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Final Report

microRNA transcription in primary vs transformed cells in response to radiation

By

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"Medical and Pharmaceutical Biotechnology"

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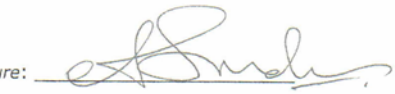
Unterschrift/*Signature*: 

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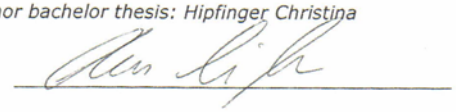
Unterschrift/*Signature*: 

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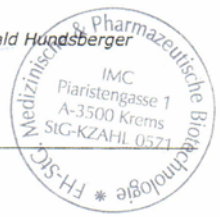

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Date: March 15th, 2016 Christina Hipfinger

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Abstract

The aim of this thesis is to find the expression levels of microRNA 494 and microRNA 103 after irradiating different cell lines and comparing those.

It is known, that miR-494 and miR-103 are up regulated in endothelial cells post radiation. These miRNAs have been discovered to cause senescence and apoptosis in response to DNA damage. Discovering this mechanism leads to the idea of combining chemotherapy and irradiation in cancer patients for thorough attack of cancer cells and tumor microenvironment.

This thesis examines the expression of miR-494 and miRNA-103 in primary cells and tumor cells and presents the results. Secondly an In-Situ Hybridization in HUVECs was introduced to compare the levels of the miRNAs expression from the results of cell culture vs. the results from the In-Situ Hybridization in HUVECs. All the experiments were carried out post radiation of 2 Gy and 20 Gy and comparing those to a control without radiation. The primary transcripts of miR-494 and miR-103 (pri-miRNA) were looked at after 1 hour and their mature forms were examined 6 hours post-radiation.

The results obtained in these experiments show that in a few cell lines there was microRNA regulation after radiation, however other cell lines do not show any significant differences. Further analysis have to be done in some cases where the pri-miR-494 and pri-miR-103 are being transcribed before radiation but the mature form is not being expressed after 6 hours post-radiation. There are many factors that need to be further analyzed if there are other transcription factors that are suppressing the expressions of these mature forms.

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List of Abbreviations

miRNA	micro-ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
snRNA	small nuclear RNA
sRNA	small RNAs
RNAi	RNA interference
GTP	guanosine triphosphate
GDP	guanosine diphosphate
bp	base pairs
RISC	RNA induced silencing complex
RLC	Risc loading complex
PACT	protein activator of PKR
PKR	protein kinase RNA-activated
MRE	miRNA recognition element
UTR	untranslated region
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase PCR
TME	Tumor microenvironment
ROS	reactive oxygen species

1. Introduction

The first microRNA discovered was in 1993 in the nematode C. elegans. This miRNA was called lin-4 as it inhibited the lin-14 messenger RNA, which is important for the nematodes development from first to second larval stage. An additional miRNA, let-7, was discovered in 2000 and had the same function as lin-4 namely suppressing gene expression. Many years after the discovery of the first miRNA over 1600 miRNAs have been found in animals, plants and viruses (Du & Zamore 2005).

MicroRNAs are small single-stranded non-coding RNAs of 20-23 nucleotides. They regulate protein-coding genes through mRNA degradation or by inhibiting the translation of mRNA. The translational repression results through the miRNA binding a complimentary sequence to its mRNA target forming a partial complimentary double RNA strand. The formation of the double strand has an effect of lower protein expression, as the translation of this mRNA is inhibited (Ventura & Jacks 2009).

The second function of miRNAs translational repression is their influence on the mRNAs stability consequently causing their degradation through deadenylation and recapping (Filipowicz et al. 2008).

Knowing that miRNAs influence gene expression, these could be used as therapeutics to target or suppress particular proteins. This results in an urge to investigate to find targets and different pathways and how miRNAs work together.

1.2 microRNAs biogenesis

microRNAs are found in a high number of different loci in the genome of vertebrates, when being transcribed (Ramalingam et al. 2014). We could classify microRNAs regarding the regions they are located as intergenic regions, introns, non-coding genes and in exons (Lee et al. 2007).

A few miRNAs form families who share the sequence nucleotides. Additionally they form clusters that have the same transcriptional process. Nevertheless due to post-transcriptional modification not all members of the same cluster are expressed equally (Slezak-Prochazka et al. 2010).

When miRNAs are being transcribed from introns they depend on the host genes transcriptional mechanism, whereas other miRNAs can be expressed freely and independently from their hosts transcriptional mechanism, as the miRNAs have their own promoters (Bartel 2004).

1.2.1 Pri-miRNA transcription

The biogenesis of the miRNA starts off with the miRNA genes transcription into primary-miRNA (pri-miRNA) (Figure 1). RNA polymerase II or RNA polymerase III initiate the transcription of the genes coding the miRNAs (Finnegan & Pasquinelli 2013).

The transcribed pri-miRNAs have a hairpin structure of ~ 80 base-pairs. The pri-miRNA has a shape of two-single strands forming a stem and ending in a terminal loop. Within the pri-miRNA is the sequence for the mature miRNA. Drosha is the enzyme which completes the maturation of the pri-miRNA into the pre-miRNA through cleavage (Ha & Kim 2014).

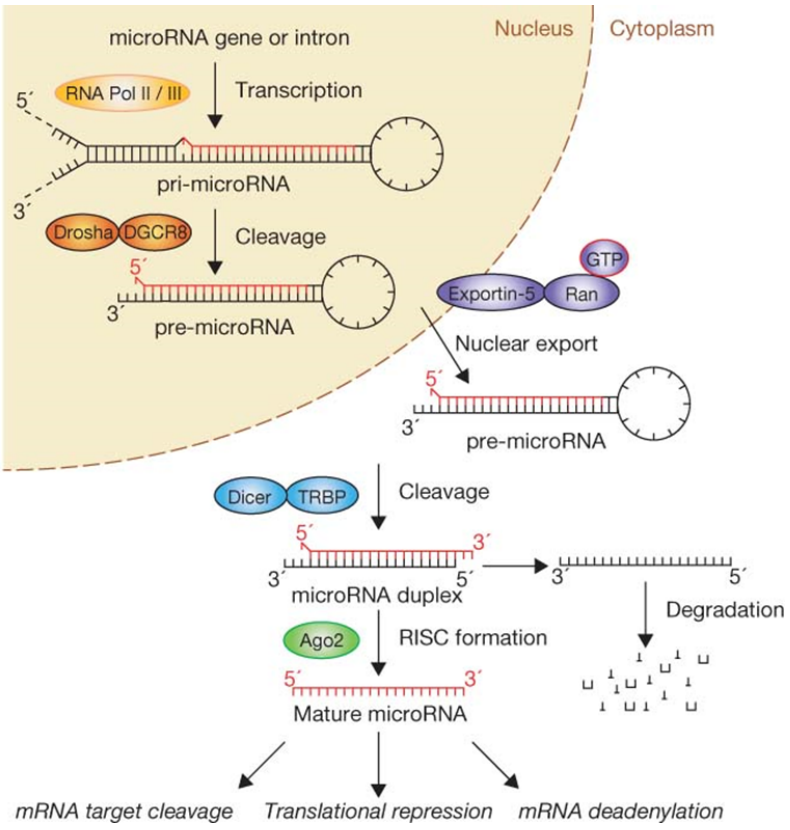

Figure 1 microRNA biogenesis

1.2.1.1 Drosha and Pasha

Drosha and Pasha (DGCR8) are RNAse III enzymes that build a complex that is known to cleave the pri-miRNA. The pri-miRNA is cleaved by Drosha at the 5´and 3´arms of the hairpin structure of the primary transcript (Winter et al. 2009). The hairpin is cleaved at about 11 base-pairs (bp) from the intersection between single and double stranded RNA and 22 bp from the position before the terminal loop (Ha & Kim 2014).

The Pasha enzyme works by defining the explicit point where the pri-miRNA is cleaved into the pre-miRNA thus resulting in a length of 60 – 70 nucleotides (Winter et al. 2009). The procedure of the pri-miRNA being cleaved into the pre-miRNA occurs within the nucleus. After the pri-miRNA is cleaved into the pre-miRNA, the pre-miRNA is transported from the nucleus into the cytoplasm where the maturation of the pre-miRNA occurs (Ha & Kim 2014).

