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Functional analysis of non-viral miRNA replacement therapy in colon carcinoma *in vitro* and *in vivo*



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To my dear family

(My Parents, my Brothers, my wife and my little Haroun)

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1. INTRODUCTION

1.1 Cancer and gene knockdown strategies

Cancer is a genetic disease in which mutational and/or epigenetic changes in the genome lead to deregulation of cell growth or cell death mechanisms. Several technologies have been used in an attempt to interfere in this aberrant gene expression. For example, antisense oligonucleotides and ribozymes have been used for more than a decade to target specific RNAs for degradation. Although these methods worked adequately in some simple experimental models, they have generally not delivered effective gene silencing in complex mammalian systems.

Nevertheless, after antisense technologies and ribozymes, in the late 1990s a novel mechanism for gene targeting was discovered, RNA interference (RNAi). It soon became clear that RNAi represents a particularly efficient and at least *-in vitro-* easy to use method for the knockdown of the expression of a selective target gene. Therefore, RNAi is now a well-established method for high throughput analysis as well as for functional studies *in vitro*, including mammalian cells. Many pathological conditions rely on the aberrant expression of the endogenous normal or mutant genes causing, e.g., variation in signal transduction pathways, cellular proliferation, apoptosis, or resistance toward external factors. Moreover, the infection of an organism can lead to the introduction and the expression of foreign genes. While the inhibition of the activity of gene products, e.g., through small molecule inhibitors or inhibitory antibodies is one major focus in therapy, much attention has now shifted to an earlier step, i.e., the initial knockdown of the specific gene of interest through RNAi.

1.2 Biology of colon cancer

Colorectal cancer (CRC) is one of the most common causes of cancer related deaths all over the world (Jemal et al., 2006), with an estimated incidence of 1 million new cases and a mortality of more than 500,000 death annually (Parkin et al., 2005). Colorectal cancer could be of sporadic origin with several intrinsic factors (e.g. age, obesity, and inflammatory bowel disease) and extrinsic factors (e.g. cigarette smoking, inadequate intake of fiber, high consumption of alcohol, and high fat diet) being associated with increased risks of colorectal cancer (Giovannucci and Michaud, 2007; Johnson and Lund, 2007; Tsoi *et al.*, 2009; Terzic *et al.*, 2010; Wei *et al.*, 2010). Familial inheritance plays also an important role, where more than one fifth of patients with colorectal cancer have a familial history. However, the diagnosis of familial syndromes, such as Lynch syndrome and familial adenomatous polyposis, can only be confirmed in 6% of these patients (Jasperson et al., 2010).

Colorectal cancer is a stepwise progression from benign adenoma to malignant adenocarcinoma and distant metastasis, giving an early diagnosis as preferred option to ease the disease burden (Lieberman, 2010). It occurs in caecum, ascending, transverse, descending and sigmoid colon and rectum. Most colorectal cancers, at least two-thirds, are adenocarcinomas, arising from the columnar surface epithelium, and few are signet-ring cell carcinoma, squamous carcinoma, undifferentiated carcinoma and medullary-type adenocarcinoma (solid carcinoma with minimal or no glandular differentiation and slight cellular pleomorphism) (Ponz de Leon and Di Gregorio, 2001).

Many molecular abnormalities have been reported in colorectal carcinoma and it is now believed that accumulated alterations of suppressor genes and proto-oncogenes are required for the development of colorectal cancers (Vogelstein *et al.*, 1988; Fearon and Vogelstein, 1990). It has been estimated that a minimum of eight to ten mutational events must accumulate during multistep carcinogenesis to produce an invasive colorectal cancer (Hamilton SR, 1998) and allowing it to escape growth and regulatory control mechanisms where, the homeostasis of the adult epithelium is controlled by a strict regulatory signaling

pathways, e.g. Wnt signaling pathway, TGF- β signaling and JNK/SAPK pathway (Parveen et al., 2010). It was illustrated that colorectal cancer arises through at least two major genetic pathways, chromosomal instability and microsatellite instability (Fukushima and Takenoshita, 2001).

Chromosomal instability refers to numerical and structure chromosomal alterations, namely gain and/or loss of whole chromosomes or chromosomal segments at a higher rate in a population of cells, such as cancer cells, compared with normal cells (Lengauer et al., 1998). This pathway, accounting for about 85% of CRCs, is characterized by allelic imbalance, chromosomal amplification and translocation. One of the key steps in this pathway is mutation of the genes, 3p21 (β -catenin gene), 5q21 (APC gene), 9p (p16 and p15 genes), 13q (retinoblastoma gene), 17p (p53 gene), 17q (BRCA1 gene), 18q (DCC and SMAD4 genes), and less frequently 16q (E-cadherin gene) (Kinzler et al., 1991).

Loss or mutation of *APC* leads to an accumulation of unphosphorylated β -catenin in the cytoplasm, translocation of β -catenin to the nucleus and subsequent activation of APC- β -catenin-T-cell factor (Tcf)/Lef. This stimulates transcription of target genes known to be involved in tumorigenesis (e.g. c-myc, cyclin D1, and c-jun) (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Sparks *et al.*, 1998). Germline mutations of the *APC* gene cause familial adenomatous polyposis, an autosomal dominant disorder characterized by the development of hundreds to thousands of colorectal adenoma appearing in adolescence or early adulthood. *APC* mutation or allelic losses of 5q are observed in 40-80% of sporadic CRCs and are found at a similar frequency in adenomas. Furthermore, mutated *APC* has been detected in the earliest adenoma, suggesting that the mutation is an initiating event for sporadic colorectal tumorigenesis (Powell et al., 1992).

Microsatellite instability (MSI) is the hallmark of hereditary nonpolyposis colorectal cancer (HNPPC) and can also be found in approximately 15% of sporadic CRCs (Soreide et al., 2009).

1.2.1 Mitogen Activated Protein Kinase (MAPK)

The mitogen activated protein kinase (MAPKs) signaling system plays an essential role in the transduction of extracellular signals to cytoplasmic and nuclear effectors that regulate various cellular processes (Chen et al., 2001; Johnson and Lapadat, 2002; Pouyssegur et al., 2002).

The MAPK cascade is highly conserved system that is involved in various cellular functions, including cell proliferation, differentiation and migration. At least four members of the MAPK family have been identified, extracellular signal regulated kinase1/2 (ERK1/2), c-jun-amino-terminal kinase (JNK), p38 and ERK5 (Sturgill and Wu, 1991; Nishida and Gotoh, 1993; Nishimoto and Nishida, 2006).

ERK5, also known as big MAP kinase1 (BMK1), is twice the size of other MAPKs (Lee et al., 1995; Zhou et al., 1995). The MAPK pathways are located downstream of many growth-factor receptors, including that for epidermal growth factor. Overexpression and activation of this receptor are commonly detected in colorectal cancer, and several lines of evidence indicate that overexpression and activation of MAPK play an important part in progression of the colorectal cancer (Fang and Richardson, 2005).

ERK5-mediated role proliferation activity may be due to activation of serum and glucocorticoid-induced kinase (SGK), a protein kinase that is closely related to the G1/S transition of the cell cycle (Buse et al., 1999). The deregulation of the Cyclin D1 gene, a key cell proliferation checkpoint, is frequently associated with tumor formation and has been shown to be regulated by ERK5 cascade (Mulloy et al., 2003).

ERK5 is essential for cardiovascular development whereas ERK5 deficient mice show cardiovascular abnormalities and angiogenic defects (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003).

In a previous study conducted in *Xenopus laevis*, the essential role of ERK5 in neuronal differentiation was shown (Nishimoto et al., 2005).

ERK5 has a unique transcriptional activation activity, where it translocates into the nucleus where the unique C-terminal region plays the most important role in this process (Kasler *et al.*, 2000; Yan *et al.*, 2001; Akaike *et al.*, 2004).

1.2.2 C-Myc

Some reports have indicated that c-Myc can play a very important role in gastric carcinogenesis, where the c-myc proto-oncogene is one of the most frequently activated oncogenes, and is estimated to be involved in 20% of all human cancers (Dang, 1999; Nesbit et al., 1999). The proto-oncogene c-myc encodes a transcription factor c-Myc, which has an important role in controlling cell growth, differentiation, apoptosis and viability. Mutations or translocations in the coding sequence of c-myc occur in a number of cancers such as Burkitt's lymphoma (Rabbitts et al., 1983; Rabbitts et al., 1984). C-myc is a member of the *myc* gene family which includes N-myc, s-myc, L-myc and B-myc. However, only the c-Myc, L-myc and N-myc have neoplastic activity (Marcu *et al.*, 1992; Lemaitre *et al.*, 1996; Facchini and Penn, 1998).

C-myc is expressed in almost all proliferating normal cells where its expression is strictly dependent on mitogenic stimuli, and is downregulated in many kinds of cells when they induced to de-nucleation or terminal differentiation, special type of apoptosis, (Evan and Littlewood, 1993).

It was reported that overexpression of c-Myc is associated with the presence of metastasis and it was also found that c-Myc mRNA was higher in metastatic than in primary lesions (Onoda *et al.*, 1996; Han *et al.*, 1999a; Han *et al.*, 1999b; Kozma *et al.*, 2001; Yang *et al.*, 2004).

