples analysed, when compared

with the controls and the inference is that this sponge was ineffective in stopping the miR-22 expression. For future trials it would therefore be necessary to use perhaps a 6X or a 9X sponge for effective miRNA silencing.

	Data generated by Luciferase Assay, using Biorad luminometer , date Sep 26th, 2010.							
		Temperature(°C)	1	2	3	4	5	6
		24.7	145.3333					
Luc			15276553	14686700	5205955	6002962	6140592	
B gal		0.058	0.699	0.709	0.427	0.426	0.431	
		correcdted	0.641	0.651	0.369	0.368	0.373	
normalized			23832376	22560216	14108280	16312397	16462712	
	avg		23196296			15627796		
			1			0.673719		
Samples 1 and 2, are the controls, normal HEK cells								
Samples 3,4 and 5 contain plasmids with the miR-22 3X sponge ligated.								

Fig 14. Luciferase assay data on 26-09-10

Conclusion : the 3 X sponge was unable to shut miR-22 off completely, for future trials it would be necessary to use 6 or 9 X sponges .

Future Possibilities of miRNA

As new roles for miRNAs in cancer continue to be discovered, their future effect on diagnosis, prognosis, and treatment of patients offer exciting possibilities. miRNA profiling holds potential for differentiating between normal and tumor cells and even between different tumor subtypes. miRNA expression profiling can classify several different human cancers, including poorly differentiated tumors, as well as distinguish them from benign tissue [(59)] . The ability to classify over 22 different tumor types using only 48 different miRNAs holds promise for patients with carcinomas of unknown primary causes. [(60)] With an incidence of up to 4% in all newly diagnosed cancers, carcinomas of unknown primary are a diagnostic and treatment challenge.

miRNAs may also be useful as prognostic or predictive tools in cancer. A five-miRNA signature has been reported to predict disease-free and overall survival in resected NSCLC (Non small cell lung cancer) patients[(60)] .These miRNA signatures may become potential tools for therapeutic decision making. A true technical advantage for miRNA profiling is that their small size lessens their susceptibility to degradation in paraffin-embedded, formalin-fixed tissue, allowing for wider application of miRNA profiling on banked tissue samples ensuring the ability to effectively utilize saved samples to validate future research [(61)] .

More important than their use as determining prognostic factors, miRNA expression may actually help oncologists determine the best course of treatment. In a retrospective study of advanced NSCLC (Non small cell lung cancer) patients treated with an epidermal growth factor receptor tyrosine kinase inhibitor, both response and survival significantly correlated with tumor miR-128b loss of heterozygosity[(62)]. Another study determined that let-7i, miR-16, and miR-21 expression levels affect the response of tumor cell lines to several different anticancer agents. [(63)]

The potential to use miRNAs as biomarkers for disease is strengthened by their stability in human serum and plasma [64][65]. Instead of using invasive procedures to extract tissue from patients' tumors, miRNAs can be measured directly from the patients' blood products. Using Solexa sequencing, the miRNA expression profile from serum of healthy individuals was shown to be significantly different from that of patients with lung cancer, colorectal cancer, and diabetes [65] . Another study investigating the expression of miRNAs in plasma found that miR-141 can serve as a biomarker for prostate cancer. [64]

miRNAs may one day even be administered in cancer therapeutics either as single agents or in combination therapies. Studies have already shown that miRNAs can play a direct role in drug effectiveness. By overexpressing let-7 in lung cancer cell lines and in C. elegans , let-7 can repress resistance to radiation therapy both in vitro and in vivo [66] delivery systems such as locked nucleic acid increase the stability of miRNAs for in vivo application [67] and may be promising as cancer therapy.

Once we are better able to understand these magical little molecules, several tools to improve cancer treatment may be on the horizon covering diagnostic, prognostic and predictive measures. A world where cancer is preventable in many instances and definitely curable in all its manifestations will truly be within our grasp.

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