1.2.1.2 microRNAs in introns

miRNAs that are found in introns use the transcription units of their host mRNA for their own development. The intronic miRNAs and their host mRNAs share the same mechanisms for regulation and expression patterns.

Other miRNAs, which are not transcribed as their host gene, may be transcribed by separate transcription units. These would be called intergenic miRNAs compared to intronic miRNAs, which have the essential difference that the intergenic miRNAs are transcribed by independent transcription units. Intergenic miRNAs have their own promoters in comparison to intronic miRNA. In the case of intergenic miRNAs these use POL II to be transcribed into pri-miRNA. However, in some cases intronic miRNAs do not transcribe at the same time that their host genes, it has been proven in

the case of human intronic miRNAs called mir-106b, mir-93 and mir-24-1 can be transcribed separately from their hosts transcription mechanisms (Altuvia et al. 2005).

1.2.1.3 miRtrons

MiRtrons lay in introns and are known to have a different pathway to develop miRNAs. They form a structure comparable to pre-miRNA skipping Drosha, the canonical pathway for microRNA processing, cleaving the primary hairpin structure into a pre-miRNA. This pathway skipping the cleavage through Drosha suggests that it was developed before Drosha starting playing a roll in the biogenesis of miRNAs (Ruby et al. 2007).

1.2.1.4 Exportin-5

The transportation of the pre-miRNA from the nucleus into the cytoplasm is carried out by the enzyme Exportin-5 (Kim 2005). In addition, exportin-5 transports short hairpin RNAs into the cytoplasm. This enzyme belongs to the Karyopherin family known to carry out transportations of noncoding RNAs, tRNAs, sRNAs and rRNAs from the nucleus into the cytoplasm. The Karyopherin members use Ran GTPase, which moves their cargo into the cytoplasm. In order to release what the Karyopherin members are carrying the RAN-GTP is hydrolyzed into RAN-GDP (Du & Zamore 2005). Exportin-5 is also expected to be a protection mechanisms for the pre-miRNA from being digested in the nucleus (Winter et al. 2009).

1.2.1.5 Primary transcripts

A primary hairpin transcription can be from one gene or even the same gene could transcribe 3 hairpins producing 3 different mi-RNA genes. This would be called Poly-cystronic translation.

Drosha additionally recognizes hairpin structures of pre-mRNAs within the introns which should be processed into mRNAs for further protein translation (Bartel 2004).

1.2.2 MicroRNA maturation: Dicer and RISC

Another enzyme contributing to miRNA biogenesis is the RNAse III enzyme, Dicer. Dicer cleaves the pre-miRNA in the cytoplasm leading to a mature miRNA (Kim 2005). The RNAse Dicer, the double-stranded RNA binding domain proteins TRBP, PACT (protein activator of PKR) and Argonaute-2 form the RISC Loading complex.

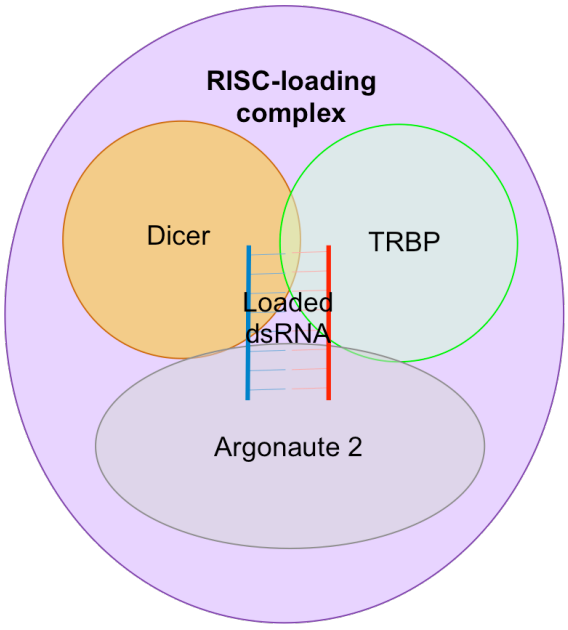

Figure 2 Components of RISC-loading complex

The role of TRBP and PACT is for them to give stabilization for Dicer and also to assist an easier cleavage of the pre-miRNA. After the cleavage through Dicer, the enzymes Dicer, TRBP or PACT remove themselves from the miRNA precursor, which at this moment is still double stranded. A helicase then unwinds the double strand leaving one guide strand and one passenger strand. The passenger strand is degraded and the passenger strand was discovered to form a complimentary strand to its target mRNA. From the two resulting strands the one with less stable base pair at the 5´end is then integrated into the RISC (Winter et al. 2009). This RNA Induced Silencing complex then transports the miRNA to its target mRNA resulting in repression or cleavage if the mRNA. This translational inhibition was discovered in plants and animals (Du & Zamore 2005).

1.2.3 Argonaute family

PAZ and PIWI are two domains that belong to the Argonautes. AGO and the PIWI form the subfamilies of the Argonaute family (Liu et al. 2004).

The subfamily PIWI control germ cell development, silencing retro-transposons and additionally cell renewing. These proteins of the PIWI family belong to the non-coding RNAs and are known as piRNAs.

RISC contains AGO proteins which are important for stabilization and work as endonucleases which in the RISC causes translational repression of the degradation of mRNA (Meister et al. 2004).

1.3 Difference between siRNA and microRNA

miRNAs are known to be single stranded and within animals they bind to their target mRNA with their complimentary strand which is not a perfect match but still acts as a repression mechanism avoiding its targets translation (He & Hannon 2004).

RNA interference (RNAi) is an additional term for translational repression induced by sRNAs. Small interfering RNAS (siRNA) are double stranded RNAs, which carry out this type of translational repression. This mechanism starts with one of the two strands integrating itself into the RNA-induced silencing complex. This RISC is similar to the one found in the mechanisms of miRNAs in the biogenesis pathway including RNAse III and Dicer. Once integrated into RISC this then cleaves the target mRNA thus resulting a post-transcriptional gene silencing.

miRNAs and siRNAs have a similar way of working but differ from their derivation. miRNAs develop from precursor pri-miRNAs within the genome which are then cleaved to form the pre-mirRNAs. These are then cleaved again within the cytoplasm into the mature miRNA.

siRNA originate from long double stranded RNAs that are artificially introduced into the cell or derive from mRNAs, transposons, viruses or heterochromatic DNA (Bartel 2004). Many siRNAs can develop from each strand of the double stranded RNAs. In contrast miRNAs can only establish one mature miRNA from one precursor.

An additional difference between siRNAs and miRNAs is how they bind to their target mRNAs. The strands of miRNAs are partially complementary but bind preferably to the 3-UTR region of their target mRNAs causing a translational repression. On the contrary, siRNAs form perfect complimentary strands to their target forming a double strand at one site causing the cleavage of the target mRNA at this specific point (He & Hannon 2004).

It has additionally been shown that miRNA sequences are mostly conserved in related organisms. Endogenous siRNAs however are hardly conserved (Bartel 2004).

1.4 miRNA function

miRNAs control protein translation by repressing the mRNAs translation in animals or by causing the mRNAs degradation in plants.