The proto-oncogene c-myc plays a role in both positive and negative growth via having an influence on proliferation, differentiation and apoptosis. Alterations in the level of myc expression or protein structure are associated with many malignancies in humans and animals (Askew et al., 1991; Evan and Littlewood, 1993).

Deregulated expression of c-myc causes cellular immortalization, but is not sufficient to induce transformation of primary cells; however, Myc and Ras have been shown to cooperate in both transformation and tumorigenesis *in vivo* (Henriksson and Luscher, 1996; Ryan and Birnie, 1997).

It was found that one third of breast and colon carcinomas have an elevated level of c-Myc gene expression (Erisman et al., 1985; Escot et al., 1986).

C-Myc and other substrates like sap1a and RSK, ERK1/2 substrates, can be phosphorylated by ERK5 (English et al., 1998; Kamakura et al., 1999; Moriguchi et al., 1999).

It was found that there is a relationship between c-Myc deregulation and gastric cancer, where c-Myc is overexpressed in over 40% of gastric cancer (Milne et al., 2007). C-Myc protein was overexpressed in all cases of both intestinal and diffuse type gastric adenocarcinoma samples of individuals from Northern Brazil (Calcagno et al., 2006).

1.3 Basic mechanism and induction of RNAi

RNAi was first described in plants as an immune response to viral infection. As early as 1928, it was noticed that tobacco plants, which were infected with tobacco ring spot virus, grew without any problems.

The upper leaves showed resistance to the effect of the virus (Wingard, 1928). It is now known that dsRNA intermediates produced during virus infection activate the RNAi machinery to silence the expression of complementary genes, thus producing immunity to the virus (Covey SN et al., 1997). This defense against foreign genetic material is one of

several physiologic pathways that are induced by naturally occurring dsRNA in a wide variety of eukaryotic organisms including fungi, plants and animals. With some variations, these responses are all mediated by a common RNAi pathway that involves processing of the dsRNA into short duplexes of about 22 base pairs with characteristic end structure. The RNAi machinery can also be induced by experimentally synthetic dsRNA, providing a valuable tool for *in vivo* gene silencing.

RNA interference (RNAi) was first characterized in the nematode worm *Caenorhabditis elegans* by Fire and colleagues (Fire et al., 1998) who found that double stranded RNA (dsRNA) induced a more potent sequence-specific silencing response than single stranded antisense RNA alone. Further investigation into this phenomenon demonstrated that injection of dsRNA into the gut of the worm caused a systemic silencing of the target gene that was passed on to the next generation offspring.

From that point forward, the emphasis has been not only on understanding how this phenomenon occurs, but also how it can be exploited as a research tool (Kamath and Ahringer, 2003). Through these investigations, RNAi has been shown to greatly facilitate both “reverse genetic” experiments (identifying the function of a known gene) and “forward genetics” experiments (identifying the gene responsible for a given phenotype).

Classic reverse genetic experiments involve designing siRNAs (chemically or enzymatically synthesized) or shRNA-expression constructs targeting a gene of interest. Following transient transfection of siRNAs or shRNA encoding plasmids or selection of shRNA-expression stable transfectants, the phenotype of the cells is assessed using an appropriate functional assay. It is critical in this type of experiments to use adequate controls to ensure that the observed phenotype is only due to targeting the gene of interest.

The discovery of dsRNA- induced gene silencing in *C. elegans* allowed genetic screens to be performed that led to the identification of genes required for RNAi in the nematode (Tabara et al., 1999). Double stranded-RNA molecules, whether introduced experimentally or as naturally occurring viral byproducts, endogenous miRNA precursors, or aberrant

transgenic transcripts, are recognized and cleaved into 21-23 nucleotide siRNA by the RNase III like enzyme termed Dicer (Bernstein et al., 2001).

Different species contain different numbers of Dicer homologous and/or associated proteins containing dsRNA binding domains that function to recognize dsRNAs from different sources. For example, in *Drosophila*, Dicer-1 processes miRNA precursors and Dicer-2 processes long dsRNAs (Lee et al., 2004). In *Arabidopsis thaliana*, there are four Dicer homologous that function together with associated proteins to cleave dsRNA of different types. To date, only one Dicer gene has been identified in mammals, and interacting proteins regulating Dicer function remain to be identified. Double stranded-RNA cleavage by Dicer generates siRNA that contain a 2-nucleotide 3' overhang and a 5'-phosphorylated terminus both of which are required for activity (Zamore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001c; Hutvagner and Zamore, 2002). Processing by *Drosophila* Dicer is adenosine triphosphate (ATP) -dependent and requires a functional RNA helicase domain (Nykanen et al., 2001). In contrast, it appears that human dicer may not require ATP (Provost et al., 2002; Zhang et al., 2002).

Introduction of 21 bp siRNA has allowed for the successful application of RNAi technology to mammalian systems. However, assays using this method are transient in nature and the suppressed phenotype can be lost within several doubling times, most likely due to the dilution of the siRNA. While this approach is reliable for short-term studies of gene expression, it cannot replace knockout mouse models or allow for precise loss-of-function genetic screens.

The first step in the RNAi pathway involves the processing of large dsRNA into small, 21-23 nucleotide dsRNA molecules (Zamore et al., 2000; Elbashir et al., 2001b). Initial studies in *Drosophila* showed that an RNase III enzyme was responsible for this processing and that the siRNA possessed 3' hydroxyl and 5' phosphate groups and, importantly, a 3' overhang of two unpaired nucleotides on each strand (Elbashir et al., 2001b).

A proposed model for the Dicer involves the ATP-dependent translocation of the enzyme along its dsRNA target. The efficiency with which Dicer cleaves a particular dsRNA molecule has also been shown to be directly proportional to the length of the target, since the longer the dsRNA, the greater is the amount of siRNA produced and hence the more potent is the silencing effect (Bernstein *et al.*, 2001). Human Dicer-mediated cleavage of dsRNA is thought to occur sequentially, beginning at the termini of the dsRNA, and the excision of small dsRNA fragments of a defined length (Ketting *et al.*, 2001; Zhang *et al.*, 2002).

Following the cleavage of dsRNA into siRNA by Dicer the second important stage of mRNA degradation occurs. This is mediated by a protein complex with nuclease activity known as RISC which is guided to its target mRNA by the siRNA into the RISC (Hammond *et al.*, 2000). This guide role of siRNA was proposed after the observation that dsRNA would only lead to the degradation of an mRNA with a homologous sequence, leaving the rest of the RNA in the cell unaffected.

Moreover, it was shown that both siRNA and RISC were required to mediate cleavage of the target (Hammond *et al.*, 2000). Following the initial discovery of the existence of a ribonucleoprotein complex as a mediator of RNAi, the components and mechanism of action of RISC began to be elucidated, and both inactive and active forms of RISC complex (the active indicated as RISC*) were found. It was shown that a second ATP-dependent step was involved in the pathway and showed the following unwinding of the siRNA duplex (Nykanen *et al.*, 2001). In another seminal study, RISC* was found to be associated only with the antisense strand of the siRNA (Martinez *et al.*, 2002). Hence, although the siRNA needs to be double stranded in order to be efficiently recognized and bound to RISC, the two siRNA strands must unwind before RISC becomes active. The efficient cleavage of the target mRNA by RISC was also shown to be dependent on the phosphorylation of the 5' siRNA duplex (Nykanen *et al.*, 2001; Martinez *et al.*, 2002).

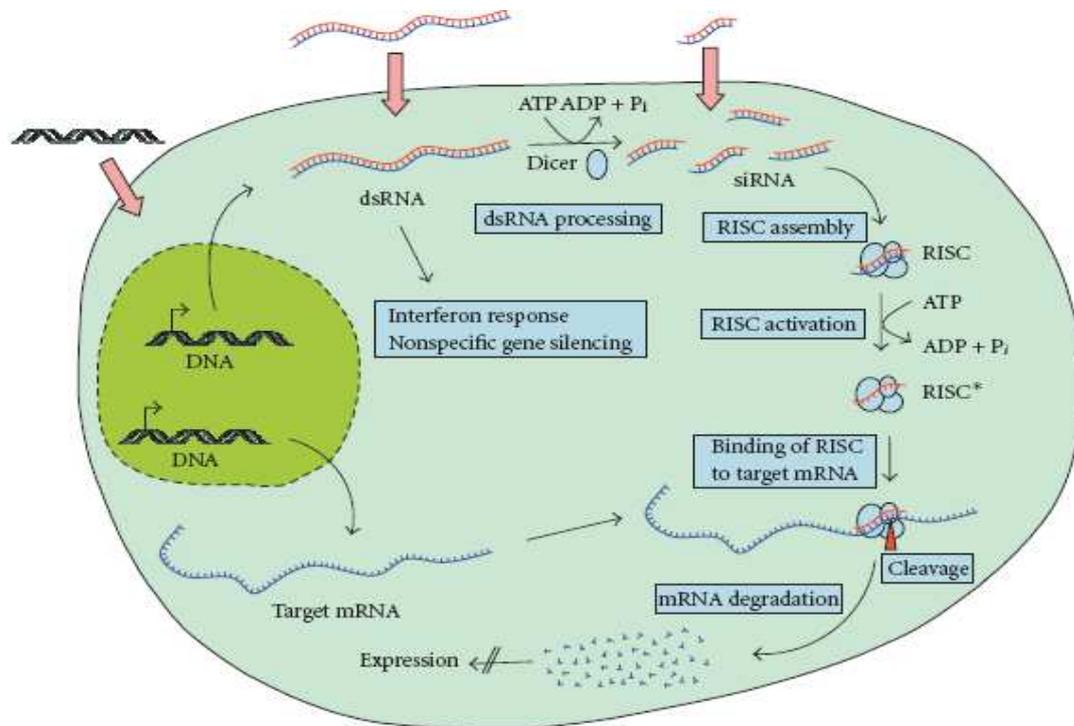


Figure 1.1 RNAi Pathway: Long dsRNA is processed by Dicer into siRNAs which then incorporates into RISC, The duplex siRNA is unwound leaving the anti-sense strand to guide RISC to complementary mRNA for subsequent endonucleolytic cleavage (Aigner, 2006).