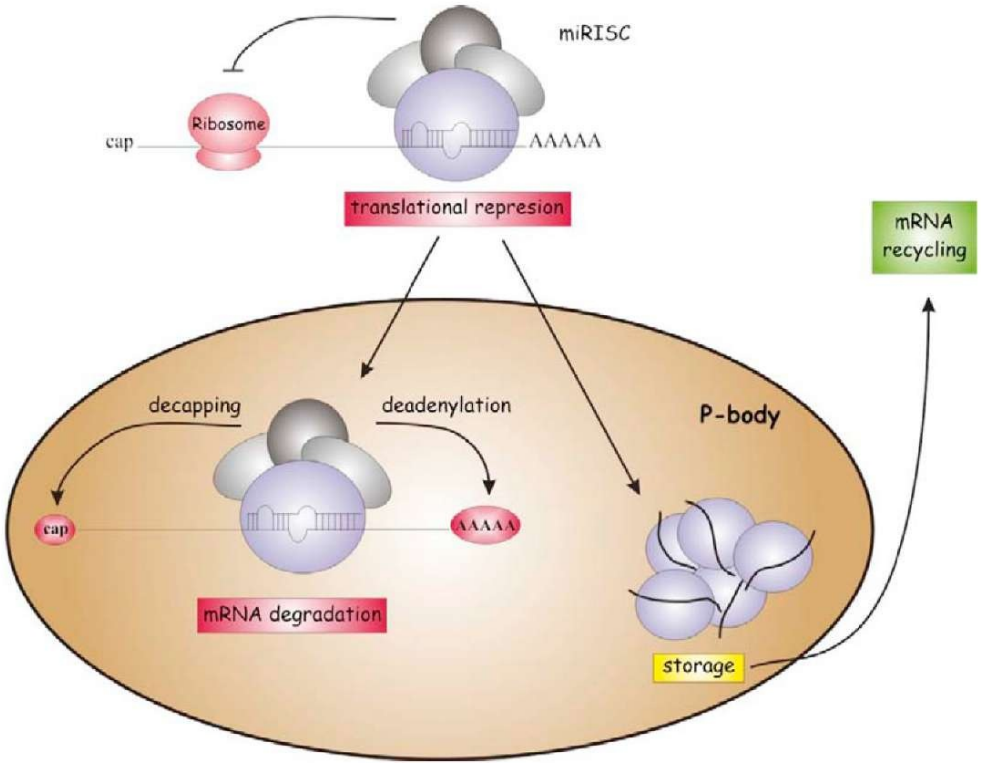

Figure 3 Translational repression and mRNA degradation through miRNA inhibtion

1.4.1 microRNA degradation

The degradation of the mRNA through the miRNA causes a down regulation of gene expression. When the mature miRNA integrates into the RISC this then determines the influence on the target mRNA. When the miRNA-mRNA is highly complimentary then the mRNA will be cleaved (Bartel 2004).

When the mRNA is deadenylated this causes the mRNA to be unstable following its degradation (Wilczynska & Bushell 2014).

After cleaving its target mRNA, the miRNA continues binding to other target sites for additional degradation.

1.4.2 Translational inhibition

It appears in various experiments, carried out by different laboratories, that the translational inhibition by miRNAs happens before mRNA degradation (Hu & Coller 2012). The miRNA integrates itself into the RISC with help of the Argonaute protein family causing the binding of this complex to the 3`-UTR of the target mRNA (Wilczynska & Bushell 2014).

1.4.3 Recognition of targets and the seeding site

The sites, where the miRNAs bind with their complimentary strand to their target mRNA in order to inhibit the mRNAs translation or to cause its degradation, are called seed sites. Where the base pairing of the strands between the miRNAs and their target mRNAs are in the 5´end is where the seed site is pairing with the 2-7 or 8 nucleotides miRNA recognition element (MRE), which is in the mRNAs 3´-UTR region.

The seed sequence is where the miRNAs will recognize and pair up with their targets but sometimes, an even larger amount of base pairing is needed between the miRNA and the MRE (Mourelatos 2008).

Presumably miRNAs can bind up to hundreds of target mRNAs and inhibit their translation. Then again numerous miRNAs can target only one mRNA. There miRNAs are then either working together to repress the translation of one target or they work individually in repressing the same target (Liu 2008).

1.5 miRNAs evolution

Genes are known to code for proteins and proteins process the genetic information. Throughout evolution there are genetically changes and therefor new proteins develop (Niwa & Slack 2007).

Many different miRNAs were discovered to regulate several functions in animals such as development, differentiation and growth. It is known that miRNAs constitute about 1-5% of all animal genes (Niwa & Slack 2007).

Higher and lower organisms have shown through analyzing them, that they both encode most protein-coding gene families like transcription factors and signal transduction molecules. Both encode most proteins even though the higher animals are morphologically more complex than lower organisms explaining that the protein coding genes within some taxa does not depend on how complex the animals became or developed during evolution. This indicates that gene expression in higher animals is regulated by another way with splicing and non-coding RNAs.

1.6. miRNAs conservation

In the cell miRNAs control a huge amount of processes for example proliferation, differentiation, apoptosis and several others. The miRNAs have in common to go through more or less the same maturation process to end up in a mature miRNA.

Many miRNAs sequences are conserved in their mature form in a variety of organisms and seem to have changed throughout evolution of multicellular organisms in controlling gene expression. This suggests that the expression of miRNAs and their different targets associate with the organisms' complexity.

Closely related species in metazoans share a homology in their miRNA sequences, pathway and structures. This indicates that the genetic information is preserved in related species. The let-7 family is found to be the most commonly shared conservation in metozoa (Lee et al. 2007).

1.7. Oncogenes and tumor suppressors

Over 50% of miRNA encoding genes lay in genomic regions that are cancer related or sites that are found to be fragile. This knowledge leads to the idea of miRNAs influencing cancer development and progression.

When miRNAs were discovered an additional experimental approach was to find out if the deletion of miRNAs promotes tumorigenesis. After examining for example miR-15a and miR-16 it was shown that these miRNAs intensify the apoptotic response by targeting the Blc-2 gene which is known to be an anti-apoptotic gene (Zhang et al. 2007).

Additionally the 3'-UTR of oncogenes are shortening and causing to loose the miRNA binding sites which results in up regulation of some oncogenes in cancer cells (Wilczynska & Bushell 2014).

The lineage is maintained in mammals during development as they are expressed in tissue-restricted pattern development. Within tumors a considerable amount of miRNAs are notable decreased leading to the thought that there is less differentiation in cancer cells, which is typical for cancer.

Very well known oncogenes come from the ras gene family in particular there are three closely related genes called N-ras, H-ras and K-ras. Ras genes promote other genes to proliferate, grow or cell-survival. When these Ras genes are mutated these could cause and up regulation resulting in cancer cells growth (Bos 1988).

In mammals the previously described Ras oncogenes have target sites for let-7 suppressing cell proliferation in differentiated cells. However in specific lineages tissue restricted miRNAs lead to cell differentiation (Hammond 2006).

1.7.1 Analysis of miRNA expression

As miRNAs have become more popular in investigating over the last few years, researchers use different methods for analyzing and interpreting the expressions of miRNAs.

One of the frequently used methods is with the quantitative Polymerase Chain Reaction (qPCR). With this technique miRNAs expressions of precursors (pri- and pre-) and their mature form are analyzed. When examining the differences of tumor cells versus primary cells the miRNA expression levels are compared.

An additional method showing the location and expression of miRNAs in tissue samples is by carrying out in situ hybridization.

A technique for detecting the miRNA-mRNA interaction is introducing artificially antisense RNAs (miRNA inhibitors) that bind to its targeting miRNA and therefor inhibiting and causing a loss of function.

When wanting to research whether miRNAs or the target genes influence cancer development point mutants in miRNAs or in their target 3'-UTRS are introduced. The binding between miRNAs and the target mRNAs occurs in the seed sequence thus causing mismatch between miRNA and mRNA decreasing the miRNAs influence on its targets gene expression (Zhang et al. 2007).

1.7.2 microRNAs as Oncogenes

In cancer cells the miRNAs expression levels influence the progression of the pathology. Comparing the expression levels of cancerous cells and primary cells we find that miRNAs are expressed differently. Taking a look at the same miRNA in a cancerous cell or in a primary cell even then they could be found to have different levels of expression. These miRNAs that have higher expression levels in tumors are re-

ferred as Oncomirs. The function of oncomirs is to inhibit tumor suppressor genes, which results in promoting tumor growth. This tumor suppressor genes would normally detect a defected cell causing this cell to undergo apoptosis or any other protection mechanism when found differentiated (Zhang et al. 2007).

1.7.3 microRNAs as tumor suppressor genes

Specific miRNAs that have lower expression levels in cancer cells are named tumor suppressor miRNAs. These tumor suppressor genes would usually inhibit the cancer cells from differentiating by blocking the cells and leading them to undergo apoptosis.

Let-7 is an example for a tumor suppressor gene. In lung cancer cells this tumor suppressor genes expression is blocked but when experimenting with an in vitro model using A549 cells (lung adenocarcinoma) it stopped proliferation. The RAS oncogene is one of this miRNAs targets. RAS is known to be involved in proliferation and enhancing tumor growth but the binding of Let-7 to the 3'-UTR of RAS reduces the tumor proliferation.

A novel idea for cancer therapy is to introduce miRNA mimics or inhibitors to either suppress oncogenes or induce apoptosis (Zhang et al. 2007).

1.7.4 The impact of irradiation on cells

It has been discovered, that radiotherapy not only has an impact on the tumor itself, but also on the tumor microenvironment (TME) which may be decisive in either promoting or decreasing the tumor growth. This is due to the fact, that radiotherapy also triggers certain responses in the TME.

When tumor endothelial cells are destroyed by radiotherapy and undergo apoptosis this triggers the inflammation cascade. Irradiation additionally destroys blood vessels affecting the vasculature and angiogenesis of the tumor. This also activates the immune response by higher production of cytokines and chemokines thus recruiting immune cells as well which is regulated by reactive oxygen species (ROS) and nuclear factor-kB(NF-kB). This stress signaling then causes the cells to either undergo apoptosis or senescence. (Barker et al. 2015)

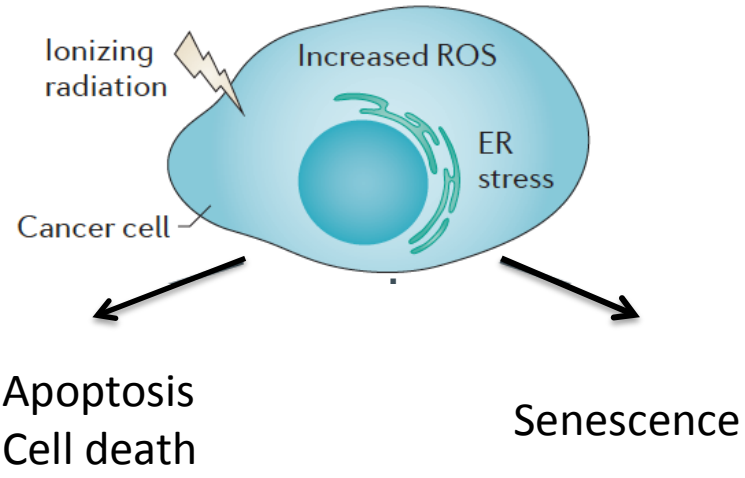

Figure 4 Impact of ionizing radiation on cells

2. Materials and Methods

2.1 Cell lines

All the cells were cultured in at 37°C and 5% CO_2 unless indicated.

HUVEC

Human umbilical vein endothelial cells are used for different research purposes such as immune response, wound healing, oxidative stress, angiogenesis, arteriosclerosis, tubule formation to name a few. They were grown in EGM2 Media (Lonza) with 10% FBS.

HEK 293-T

Human embryonic kidney cells that contain the SV40 T-antigen. These cells can replicate vectors carrying the SV40 region. Additionally they are used for gene expression and protein production. They were cultured in DMEM 10% FBS with 5% Penicillin/Streptomycin

HCT-116

HCT-116 cells derive from a human adult male with colorectal carcinoma. They were grown in McCoy's Media with 10% FBS.

SW480

The SW480 cells originate from a 50 year old Caucasian male having the Dukes' type B disease which is a colorectal adenocarcinoma. They were cultured in L15 Media (Lonza) at 37°C and 0,5% CO2.

SW 620

SW 620 cells stem from a 51 year old Caucasian male carrying the Dukes# type C disease, a colorectal adenocarcinoma. They were cultured in L15 Media (Lonza) at 37°C and 0,5% CO2.

2.2 VWR Symphony Air –Jacketed CO2 Incubator

The VWR Symphony Air-Jacketed CO2 Incubator allows you to control the temperature (37°C) and the CO2 (normally 5%). Laboratories who use this particular incubator can expect advanced air-jacketed temperature control technology assuring optimal and stable growing environment for cell and tissue cultures at low maintenance. The interface panel features large LED displays for temperature and CO2 parameters which makes it easy to use and keeps you always informed about the incubators current status.

Figure 5: VWR Symphony Air Jacketed CO2 Incubator

Specifications:

- Dimensions (WxHxD): 63.7 x 86.7 x 76.6 cm (25 x 34 x 307/32")
- Interior Dimensions(WxHxD): 47cm x 60.7cm x 53cm
- Temperature Control: +/- 0.1°C
- Temperature Uniformity: +/- 0.3°C at 37°C (99°F)
- CO2,, Range: 0-20%
- Electrical: 120V, 60Hz

2.3 Caesium Irradiator

The Caesium irradiator we used is a self-shielded irradiator that is a self-contained device that needs and integrated shielding when being operated. During the operating machine the irradiation chamber is not accessible. The irradiator has a Cs-137 source and can be used for irradiating different materials and products.

A large amount of irradiators are not designed for large throughputs. It is not allowed to irradiate explosives or flammable materials.

The irradiation source is fixed in this type of irradiator and has an irradiation shield. The shield contains a rotor that is operated by an electric drive. The irradiator also has a turntable inside which turns 180 degrees letting the product be exposed to radiation from all sides. Most irradiators are of this type.

2.4 mirVana miRNA Isolation Kit

This kit is used to isolate small RNAs from tissue and cells. Through the glass fiber filter total RNA in the range of kilo bases to 10-mers are isolated. There are enough reagents within the kit for 40 isolations of total RNA.

Procedure to be followed as in mirVana kit manual:

- Cells disrupted and collected using lysis binding buffer
- Adding microRNA homogenate and vortex
- Acid phenol: Chloroform was added to remove other cellular constituents, which also removes most DNA and spin down 5min.
- 2 phases to be seen in the tube
- Extract upper phase, since bottom phase is phenol waste
- Add extracted upper phase to separate tube with filter
- Washing with different ethanol based washing buffers and spinning of product in between
- Last spin after diluting sample with water to obtain best results of pure RNA

-

2.5 RT-PCR

The RNA that was obtained through the RNA isolation with the mirVana Kit is further processed by RT-PCR. Through RT-PCR a complementary DNA strand, which is also known as the cDNA, is formed to the RNA template. The reverse transcriptase, which is a DNA-polymerase, is responsible for the transcription into cDNA. This method is used for quantification of the DNA through the polymerase chain reaction.

2.6 QPCR

This method is used to detect and quantify DNA samples to show whether a particular DNA sequence is present and how much is found through its amplification. By using sequence-specific probes containing fluorescent dyes, this could then be measured. Through calculations we could then find the gene expression of the particular miR-103 or miR-494 in its pri-miR or mature form depending of what probes were used to prepare the RT-PCR and the QPCR.

2.7 In Situ Hybridization

HUVECs were platted on various glass plates in 6- well plates to grow overnight. Then the cells were irradiated at 2 Gy and 20 Gy and compared to a control of HUVECs that were not irradiated. Through In Situ Hybridization we wanted to see, whether miR-103 or miR-494 was up regulated after different irradiation doses. The up regulation of the miR-103 and miR-494 would then appear as red accumulation around the specific nucleus blue staining.

Protocol:

Step 1: Deparaffinize Slides in Xylene and EtOH

Xylene 3 X 5min

100% EtOH – Immerse 10X

100% EtOH – Immerse 10X

100% EtOH 5 min

96% EtOH – Immerse 10X

96% EtOH 5 min

70% EtOH – Immerse 10X

70% EtOH 5 min

PBS - 2-5 min

Step 2: Incubate with Proteinase-K for 10 min at 37C

Immediately before use, add Proteinase-K to Proteinase-K buffer (See recipe at end of text), ~300ul/slide to fully cover the section and incubate slides for 10min at 37°C in the hybridizer oven

Step 3: Place slides into a slide rack in a jar with PBS, wash 2 x in PBS

Step 3a: Block Endogenous peroxidase activity with 3%H2O2 for 2 X 3 min at RT, put in a rack in a jar with PBS. Wash 2 X with PBS.