In non-mammalian cells, there is evidence that an alternative branch of the RNAi pathway which results in the amplification of the original message, can account for the efficiency of gene silencing (Sijen et al., 2001). In this case, the unwound siRNA no longer acts as a guide to bring RISC to the target mRNA but simply functions as a primer for an RNA-dependent RNA polymerase (RdRP), which uses the target mRNA as a template to produce new dsRNA. This can be subsequently recognized and cleaved by Dicer, thus re-entering the RNAi pathway and initiating a new round of silencing. Therefore, not only is the mRNA targeted via the specific oligonucleotide sequence (and hence gene expression silenced) but also new dsRNAs arising from the entire mRNA sequence are created and thus amplify the original RNAi trigger. Several RdRPs participating in RNAi have been identified in fungi,

plants and invertebrates (Cogoni and Macino, 1999; Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Smardon *et al.*, 2000; Sijen *et al.*, 2001; Martens *et al.*, 2002).

1.4 MicroRNAs

MicroRNAs are small, double-stranded RNA molecules possessing the reverse complement of the mRNA transcript of another protein-coding gene. These miRNAs can inhibit the expression of the target gene. MiRNAs were first observed in *C. elegans* as RNA molecules of 18- to 23 nucleotides that are complementary to the 3' untranslated region of the target transcripts, including the *lin-4* (Lee *et al.*, 1993) and *let-7* (Lau *et al.*, 2001). As a result, these small RNA molecules regulated the development of the worm. Subsequently, miRNAs were found to occur in diverse organisms, ranging from worms, to flies, to humans (Lagos-Quintana *et al.*, 2003), suggesting that these molecules represent a gene family that has evolved from an ancient ancestral gene.

The miRNAs are thought to be transcribed from DNA that is not translated, but regulates the expression of other genes. Primary transcripts of the miRNA genes (pri-miRNAs) are long RNA transcripts consisting of at least one hairpin-like miRNA precursor. Pri-miRNAs are processed from the non-loop region in the nucleus into ~70 nucleotide hairpin-like precursor pre-miRNAs by a microprocessor (Lee *et al.*, 2003).

The core components of the microprocessor are the RNase III enzyme Drosha and a double stranded RNA binding protein termed DGCR8/Pasha (Lee *et al.*, 2003; Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Landthaler *et al.*, 2004). The pre-miRNAs are exported from the nucleus by Exportin-5 (Lund *et al.*, 2004). The cytoplasmic RNase III enzyme, Dicer, excises the miRNA from the pre-miRNA loop region. MiRNAs and siRNAs seem to be closely related, especially taking the dsRNA and hairpin structures into account.

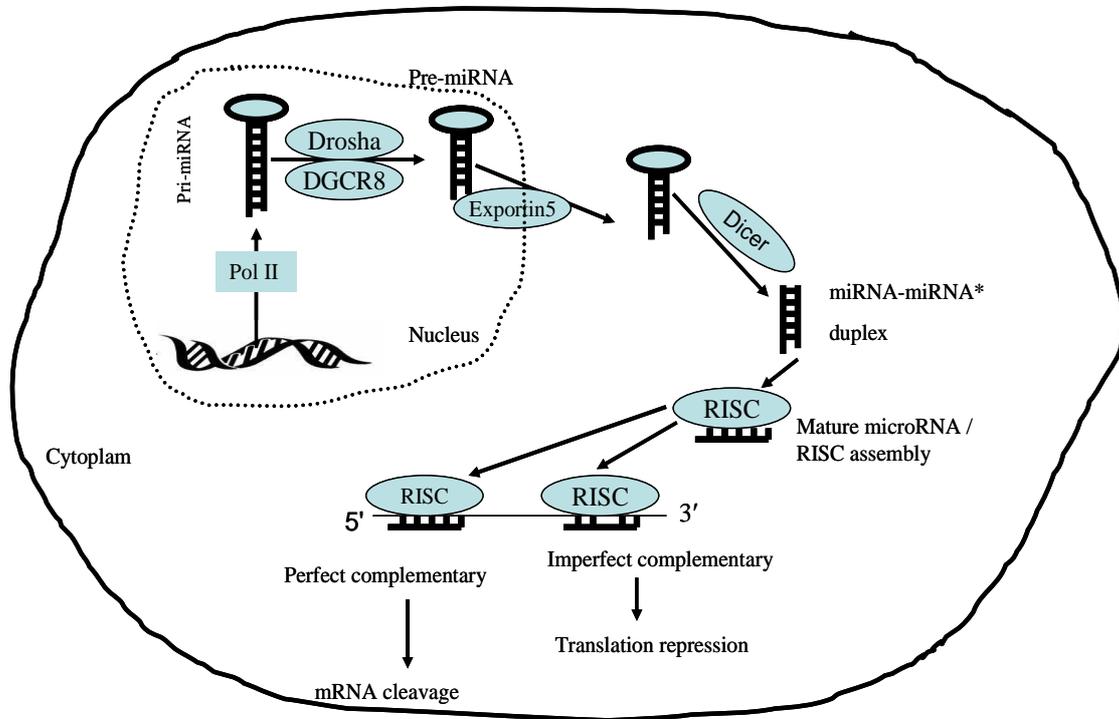


Figure 1.2 miRNA Biogenesis: Shows Pri-miRNA processing by Drosha/DGCR8, translocation of pre-miRNA by Exportin5 into the cytoplasm where mature miRNA processed by Dicer.

The annealing of the miRNA to the target mRNA inhibits protein translation (imperfect base pairing). In some cases, the formation of dsRNA through the binding of miRNA triggers the degradation of the mRNA transcript through a process similar to RNAi (perfect base pairing), although, in other cases, it was thought that the miRNA complex blocks the protein translation machinery or otherwise prevents protein translation without causing the mRNA to be degraded. Because most of the miRNA suppresses gene function based on partial complementarity, conceivably, one miRNA may target more than one mRNA, and many miRNAs may act on one mRNA, coordinately modulating the intensity of gene expression in various tissues and cells. Therefore, miRNAs may have a broad function in

fine-tuning the protein-coding genes. Indeed, the discovery of miRNAs has revolutionized our understanding of gene regulation in the postgenome era.

It has been observed that plant miRNAs follow the strategy of perfect complementarity while animal miRNAs primarily employ the latter mechanism (Vandenboom Li, 2008) (see Figure 1.3). The imperfect miRNA-mRNA complementarity in human cells is usually composed of matched nucleotides in the 5' portion of the miRNA, termed the seed sequence (positions 2-7), with mismatches at positions 10 and 11 in the 3' UTR of the transcripts (Elbashir *et al.*, 2001a; Lewis *et al.*, 2005; Berkhout and Jeang, 2007). In humans, this complex leads to the inhibition of target gene translation and only very rarely causes degradation of the mRNA.

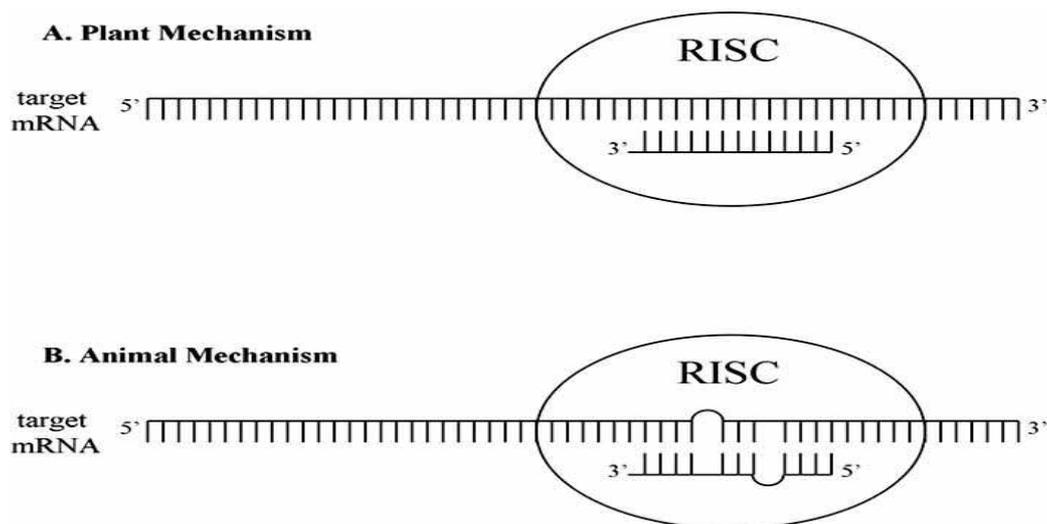


Figure 1.3 Modes of miRNA Action: **A)** complimentary base pairing of miRNA with mRNA, which results in the degradation of mRNAs, a common mechanism in plants. **B)** imperfect base pairing of miRNA with mRNA results in translational inhibition, the typical mode of action in animals (Vandenboom Li, 2008).