Step 4: Dehydrate Slides

70% EtOH – Immerse 10 X

70% EtOH – Immerse 1 min.

96% EtOH – Immerse 10 X

96% EtOH – Immerse 1 min.

100% EtOH – Immerse 10X

100% EtOH – Immerse 1 min.

Step 5: Apply Hybridization mix and hyb. for 1 hour at 50-60C

Apply 25ul hyb. mix (See below for directions).

Step 6: 5 X SSC

Place the slides in 5 X SSC.

Step 7: Wash slides in SSC buffers

5 X SSC – 5 min @ Hyb temp

1 X SSC – 5 min @ Hyb temp

1 X SSC – 5 min @ Hyb temp

0.2 X SSC – 5 min @ Hyb temp

0.2 X SSC – 5 min @ Hyb temp

0.2 X SSC – 5 min @ RT

Step 8: Apply Hydrophobic Barrier

Transfer slides to glass jars with PBS. Remove slides from PBS, do not allow slides to dry out. Apply a hydrophobic barrier around tissue sections using a Dako-Pen following the manufacturer's instructions. Do not allow sample to dry out during or after this step.

Step 9: Incubate with antibody blocking solution for 15 minutes

Place the slides in a humidifying chamber and incubate with blocking solution for 15 min. at RT

Step 10: Apply anti-DIG-POD antibody

Remove blocking solution and incubate slides with anti-DIG-POD 1:400 for 60 min at RT.

Step 11: 3 X 3 min wash in PBS

Step12: Apply TSA-plus FITC substrate (1:50) to the sections and incubate slides 2 X 5 min at RT, wash 1 x with PBS

Step 12a: Apply NucBlue Fixed cell Stain (Molecular Probes, #R37606) about 400ul per slide (to prepare, add two drops to 1ml PBS)

Step 13: Wash slides in 3 X 5 min in PBS buffer to stop the RXN

SOLUTIONS:

Antibody Blocking Solution: PBS, 0.1% Tween, 2% Sheep/Goat Serum, 1%BSA

Antibody Dilutant Solution: PBS, 0.05% Tween, 1% Sheep/Goat Serum, 1% BSA

Proteinase-K: Prepare immediately before use. For a Proteinase-K concentration of 15ug/ml: Add 7.5ul Proteinase K stock to 10ml Proteinase-K buffer

Proteinase-K Buffer: 900ml RNase-free H2O, 5ml 1M Tris-HCl (pH 7.4), 2ml of 0.5M EDTA, 0.2ml 5M NaCl. Adjust volume to 1000ml. Autoclave.

Hybridization mix (microRNA ISH buffer and LNA Detection probes):

1) Dilute the 2x microRNA ISH buffer 1:1 with RNase-free water, e.g. mix 1 mL 2x microRNA ISH buffer with 1 mL RNAse-free water to give 2 mL 1x buffer.
2) For each probe to be used in the experiment, place the appropriate amount of LNA™ probe in a 2 mL non-stick RNase-free tube
3) Denature the probes at 90°C for 4 minutes.
4) Place the tubes in table-top microfuge and spin down shortly.
5) Immediately add the 2 mL 1x microRNA ISH buffer to each of the tubes with the different LNA™ probes.

Probe	Final probe conc.	Probe vol.	Dilution factor	1x ISH buffer vol.
LNA™ U6 snRNA (0,5µM)	1 nM	4 µl	"1:500"	2 ml
LNA™ microRNA probe (25µM)*	20 nM	1.6 µl	"1:1250"	2 ml
LNA™ microRNA probe (25µM)*	40 nM	3.2 ul	"1:625"	2 ml
LNA™ scrambled microRNA probe (25µM)	40 nM	3.2 ul	"1:625"	2 ml

Experimental procedure

Firstly the cells were platted 24 hours before irradiation. The cells were counted to be platted approximately 300 000 cells per well. After leaving the cells to grow overnight in the incubator, they were then irradiated at 2 and 20 Gy. Afterwards the irradiated cells were placed in the incubator again. Counting the time after placing the cells in the incubator, the RNA isolation was carried out at 1 hour (pri-miRNA) and at 6 hours (mature miRNA). For a control we used 0 time points for 2 and 20 Gy and additionally same time points with non-irradiated cells.

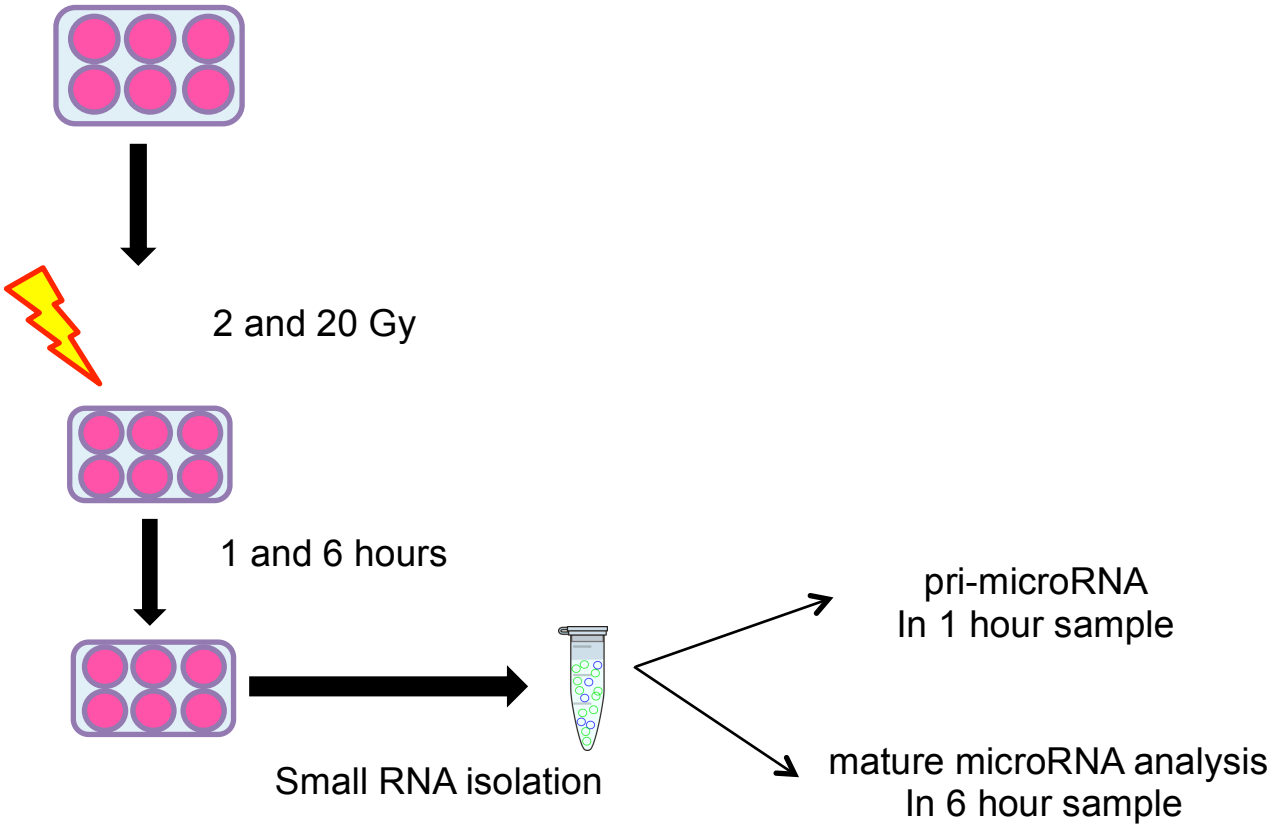

Figure 6 Experimental procedure

3. Aims

1. To analyze differences in microRNA regulation, comparing non-tumor to tumor cells after radiation (2 and 20 Gy dose).
2. To study difference behavior of miR-494 and miR-103 in: HUVEC, HEK293T, HCT-116, SW480 and SW620.
3. To compare qPCR data with *in situ* hybridization in HUVEC

4. Results

4.1 Primary cells and non-tumor cells analysis

To analyze microRNA levels in HUVEC (Figure X) and non-tumor cells as HEK293T (Figure Y) we cultured them, as it is described in the methods. We analyzed miR-103 and miR-494. In the samples isolated 1 hour after radiation we analyzed the primary microRNA precursor, and we observed that there is not significant regulation of these microRNAs on HUVEC at this time point. However when we analyze the mature forms of both microRNAs at 6 hours post-treatment we saw 3 fold up regulation of miR-103 and miR-494 (Figure 7).