1.4.1 MicroRNAs and cancer.

When living cells exhibit abnormal growth and loss of apoptosis, it may result in cancer formation. Several recent studies indicate that miRNAs regulate cell growth and other cellular processes (Cheng et al., 2005).

A more direct link between miRNA function and cancer pathogenesis was supported by studies examining the expression of miRNAs in clinical samples. The first study documenting abnormalities in miRNA expression in tumor samples focused on B-cell chronic lymphocytic leukemia (B-CLL). Deletion of chromosome 13q14 is the most frequent chromosomal abnormality in this disorder, and it has been postulated that a tumor suppressor gene resides in this region. It was demonstrated that this tumor suppressor activity is likely provided by two miRNAs, miR-15a and miR-16-1, which are localized in the minimally deleted region and are frequently deleted or downregulated in CLL patients (Calin et al., 2002).

It was found that microRNA expression correlates with various cancers, and these regulatory genes are thought to function as tumor suppressors or oncogenes (see Table 1).

More than half of miRNAs are located at sites in the human genome which are frequently amplified, deleted, or rearranged in cancer, suggesting that miRNA abnormalities play a broad role in cancer pathogenesis (Calin et al., 2004). Also consistent with this notion, is the observed dysregulation of miRNA expression in diverse cancer subtypes including Burkitt's lymphoma (Metzler et al., 2004), colorectal cancer (Michael *et al.*, 2003), lung cancer (Takamizawa et al., 2004), breast cancer (Iorio et al., 2005), and glioblastoma (Chan et al., 2005).

Recently, Golub and co-workers used a bead-based flow cytometric method to profile 217 mammalian miRNAs across a large panel of samples representing diverse human tissues and tumors (Lu et al., 2005a). They found that miRNA profiles were highly informative, reflecting the developmental lineage and differentiation state of the tumors.

Furthermore, tumors within the same lineage harboring distinct chromosomal rearrangements were found to exhibit distinct miRNA expression profiles. Thus, the developmental history of a tumor is reflected by its miRNA expression pattern.

Table.1: Examples of some microRNAs involved in cancer.

miRNA	Cancer	Function	References
miR-15a, miR-16	Downregulated in B-cell chronic lymphocytic leukemia, negatively regulate BCL2	Tumor suppressor	(Calin <i>et al.</i> , 2002; Cimmino <i>et al.</i> , 2005)
miR-143,miR-145	Downregulated in colorectal cancers, breast, prostate	Tumor suppressor	(Michael <i>et al.</i> , 2003; Iorio <i>et al.</i> , 2005)
miR-21	Upregulated in Glioblastoma and breast cancer	Oncogenic	(Ciafre <i>et al.</i> , 2005; Iorio <i>et al.</i> , 2005)
Let-7 family	Decreased in Lung cancer	Tumor suppressor	(Takamizawa <i>et al.</i> , 2004; Johnson <i>et al.</i> , 2005)

Irrespective of cell type, more than half of the miRNAs examined in the Golub study were expressed at significantly lower levels in tumors compared with normal tissues. This likely reflects a role for miRNAs in terminal differentiation and the relatively incomplete differentiation status of cancer cells. Consistent with this hypothesis, inducing differentiation of a myeloid leukaemia cell line with trans- retinoic acid increased the expression of many miRNAs. A similar induction of miRNA expression was also observed in primary human haematopoietic progenitor cells undergoing erythroid differentiation. The emerging view from these studies is that deregulation of miRNA expression is a frequent occurrence in diverse types of cancer. These findings highlight the potential utility of miRNA profiling for diagnostic and prognostic applications. These studies also emphasize a need for more direct functional analyses of the roles of miRNAs in regulating pathways relevant to tumor pathogenesis.

Colorectal cancer is associated with alterations in miRNA expression where it was reported that miR-143 and miR-145 was significantly reduced at the adenomatous and cancer stages

of colorectal cancer (Michael *et al.*, 2003). miRNAs are also involved in brain cancer where Glioblastoma multiforme is the most frequent malignant brain tumor and these tumors are highly aggressive, invasive and one of the most incurable cancer in human (Ciafre *et al.*, 2005). It was found by the same investigators, that miR-221 was strongly upregulated in glioblastoma samples from patients while miR-181a, miR-181b and miR-181c were downregulated in glioblastoma compare to normal samples.

MiR-372 and miR-373 were reported to function as oncogenes in human testicular germ cancers, which induce the proliferation and tumorigenesis of primary human cells that have both oncogenic RAS and active wild type p53 (Voorhoeve *et al.*, 2006).

1.4.2 MicroRNAs and apoptosis

Apoptosis is a programmed, physiological mode of cell death that plays an essential role in tissue homeostasis. Deregulation of apoptosis is a critical step in cancer as it allows the genetically unstable cells to survive and accumulate further mutations that eventually lead to tumorigenesis. Multiple triggers of apoptosis are known, such as withdrawal of growth factors, DNA damage, Fas-ligand binding, or application of chemotherapeutic drugs (Collins *et al.*, 1994; Berke, 1995).

All of these triggers lead to cell death but they differ in the length of the period after which the cells shows the first morphological signs of apoptosis. This period is called trigger phase. The duration of this phase depends on the type of triggers, the cell type and the surrounding environment. Some genes were reported to control this phase, such as BCL2 that protects the cells against apoptosis (Martin and Green, 1995; Nagata, 1997).

It was reported that microRNAs play an important role in regulating the expression level of these genes such as the miR-15-16 cluster which induces apoptosis by targeting the anti-apoptotic gene BCL2 at the translational level (Cimmino *et al.*, 2005). Enhanced expression of miR-145 in urothelial carcinoma cell lines (T24 and SW780) induced apoptosis as

determined by Caspase activity, DNA fragmentation and nuclear condensation (Ostenfeld et al., 2010).

It was demonstrated that miR-26a and miR-145 have an important role in TRAIL-induced apoptosis (Sudbery et al., 2010).

MiR-21 was reported as the most upregulated miRNAs in many cancer types. miR-21 was established as an antiapoptotic factor by the observation that knock-out of miR-21 increased apoptotic cell death in human glioblastoma cells (Chan *et al.*, 2005).

1.5 SiRNA delivery

RNA interference has been regarded not only as an innovative approach to suppress the expression of a target gene, but also as a new therapeutic strategy to combat many diseases such as cancer, autoimmune diseases and viral infections (Iorns et al., 2007). SiRNAs and miRNAs can theoretically interfere with the expression of many genes through transcriptional or translational repression (de Fougères et al., 2007). Much progress has been made in clinical trials using siRNAs to treat many diseases such as age-related macular degeneration and respiratory syncytial virus infection (Melnikova, 2007). Moreover, the first evidence of targeted *in vivo* gene silencing for human cancer therapy through systemic delivery of siRNA using transferrin-tagged, cyclodextrin-based polymeric nanoparticles has been recently presented (Oh and Park, 2009).

The most important challenge in the use of siRNA-based therapies is the difficulty of delivery. Therefore, many strategies of siRNA delivery have been developed either *in vitro* or *in vivo*. Some strategies use viruses as natural vehicles while others use liposomes, nanoparticles or bacteria (transkingdom RNAi) (Li, 2006; Aigner, 2009; Kruhn *et al.*, 2009; Nguyen and Fruehauf, 2009).

Five types of viral vectors are currently in use for RNAi, including the Retrovirus, Lentivirus, Adenovirus, Adeno-Associated-Virus (AAV), and Baculovirus (Ong et al.,

2005), and have been used efficiently to deliver siRNAs and induce gene silencing in a wide range of mammalian cells (Brummelkamp et al., 2002). Adenoviral vectors encoding siRNA against pituitary transforming gene 1, which lead to inhibition of the growth of the pituitary tumor *in vitro* and *in vivo* (Cho-Rok et al., 2006). Adeno-associated virus (AAV) has been approved as a promising and effective vector for nucleic acid delivery because of its non-pathogenicity and has a broad range of possible target cells, including non-dividing cells. It was reported that Adeno-associated virus has been efficiently used for *in vitro* siRNA delivery (Tomar et al., 2003; Moore et al., 2005) as well as it has been used *in vivo* transfection via local stereotactic injection into the brain (Xia et al., 2004; Babcock et al., 2005).

Cationic liposomes formulated with anisamide-conjugated poly ethylene glycol penetrated effectively into lung metastases of melanoma tumors in mice and resulted in 70-80% gene silencing after a single intravenous injection (Lia SD, 2005). Chemically modified siRNA for enhanced RNA interference has been established, where several positions in siRNA have been modified or replaced in order to increase the efficiency of RNAi. For example, phosphodiester (PO₄) linkages were replaced with phosphothioate at the 3' end, and introducing O-methyl group greatly extended half-lives in plasma and enhanced RNAi efficiency in cultured cells (Braasch et al., 2003; Chiu and Rana, 2003; Czauderna et al., 2003; Harborth et al., 2003).

SiRNAs can be easily complexed with synthetic polymers e.g., polyethylenimine (PEI), biodegradable cationic polysaccharides, chitosan, and cationic polypeptides, atelocollagen or protamine, through electrostatic interactions. For example, an endothelial growth factor siRNA/atelocollagen complex significantly inhibited tumor angiogenesis and growth in prostate xenografts in mice (Takei et al., 2004). Intravenous injection of RhoA siRNA/chitosan complex resulted in effective gene silencing in subcutaneous breast cancer xenografts in mice (Pille et al., 2006).

In order to achieve RNAi *in vivo* via systemic delivery, it is crucial for siRNA to be efficiently delivered into the desired tissues or cells. This requires three important

processes: prolonged circulation in the body, high accessibility to target tissues and specific binding to target cells. Targeted siRNA delivery maximizes the local concentration in the desired tissue and prevents nonspecific siRNA distribution. Recent studies have reported tumor-targeted siRNA delivery using nanoparticles that specifically bind to cancer-specific or cancer-associated antigens and receptors (Dubey et al., 2004; Lu et al., 2005b).

Polyethylenimine is one of the most important nanoparticles used in nucleic acid delivery (Boussif et al., 1995).

1.5.1 Polyethylenimine (PEI)

Polyethylenimine (PEI) is one of the most widely examined synthetic cationic polymers for nucleic acid delivery *in vitro* and *in vivo*. Polyethylenimines are synthetic linear or branched polymers available in a wide range of molecular weights (Tang and Szoka, 1997; Godbey *et al.*, 1999). PEI is very efficient in nucleic acid delivery with its distinctively high buffering capacity at endosomal pH ‘proton sponge effect’ which releases ‘the nucleic acid payload’ into the cytoplasm (Boussif *et al.*, 1995).

PEI exists as branched or linear morphological isomers. Branched polyethylenimine (bPEI) has been used to deliver oligonucleotides (Bandyopadhyay et al., 1999), plasmid DNA (Iwai et al., 2002) as well as RNA and intact ribozymes (Aigner et al., 2002). The efficacy of branched-PEIs as non-viral vehicles and their cytotoxic effects depend on material characteristics like the molecular weight, the degree of branching, the cationic charge density and buffer capacity (Fischer *et al.*, 1999; A. von Harpe, 2000; Kunath *et al.*, 2003)

The efficiency of transfection is depending on some factors e.g. the N/P ratio, which refers to the ratio of the nitrogen atoms of PEI to nucleic acid phosphates and simply describes the amount of polymer used for polyplex formation (Lungwitz et al., 2005). The N/P ratio influences the efficiency of the nucleic acid delivery, it has been shown that every fifth or sixth amino nitrogen of branched-PEI is protonated at physiological pH (J. Suh, 1994), and

these positively charged amino groups can interact with the negative charge of nucleic acids. Therefore at high N/P ratio and the positive net charge of the corresponding complexes increases, which leads to improvement of cell interaction and enhancement of the cellular and nuclear uptake (Oh et al., 2002). Particle size of the polyethylenimine plays an important role in efficacy of transfection process, which can be controlled by the N/P ratio, the molecular weight and during the particle preparation (Ogris et al., 1998).

In contrast to branched-PEI, linear polyethylenimine (l-PEI) is characterized by presence of all secondary amino groups within the random copolymers which increase the buffering capacity and enhance the nucleic acid compaction (Lungwitz *et al.*, 2005), while b-PEI contains primary, secondary, and tertiary amino groups.

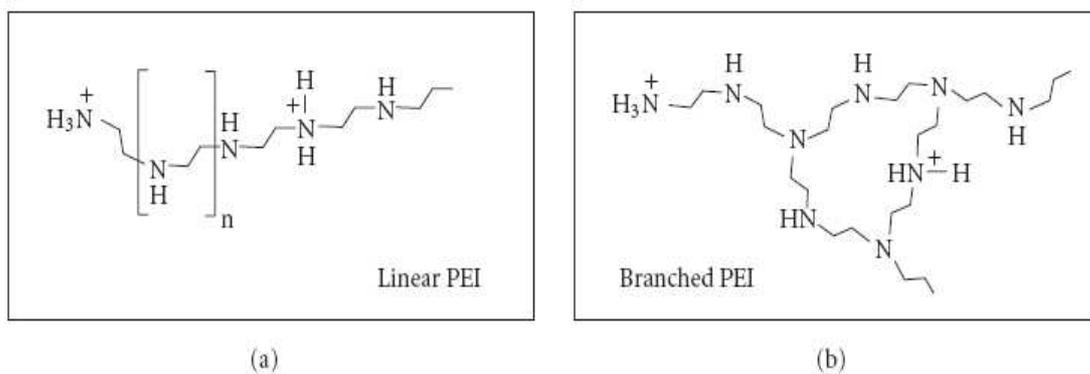


Figure 1.4: Chemical structure of linear-PEI and branched-PEI

It was shown that l-PEI is an efficient transfection agent *in vitro* and *ex vivo* (Louis et al., 2006). In this study, they proved that intraperitoneal injection of ovarian tumor nodes developed in mice with l-PEI/DNA complex led to straightforward distribution of plasmid in the complete peritoneal cavity.

In vivo studies, in xenografted mice, have established that intraperitoneal injection of PEI-complexed siRNAs lead to delivery of intact siRNAs into subcutaneous tumors and efficient suppression of tumor growth as illustrated in studies carried out by (Urban-Klein et al., 2005).

Polyethylenimine complexes with the nucleic acids in an anionic interaction and introduce them into a variety of different cells via endocytosis (Klemm et al., 1998).

Proton sponge hypothesis was postulated by (Behr, 1994), where it allows the osmotic swelling of endosomal vesicles which lead to rupture of the vesicles and release of the PEI/complex in the cytoplasm.

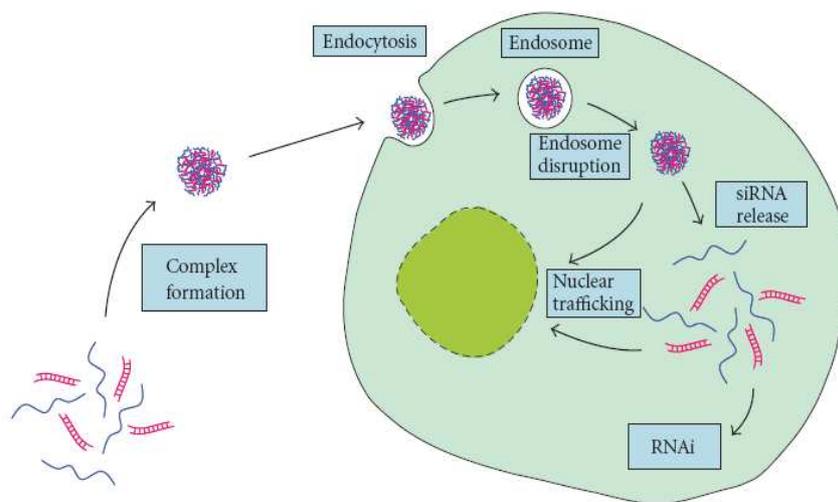


Figure 1.5: Proposed mechanism of PEI-mediated siRNA delivery (Aigner, 2006).

2. Objectives of the thesis

The aims of this work were to study the non-viral delivery of microRNA in colon cancer *in vitro* and *in vivo*. To this end, this thesis focused on the following points:

- 1- Selection of appropriate microRNAs that are already known to be downregulated in colorectal cancer. This step was done by searching several databases such as <http://www.targetscan.org/>, <http://www.microrna.org/microrna/home.do>.
- 2- Evaluation of the expression level of the mature and precursor forms of miR-143 and miR-145 in different cell lines of different origins.
- 3- Establishment of *in vitro* miRNA delivery by different transfection reagents through variations in transfection conditions.
- 4- Analysis of cellular effects of miR-143 and miR-145 delivery in colon cancer cells (proliferation assay, apoptosis assay, soft agar assay, biochemical and immunochemical analysis).
- 5- Evaluation of the antitumor effects of Polyethylenimine (PEI)-mediated miR-145 delivery *in vivo* using athymic nude tumor xenograft models.
- 6- Analysis of cellular effects of the therapeutic miRNA replacement *in vivo* in the s.c tumor xenografts.

3. Materials and Methods

3.1 Materials

3.1.1 Reagents

Standard chemicals and reagents were purchased from well-known companies.