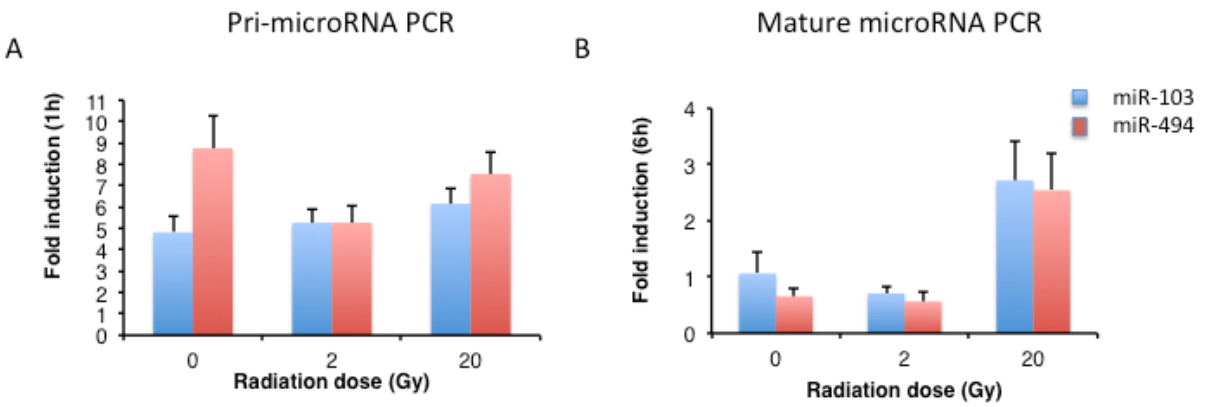

Figure 7: MicroRNA expression in HUVEC after radiation. HUVEC were treated in a Cesium Irradiator with 2 Gy and 20 Gy doses for 1 hour and 6 hour. A) Analysis of primicroRNA-103 and primicroRNA-494. B) Analysis of miR-103 and miR-494 after 6 hours treatment. Bar graphs represent fold values of the microRNA relative to their housekeeping controls GADPH and U6 respectively.

We used HEK293T cell line, as a non-primary and non-tumor cell line control. Similarly to the results we saw in primicroRNA analysis with HUVEC, as it is indicated in Figure 8 we did not find significant changes. Interestingly in this case when we analyzed mature microRNAs we did not find miR-494 increase with any radiation dose, but miR-103 had a 6-fold increase with 2 Gy and a slight up regulation with 20 Gy as well.

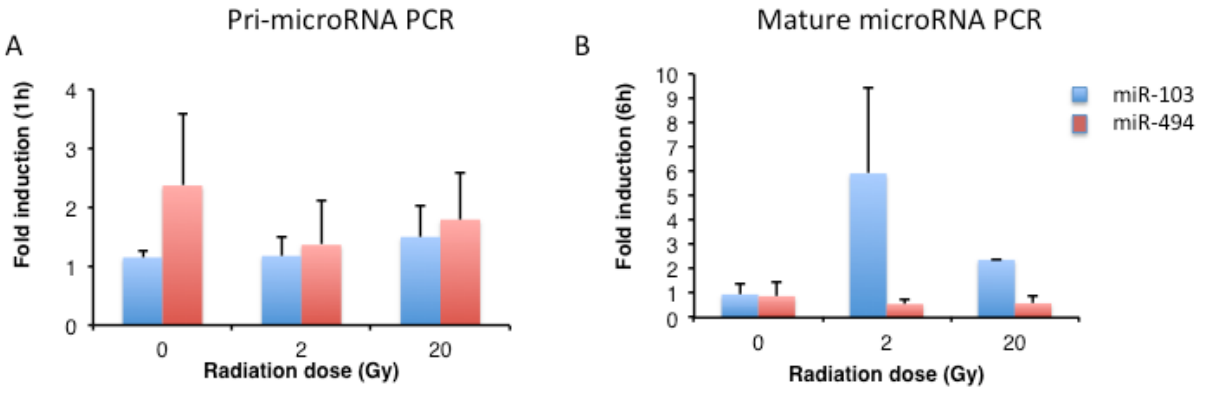

Figure 8: MicroRNA expression in HEK293T after radiation. HEK293T were treated in a Cesium Irradiator with 2 Gy and 20 Gy doses for 1 hour and 6 hour. A) Analysis of primicroRNA-103 and primicroRNA-494. B) Analysis of miR-103 and miR-494 after 6 hours treatment. Bar graphs depict fold the levels of the microRNA relative to their control.

4.2 Tumor cells analysis

We wanted to compare if different colorectal carcinoma tumor cells behaved as primary cells after radiation. For that we used three different cell lines HCT116, SW620 and SW480. First line we analyze was HCT116. As shown in figure 9, there was a tendency to up regulate the pri- forms of these two microRNAs, however when we analyzed the mature form 6 hours after treatment, we did not see any increase in microRNA levels, even in miR-494 levels we saw a tendency to decrease the expression

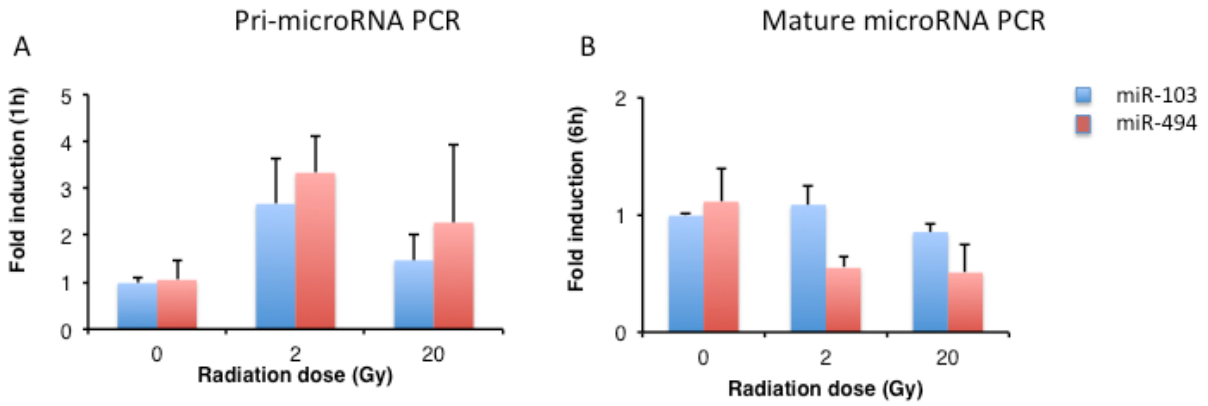

Figure 9: MicroRNA expression in HCT116 after radiation. HCT116 were treated in a Cesium Irradiator with 2 Gy and 20 Gy doses for 1 hour and 6 hour. A) Analysis of primicroRNA-103 and primicroRNA-494. B) Analysis of miR-103 and miR-494 after 6 hours treatment. Bar graphs depict fold the levels of the microRNA relative to their control.

Analyzing other colorectal cancer cell line, SW480 we saw similar results. In this case pri-miR-494 was up regulated after 1 h with 2 Gy radiation, and completely depleted with 20 Gy doses, no changes were observed with pri-miR-103. In the analysis of the mature form non-significant up regulation was observed.