Name	Supplier
Ammonium Peroxodisulfate (APS)	Merck
Bacto-Agar	Becton
BromphenolBlue	ROTH
BSA	ROTH
Cell Proliferation Reagent WST-1	Roche
3,3'-Diaminobenzidinetetrahydrochloride (DAB)	SIGMA-Aldrich
Diethylpyrocarbonate (DEPC)	SIGMA-Aldrich
Harris hematoxylin solution	SIGMA-Aldrich
Jet-PEI	Polyplus
PEI F25-LMW (LMW 4-10 kDa)	fractionated and purified in the lab
INTERFERin TM	Polyplus
2-Mercaptoethanol	SIGMA-Aldrich
Milk powder	SIGMA-Aldrich
Normal goat serum	Vector
Normal horse serum	Vector
N,N,N',N',-Tetramethylethylenediamine (TEMED)	SIGMA-Aldrich
Prestained protein ladder, 11-250 kDa	Fermentas
RotiPhorese® Gel 30	ROTH
Trizol Reagent	SIGMA-Aldrich
Tween 20	SIGMA-Aldrich

3.1.2 Kits and enzymes

Name	Supplier
mirVana™ microRNA isolation kit	Ambion
LumiGlo HRP Western blot kit	Amersham
DC protein assay	Bio-Rad
Caspase-Glo® 3/7 assay	Promega
RevertAid™ H Minus First Strand cDNA synthesis kit	Fermentas
RevertAid™ H Minus Reverse Transcriptase	
RiboLock™ RNase inhibitor	
5X Reaction buffer	
dNTP Mix, 10 mM each	
ABsolute™ QPCR SYBER® GREEN Capillary MIX	Thermo Scientific
FITC Annexin V apoptosis detection kit I	BD Pharmingen™
10X annexin V binding buffer	
FITC annexin V	
Propidium iodide staining solution	

3.1.3 Antibodies

Name	Supplier
Rabbit polyclonal C-Myc antibody	Santa Cruz Biotechnology
Rabbit polyclonal ERK5 antibody	Cell Signaling
Rabbit polyclonal β -actin antibody	Santa Cruz Biotechnology
Anti-Rabbit IgG horseradish peroxidase linked antibody	GE Healthcare
Biotinylated horse anti-mouse IgG antibody	Vector laboratories
Mouse monoclonal PCNA antibody	Cell Signaling

3.1.4 Oligonucleotides and primers

Name	Sequence
hsa-miR-143 for	5'-GGUGCAGUGCUGCAUCUCUGGU-3'
hsa-miR-143 rev	5'-UGAGAUGAAGCACUGUAGCUC-3'
hsa-miR-145 for	5'-GUCCAGUUUCCCAGGAAUCCCU-3'
hsa-miR-145 rev	5'-GGAUUCCUGGAAAUACUGUUCU-3'
ERK5 for	5'-AATGGCGGACACAATTCC-3'
ERK5 rev	5'-GCCGTCTTCCTCCTTCAGA-3'
c-Myc for	5'-CACCAGCAGCGACTCTGA-3'
c-Myc rev	5'-GATCCAGACTCTGACCTTTTGC-3'
PCNA for	5'-TGGAGAACTTGGAAATGGAAA-3'
PCNA rev	5'-GAACTGGTTCATTCATCTCTATGG-3'
Actin for	5'-CCAACCGCGAGAGAGTGA-3'
Actin rev	5'-CCAGACGCGTACAGGGATAG-3'
Stem loop RT primer miR-145	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACAGGATT-3'
hsa-miR-145 for	5'-CGCGCGTCCAGTTTTCCAGG-3'
Stem loop RT primer miR-143	5'-CGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGAGCTAC-3'
hsa-miR-143 for	5'-CGCGCGTGAGATGAAGCACT-3'
Universal Reverse PCR Primer	5'-GTGCAGGGTCCGAGGT-3'

3.1.5 MicroRNAs

Name	Sequence
hsa-miR-143 for	5'-GGUGCAGUGCUGCAUCUCUGGU-3'
hsa-miR-143 rev	5'-UGAGAUGAAGCACUGUAGCUC-3'
hsa-miR-145 for	5'-GUCCAGUUUCCCAGGAAUCCCU-3'
hsa-miR-145 rev	5'-GGAUUCCUGGAAAUACUGUUCU-3'

3.1.6 Tissue culture media and reagents

Name	Supplier
IMDM + Glutamine	PAA
Fetal Calf Serum (FCS)	Gibco
Trypsin/EDTA	PAA
Phosphate buffered Saline (PBS)	PAA
Keratinocyte SFM with Glutamine	Gibco

3.1.7 Cell lines

Cell Line	Tissue	Cell Type	Source
A549	Lung	Adenocarcinoma	ATCC
K562	Bone marrow	Chronic myelogenous leukemia	ATCC
SKOV3	Ovary	Adenocarcinoma	ATCC
OVCAR-3	Ovary	Adenocarcinoma	ATCC
DU145	Prostate	Adenocarcinoma	ATCC
PC3	Prostate	Adenocarcinoma	ATCC
HCT116	Colon	Colorectal carcinoma	ATCC
SW620	Colon	Colorectal adenocarcinoma	ATCC
SW480	Colon	Colorectal adenocarcinoma	ATCC
LS180	Colon	Colorectal adenocarcinoma	ATCC
LS174t	Colon	Colorectal adenocarcinoma	ATCC
LoVo	Colon	Colorectal adenocarcinoma	ATCC
Colo201	Colon	Colorectal adenocarcinoma	ATCC
U87	Brain	Glioblastoma	ATCC
1205LU	Skin	Melanoma	ATCC
NIH/3T3	Embryo	Fibroblast	ATCC

3.1.8 Devices and working materials

Name	Supplier
Adjustable air-displacement pipettes Pipetman®	Gilson
Autoclave Varioklav®	H+P Labortechnik
Balance ERN572	KERN&Sohn
Centricon-10 vials	Amicon
Centrifuge Biofuge	Heraeus
Centrifuge Megafuge 1.0 R	Heraeus
Centrifuge Sorvall RC 5B	Du Pont
CO ₂ -incubator Hera Cell	Heraeus
Digital Camera Digital Science DC 120	Zoom Kodak
Dounce homogenizer	B.Braun
ELISA reader	BIO-TEK
Exposition cassette (Hypercassette™)	Amersham Pharmacia
FLUOstar OPTIMA	BMG LABTECH
FACSCalibur	Becton-Dickinson
Heat block	PeQLab
Hyperfilm ECL	Amersham Pharmacia
Laminar flow hood Hera Safe	Heraeus
Light microscope Wilovert A	Hund
Light cycler	Roche Diagnostics, Penzberg
Magnetmixer Variomag®	H+P Labortechnik
PCR T3 thermal cycler	Biometra
pH-meter	GREISINGER Electronic
Orbital shaker	Forma Scientific
Power supply PAC300	Bio-RAD
PVDF 0.45um membrane	Schleicher & Schuell
-80°C freezer	Forma Scientific
Rotor SS-34	Sorvall
Ultracentrifuge L7-55	Beckman

UV-Bank UV Transilluminator 2000	Bio-Rad
Vortex	Janke&Kunkel

3.1.9 Standard solutions and buffers

<u>Name</u>	<u>Components and Concentration</u>
Cell culture freezing medium	
DMSO	5% (w/v)
FCS	2 0% (v/v)
IMDM	ad 100% (v/v)
=====	
10x PBS (Phosphate-buffered saline)	
NaCl	1.5 M
Na ₂ HPO ₄	52 mM
KH ₂ PO ₄	17 mM
=====	
PBS-T	
NaCl	150 mM
Na ₂ HPO ₄	5.2 mM
KH ₂ PO ₄	1.7 mM
Tween 20	0.05% (v/v)
=====	
10xTBS-T	
Tris-HCl	1 M
NaCl	1.5 M
Tween 20	0.5% (v/v)
=====	
SDS-Loading Buffer	
SDS	4 % (v/v)
2-Mercaptoethanol	40 % (v/v)

Glycerol	20 % (v/v)
Bromphenol blue	0.01 % (w/v)

10x SDS electrophoresis buffer

Tris-HCl	2.9 % (w/v)
Glycine	14.4 % (w/v)
SDS	1 % (w/v)

10x transfer buffer

Tris-HCl	2.9 % (w/v)
Glycine	14.4 % (w/v)
pH adjusted to 8.5	

10x Western blot electrophoresis transfer buffer

Tris-HCl	2.9 % (w/v)
Glycine	14.4 % (w/v)
pH adjusted to 8.5	

Western blot stripping buffer

Glycine	0.1M
pH adjusted to 2.9	

1XCell Lysis buffer

Tris-HCl	20 mM
NaCl	150 mM
EDTA	1 mM
Triton X-100	1% (v/v)
Sodium pyrophosphate	2.5 mM
NP40	1 % (v/v)

SDS	0.1% (w/v)
PMSF immediately prior to use	1 mM
Complete mini EDTA free Protease inhibitor cocktail	
pH adjusted to 7.5	

=====

Blocking Buffer

Milk powder	5% (w/v) in TBST
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=====

Primary Antibody Dilution Buffers

BSA	5% (w/v) in TBST
Milk powder	5% or 3% (w/v) in TBST

=====

Secondary Antibody Dilution Buffer

Milk powder	5% (w/v) in TBST
-------------	------------------

3.2 Methods

3.2.1 Cell culture

All cell lines were cultured in 75 cm² flasks in Dulbecco's modified Eagle's medium supplemented with glutamine 10% fetal calf serum, with the exception of 1205LU, which were maintained in Keratinocyte SFM supplemented with Glutamine and 10% fetal calf serum. Handling and propagation of all cell lines were done under sterile conditions. All solutions were stored at 4°C and pre-warmed to 37°C in a water-bath prior to use. All cell lines were grown under standard conditions (37°C, 5% CO₂) in a humidified incubator.

3.2.2 Freezing and Thawing of cultured cell lines

To store the cell lines for long term, it was recommended to dilute them in freezing medium and to keep them at –80°C or in liquid nitrogen. Cells were harvested (at least at 80 % confluency) and centrifuged at 1000g for 5 min. The media was then aspirated and the cells were resuspended in 1.5 ml of cell culture freezing medium (IMDM culture medium, 20% FCS and 5% DMSO). The resuspended cell solution was transferred to cryo-tubes and placed at –80°C in an isopropanol-containing cell-freezing container, which guarantees a slow freezing process. After 24 h, the tubes were transferred to a storage box at –80°C or in liquid nitrogen.

To grow up a cell line, the freeze stock of the cells was thawed quickly in a 37°C water-bath then, added to 5ml fresh medium and centrifuged at 800g for 10 min to get rid of the traces of the cryoprotectant (DMSO). Then, the supernatant medium was aspirated and a fresh growth medium was immediately added to the pellet, and the suspension was transferred to a culture flask containing 10 ml medium and incubated under standard conditions (5% CO₂, 37°C).

3.2.3 Maintenance of cells in culture

All cell lines were grown in a 37°C, 5% CO₂ incubator and split in certain ratios depending on the stage of confluency and the proliferation rate of each cell line. The medium was changed every three days (according to the confluency). To split the cell lines, the medium was completely aspirated from the flask and the cells were washed briefly with 5-10 ml of PBS to remove traces of FCS. After removing the PBS, 2 ml trypsin was added to the flasks and then incubated at 37°C. The cells were detached after about 1-2 min with occasional gentle tapping. The trypsin was then immediately inactivated by adding 8 ml of serum-containing medium to each flask. This suspension was transferred into a 15 ml tube and centrifuged at 1000g for 5 min. The supernatant was aspirated and the cell pellet was resuspended in medium and split into new flasks. The amount of medium added to the flask was dependent on its size. For 75 cm² flasks, 10 ml medium was added

3.2.4 Cell Transfection

Transient transfections were performed using Jet-PEI, PEI F25-LMW and INTERFERin as transfection reagents. Each reagent has its own protocol of transfection as indicated by the manufacturers. Prior to transfection, cells were grown to 50-70% confluency in culture flasks in 10ml IMDM medium containing 10 % FCS.

The cells were seeded on the day before transfection in 96-well plates and the complexes were added subsequently to the cells. The transfection was done according to the type of the reagents where Jet-PEI was used at (N/P ratio 5), PEI F25-LMW was used at (N/P ratio 33) while INTERFERin was added in (a volume manner) as follows.

Jet-PEI complexation of 50 nM miRNA

Culture dish	Number of cells/ well	Volume of medium/ well	Amount of miRNA+ HEPES/NaCl for 20 wells	Volume of Jet-PEI + HEPES/NaCl for 20 wells	Total volume in well
96-well	1000	100 μ l	2 μ l +200 μ l	2.6 μ l+200 μ l	120 μ l
6-well	200-300x10 ³	2 ml	2 μ l+ 50 μ l (for one well)	2.6 μ l+50 μ l	2.1 ml

PEI F25-LMW complexation of 5 nM miRNA

Culture dish	Number of cells/well	Volume of medium/well	Amount of miRNA+ HEPES/NaCl for 20 wells	Volume of PEI-F25+ HEPES/NaCl for 20 wells	Total volume in well
96-well	1000	100 μ l	10 μ l +200 μ l	0.13 μ l+ 200 μ l	120 μ l
6-well	200-300x10 ³	2 ml	2 μ l+ 50 μ l (for one well)	0.13 μ l+50 μ l	2.1 ml

INTERFERin complexation with 20nM miRNA

Culture dish	Number of cells/well	Volume of medium/well	Amount of miRNA+ medium without serum +INTERFERin for 20 wells	Total volume in well
96-well	1000	100 μ l	0.8 μ l + 1000 μ l+ 8 μ l	150 μ l
6-well	200-300x10 ³	2 ml	8 μ l+ 200 μ l + 10 μ l (for one well)	2.2 ml

3.2.5 *In Vitro* analysis

3.2.5.1 Anchorage-dependent proliferation assay

The measurement of cell proliferation and cell viability has become a key technology in life science. A proliferation assay has become available for analyzing the number of viable cells by cleavage of the tetrazolium salt (WST-1: 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) added to the culture medium. This technique requires neither washing nor harvesting of cells and the complete assay from the beginning to measurement by ELISA reader was performed in the same plate.

The principle of this assay is that the tetrazolium salt is cleaved to form a water-soluble formazan red dye by cellular enzymes (mitochondrial dehydrogenases). The soluble formazan produced by metabolically active cells was quantified through a scanning in a multiwell spectrophotometer (ELISA reader) by measuring the absorbance of the dye at 450nm.

The determination of anchorage-dependent proliferation of LS174t and HCT-116 was carried out in a humidified incubator under standard conditions. LS174t cells were seeded in triplicates in 96-well plates at 1000 cells per well on the day before transfection (100 μ l IMDM culture medium supplemented with 10% FCS, while HCT-116 were seeded at 200 to 300 cells per well in the same medium.

The cell density was measured at various time points (4 - 5 days) to obtain a growth curve.

At the time of measurement, the medium was aspirated and the WST-1 was diluted in IMDM culture medium 1:10 and 50 μ l was added to each well and incubated for 1 h at 37°C in 5% CO₂ humidified incubator. For measuring the background, an empty well was filled with 50 μ l diluted WST-1 at every time point.

3.2.5.2 Anchorage-independent Proliferation assay

The anchorage-independent growth assay (soft agar assay) resembles more closely the *in vivo* conditions since the transformed cells have the ability to form three-dimensional colonies in soft agar.

LS174t and HCT116 cells were seeded in 6-well plates at $200\text{-}300 \times 10^3$ per well on the day before transfection. The LS174t cells were transfected with 100 nM miRNAs using JET-PEI while HCT-116 were transfected with 20nM miRNAs using INTERFERin as transfection reagent, the cells were then incubated for 24 h in 37°C, 5% CO₂ humidified incubator.

Before doing the experiments, 2.4% agarose was prepared (2.4 g Bacto-agar was dissolved in 100 ml mono-distilled water and autoclaved).

In the laminar flow, a water bath at 42°C was prepared in which an empty sterilized 50 ml bottle was placed with 1.25 ml 10x IMDM-medium and 37.5 ml 1x IMDM-medium with 10% FCS.

The 2.4% agarose was melted completely in a microwave and then cooled to about 50°C. Then 12.5 ml was added to the bottle in the water bath (resulting in a final agarose concentration of 0.6%).

1 ml of 0.6 agarose solution was pipetted into a well of a 6-well plate (bottom layer), air bubbles were avoided, and the plates were then allowed to cool and solidify.

During solidification of the bottom layer, the cells were trypsinized, harvested, counted and adjusted to 6×10^4 cells/ml.

In a 12-well plate, 1 ml of this cell suspension was mixed with 1.5 ml of the 0.6 % agarose mixture was added to the same well.

Then 800 μl (about 20×10^3 cells per well) of this mixture was immediately overlaid on the bottom layer of 6-well plates (the experiment was done in triplicates).

Depending on the cell line, the plates were incubated 1-3 weeks at 37°C , 5% CO_2 in a humidified incubator. Colonies larger than 50 μm in diameter were counted independently by two blinded investigators.

3.2.6 Biochemical and immunochemical methods

3.2.6.1 Protein and measurement of protein concentration

To analyze expression level of our targets at the protein level, transient transfections were performed as described above in LS174t and HCT-116 with 100 nM or 20 nM miRNAs, respectively. At 96 h post transfection, cells were lysed by addition of 200 μl denaturing lysis buffer, sonicated for 20 sec, then incubated on ice for 30 min and then centrifuged at 13,000 rpm for 25 min. The supernatant was pipetted into a new tube and stored at -20°C .

The protein concentration in lysates either from cell lines or from tumor xenografts were measured by the Bio-Rad DC protein assay kit by Elisa Reader at 630 nm using standard curve of different concentration of bovine albumin as standard. Measurement of protein concentration was performed in 96-well plates in triplicates and in comparison to a blank as following:

10 μl sample (cell or tumor lysate)

20 μl solution A* (1 ml solution A + 20 μl solution S)

Mix well, then

100 μl solution B

Incubation for 15 min.