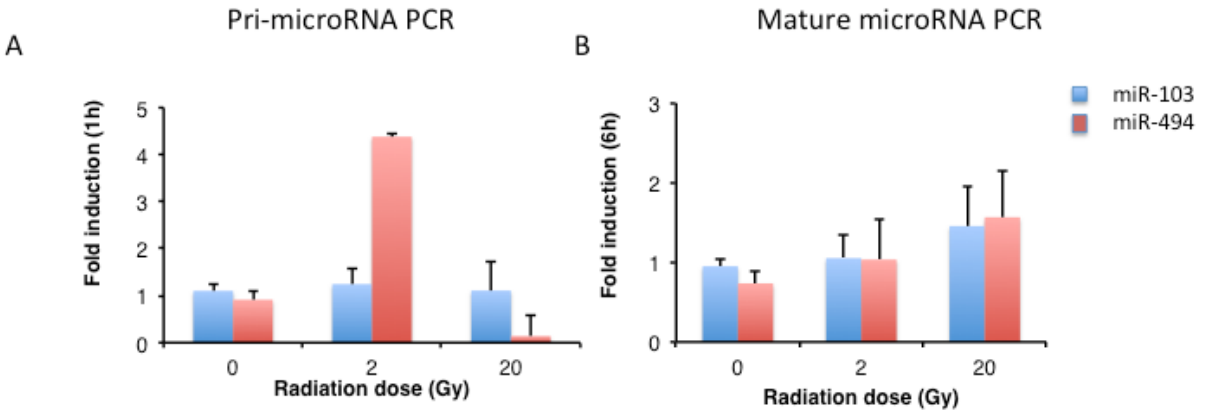

Figure 10: MicroRNA expression in SW480 after radiation. SW480 were treated in a Cesium Irradiator with 2 Gy and 20 Gy doses for 1 hour and 6 hour. A) Analysis of primicroRNA-103 and primicroRNA-494. B) Analysis of miR-103 and miR-494 after 6 hours treatment. Bar graphs depict fold the levels of the microRNA relative to their control.

In the last cell line we tested SW620, we saw an increase in pri-miR-103 (Figure 11), however when we analyzed the mature form of these microRNAs is miR-494 which is increase with 20 Gy (Figure 11)

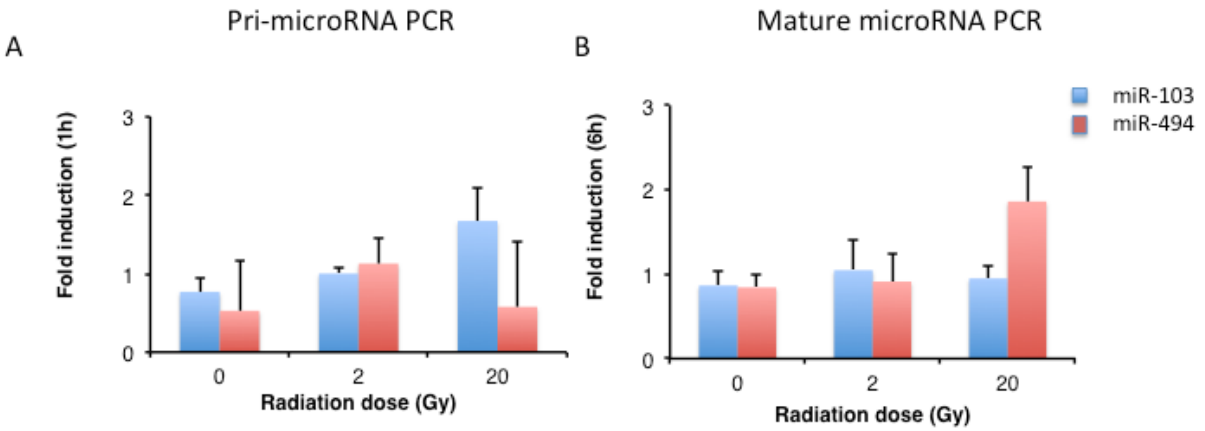

Figure 11: MicroRNA expression in SW620 after radiation. SW620 were treated in a Cesium Irradiator with 2 Gy and 20 Gy doses for 1 hour and 6 hour. A) Analysis of primicroRNA-103 and primicroRNA-494. B) Analysis of miR-103 and miR-494 after 6 hours treatment. Bar graphs depict fold the levels of the microRNA relative to their control.

4.3 In situ hybridization

To validate our previous results in primary cells, in this case HUVEC we used a different method recently set up in the lab. We analyze by *in situ* hybridization miR-103 (Figure 12) and miR-494.

The miR-103 analysis showed that there was a high increase in miR-103 expression in the cells after 20 Gy treatment.

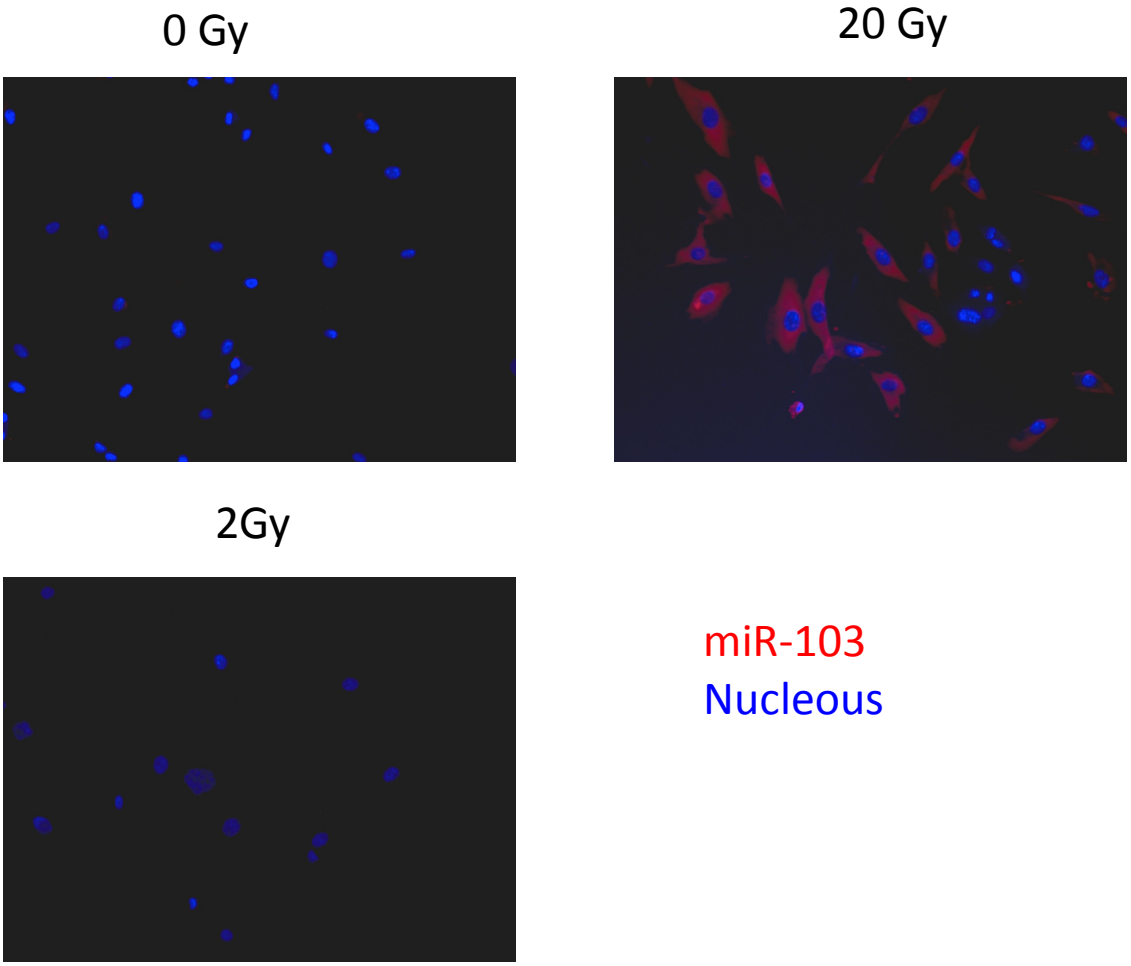

Figure 12: miR-103 in situ hybridization in HUVEC. HUVEC were plate in coverslips in a 24 well plate overnight. When cells were attached, were treated with 2 and 20 Gy. After 6 hours incubation cells were fixed with Neutral formalin solution and then analyzed against miR-103 in situ probe.

However miR-494 was slightly different. miR-494 levels in HUVEC were higher than for miR-103 (Figure 13) and the highest expression found was at 2 Gy instead of 20 Gy, although at 20 Gy miR-494 expression is still high.

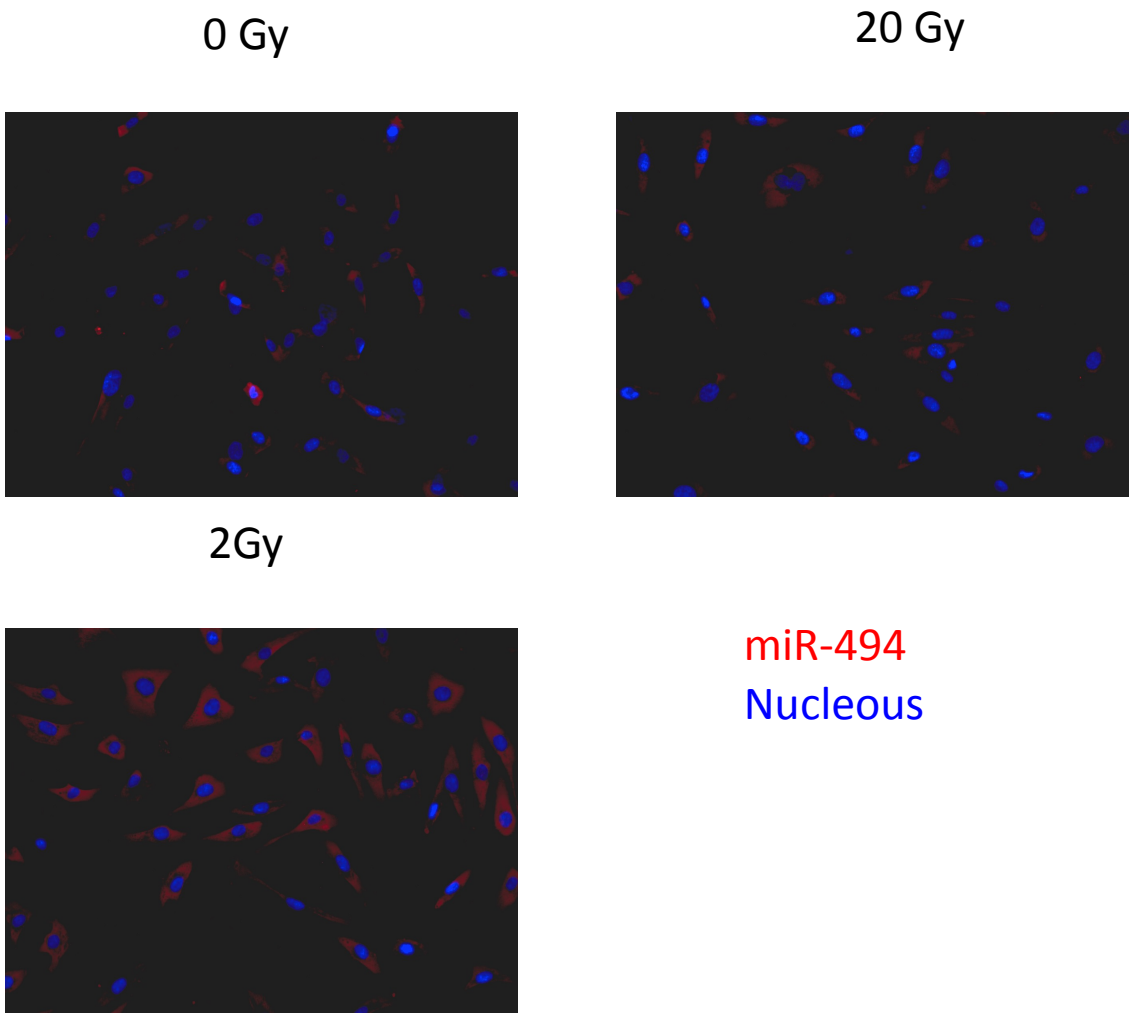

Figure 13: miR-494 in situ hybridization in HUVEC. HUVEC were plate in coverslips in a 24 well plate overnight. When cells were attached, were treated with 2 and 20 Gy. After 6 hours incubation cells were fixed with Neutral formalin solution and then analyzed against miR-103 in situ probe.

5. Discussion

In graph 1 there are no significant results for pri-mir-494 and pri-mir-103 after any radiation doses. When taking a look at graph 2 we can see an up regulation of both miR-494 and miR-103 at a dose of 20 Gy. One dose not find yet an up regulation of pri-miRNA after 1 hour time point so for future investigation the idea is to see whether there are more pri-miRNAs at a later time point as we see in graph 2 that there are mature forms after 6 hour time points, so we expect an accumulation of pri-miRNAs beforehand, so the mature form can develop from these.

There are no pri-miRNA differences to be seen in graph 3 for the HEK293T cells. miR-103 however is up regulated at 2 and 20 Gy post-treatment at a time point of 6 hours as can be seen in graph 4. miR-494 shows no differences at any dose of radiation at all.

Graph 5 shows increased levels of pri-miR-494 and pri-miR-103 1 hour after a dose of 2 and 20 Gy for HCT-116 cells. After 6 hours there are no significant results for the mature miRNAs (graph 6). The next step for this would be to investigate why the pri-miR-494 and pri-miR-103 that can be seen up regulated in graph 5 are not being transcribed into their mature forms after 6 hours time point. One would expect these pri-miRNAS to be transcribed so further investigation has to be done to see whether there are other miRNAs blocking the transcription or if there are other transcription factors involved which do not let the completion to be done.

For the SW480 cells we found pri-miR-494 was up regulated 1 hours after a dose of 2 Gy, however at a dose of 20 Gy it looks like it is down regulated. pri-miR-103 does not show any significant results for 1 hour after 2 and 20 Gy. The mature miRNAs are not significant 6 hours post-radiation. This would have to be investigated further on to find out why miR-494 is not being transcribed into its mature form after 6 hours whether there are other transcription factors involved or if they are being transcribed at a later time point.

Figure 9 shows an up regulation of pri-miR-103 1 hour after a treatment of the SW620 cells with 20 Gy. We do not see any differences in pri-miR-494 at any dose of radiation. After 6 hours there are no mature miR-103 to be seen in graph 10 but for miR-494 we see a 2-fold increase at 20 Gy after 6 hours.

When comparing the results from the in-situ hybridization of HUVECS with the QPCR results we can see that the results for miR-103 coincide. Graph 13 shows in red an accumulation of miR-103 in the cytoplasm after a dose of 20 Gy. In graph 11 and graph 12 there is no red color, which would indicate the presence of miR-103. These are the results that were expected, when taking a look at graph 2 of HUVECS.

The in-situ-hybridization for miR-494 shows already a presence of this miR from the beginning without any treatment (graph 14). Graph 15 shows a higher amount of miR-494 at a dose of 2 Gy. At a dose of 20 Gy there are lower amounts to be seen in graph 16. The results of miR-494 are different to the results of the QPCR. This has to be investigated further on since we would have expected only a higher amount of miR-494 at a dose of 20 Gy.

Through all the results that have been obtained by the different experiments we can see that tumor cells have a higher transcriptional response and accumulate more pri-miRNA than primary and non tumor cells. We also see, that there are different miRNA expressions in primary cells comparing to tumor cells. miR-494 and miR-103 is increased in primary cells but not in transformed cells.

The next step would be to investigate whether this is due to the fact that there are different epigenetic modifications or to look at the biogenesis whether there are lower levels of Drosha and Dicer resulting in lower transcriptions into the mature form of the miRNAs.

The future idea with these results would be to make a combination therapy of radiation and microRNAs, which would amplify the benefits since miR-103 is known to in-

duce apoptosis and miR-494 has a senescence phenotype. So when irradiating the cells these miRNAs would be up regulated to either undergo apoptosis or senescence.

6. Conclusion

1. microRNA expression is different in primary cells vs. tumor cells. miR-X and miR-Y are increased in primary cells, but not in transformed cells
2. Tumor cells have a higher transcriptional response or they acummulate more pri-microRNA than primary and non tumor cells

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