Before electrophoretic separation, the tertiary structure of protein was denatured through thermal denaturation at 95°C in SDS-containing sample loading buffer. By Addition of this anionic detergent, the protein chains were covered with negative net charge.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate complex protein mixtures by their molecular size. The standard Laemmli method is applied for discontinuous gel electrophoresis under denaturing conditions in the presence of SDS. Western blotting (immunoblotting) is applied to identify specific proteins (antigens) recognized by polyclonal or monoclonal antibodies. After separation by SDS-PAGE, the proteins are electrically transferred onto PVDF membranes. The transferred proteins are bound to the surface of the membrane providing access for immunodetection reagents.

All remaining binding sites are blocked by immersing the membrane in a solution containing either a protein or a detergent-based blocking agent. After probing with the primary antibody, the membrane is washed and the antigen is identified by detection with a secondary horseradish peroxidase-conjugated anti-IgG antibodies. Visualization of the antigen/antibody complex is performed by enhanced chemiluminescence using sensitive light-films.

Sample preparation

According to the protein concentration, 100 µg of protein lysate was mixed with 4x SDS loading buffer then diluted to 1x by adding distilled sterilized water. Then the samples were incubated in a hot plate at 90°C for 5 min.

Preparation of SDS gels.

Separation gel 7%	3.5 ml 30% acrylamid/0.8% bis-acrylamide 3.75 ml 4x Tris-HCl/SDS, pH8.8 7.75 ml H ₂ O 50 µl 10%(w/v) ammonium persulphate (APS) 10 µl TEMED (N,N,N,N-Tetramethylendiamin)
Stacking gel 4%	1ml 30% acrylamid/0.8% bis-acrylamide 1.25 ml 4x Tris-HCl/SDS, pH8.6 3.05 ml H ₂ O 50 µl 10% (w/v) ammonium persulphate (APS) 20 µl TEMED (N,N,N,N-Tetramethylendiamin)

The separation gel solution was prepared and mixed well. It was poured between the glass plates and then overlaid with isopropanol to straighten and even the level of the gel. After polymerization, the isopropanol was aspirated, the stacking gel was added and a suitable comb was directly inserted.

For c-Myc, a 15 % gel was performed.

Separation gel 7%	7.5 ml 30% acrylamid/0.8% bis-acrylamide 3.75 ml 4x Tris-HCl/SDS, pH 8.8 3.75 ml H ₂ O 50 µl 10%(w/v) ammonium persulphate (APS) 10 µl TEMED (N,N,N,N-Tetramethylendiamin)
Stacking gel 4%	1ml 30% acrylamid/0.8% bis-acrylamide 1.25 ml 4x Tris-HCl/SDS, pH8.6 3.05 ml H ₂ O 50 µl 10% (w/v) ammonium persulphate (APS) 20 µl TEMED (N,N,N,N-Tetramethylendiamin)

Electrophoresis and Western Blot

The comb was carefully removed from the gel cassette and the whole gel was placed in a chamber filled with an adequate volume of gel running buffer. 40 µg of denatured protein was injected smoothly, using a suitable syringe, into the wells. Likewise, in one lane a protein ladder (11-250 kDa) was added. The gel was run for at 200V until the bromophenolblue reached the end of the gel.

The gel was removed from the apparatus and the upper stacking gel was discarded, and the separation gel was incubated in about 100 ml transfer buffer for 5 min with shaking. In the mean time, one piece of PVDF per gel cut to the size of the gel, was incubated in methanol for 1 min, washed in bidistilled water and then incubated in 100 ml transfer buffer for at least 10 min.

The transfer sandwich was prepared in the following order from the negative cathode to the positive anode.

Three pads wetted in the transfer buffer

Three wetted filter papers

The gel

The PVDF membrane

Three wetted filter papers

Three wetted pads

The transfer cassette was tightly put into the transfer chamber, while was filled with 1X transfer buffer. The transfer was performed for 90 min. The PVDF membrane was then removed. Optionally, it was incubated in Ponceau S solution (0.1 % ponceau S (w/v) in 5 % acetic acid (v/v) for 30 sec to check the efficiency of blotting process, and then washed under tap water several time to remove the stain remaining.

The membrane was then incubated in blocking solution (5 % non-fat dry milk in 1X TBST) for one hour at room temperature with shaking.

The blot was washed three times 5 min each, and then incubated in the primary antibody dilution at 4°C overnight (1:1000 for ERK5 and 1:500 for c-Myc). It was then washed three times 5 min each in TBST and incubated in a 1:2000 dilution of donkey anti-rabbit secondary antibody coupled to horseradish peroxidase for 1 h at RT. After additional washing steps in TBST as above, bound antibody was visualized as bands using the enhanced chemiluminescence reagents system from Amersham at different exposure times.

3.2.6.2 Immunohistochemistry

This experiment was performed over two days, using a standard streptavidin biotinperoxidase complex method. On first day The tumor sections (3 μ M thickness) were firstly deparaffinized by immersing them two times in Xylol for 10 min each, then

rehydrated by washing in 100% ethanol two times 5 min each and then 2 min for one time in a gradual concentrations of ethanol (90%, 80%, 70%). The slides were washed for 1 min in bi-distilled water and then washed one time for 5 min in phosphate buffered saline-Tween (PBST).

For antigen retrieval, the slides were immersed in a 90°C 1% acetic acid (v/v PBS) for 15-20 min. The slides were left to cool down for 20 min at room temperature then washed in PBST for 5 min.

For blocking endogenous peroxidase, the slides were incubated in 3% peroxide solution (10 ml 30% H₂O₂ to 90 ml ddH₂O) at 4°C for 30 min; the slides were washed three times in PBST for 5 min each. Manually the slides were dried and carefully the surrounding area around the tumor sections. By a grease pencil, a thick circle was lined around the sections to prevent the leakage of subsequent added solutions.

For blocking of the non-specific sites on the sections, 100-200 µl of blocking solution (10% horse serum in 1X PBST and 2% BSA) was added, the blocking solution which was used was from the species the secondary antibody was derived from, and incubated for one h at room temperature. The blocking solution was aspirated (not washed) and directly (avoid dryness) 100 µl of the primary antibody dilution 1:400 (diluted in PBST and 2% BSA) was added. The slides were incubated in a humidified chamber overnight at room temperature.

On the second day, the slides were washed three times 5 min each, then the secondary antibody was added which was diluted in PBST and 2% BSA at 1:3000 and incubated for one h at room temperature.

The slides were washed three times in PBST 5 min each. During washing step, the avidin/biotin complex (ABC) was prepared as follow (10 ml PBST+ 2 drops avidin and vortex well and then 2 drops biotin and vortex). The ABC was added to the section and incubates for 30 min at room temperature. During last 5 min of incubation, the 3,3'-diaminobenzidine (DAB) solution was prepared (250 mg DAB was dissolved in 50ml

ddH₂O), 180 ml PBST and 20 ml 1M Tris-HCl pH 7.5 was added, then 25 µl H₂O₂ was added just before use.

Before doing staining step, the slides were washed in PBST three times 5 min each, and then the slides were incubated at room temperature in DAB solution for adequate time to get enough staining. The staining was stopped by immersing the slides in ddH₂O. The slides were counter-stained by a blue hematoxylen for 30 sec to one min. Then the slides were washed in tap water for 5 min and checked under microscope.

The slides were mounted with organic mounting media but previously the slides were dehydrated by immersing them in gradual concentrations of ethanol but in reverse manner (i.e.; 70%, 80%, 90% and 100%), then in Xylol for 10 min, the slides were well dried, the mounting media was added and finally covered by a glass cover.

3.2.7 Apoptosis analysis

3.2.7.1 Caspase Glo3/7 assay

The Caspase-Glo® 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. These members of the cysteine **aspartic** acid-specific protease (Caspase) family play effective roles in the apoptosis process in mammalian cells.

This assay was measured at various time points (24, 72 and 120 h) after miRNA transfection of cells.

The cells were seeded in a 96 well plates. The media was aspirated and 100 µl of fresh Caspase-Glo® 3/7 Reagent to each well. In addition, 100 µl of fresh caspase reagent was measured in an empty well as a blank.

The plates were incubated for 1 h at room temperature in the dark. The caspase level was measured in a plate reading luminometer.

In parallel, the number of viable cells was determined by a WST-1 assay as described above.

The readings of caspase levels in each well were normalized to the values of viable cells as determined by WST-1.

3.2.7.2 FITC-Annexin assay

This assay provides a simple and efficient method to detect apoptosis at a very early stage. It takes advantage of the fact that phosphatidylserine (PS) is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis, and that the annexin V protein has a strong, specific affinity for PS.

Phosphatidylserine on the outer leaflet is then available to bind labeled annexin V, providing the basis for a simple staining assay.

The LS174t cells were seeded and transfected in 6-well plates with miRNAs as described above. The cells were harvested after 120 h, were washed twice with cold PBS and resuspended in 1X Binding buffer at a concentration 10^6 cells/ml. 100 μ l were transferred to a new Eppendorf tube, 5 μ l FITC-annexin and 5 μ l of propidium iodide were added, the mixture was gently vortexed and incubated for 15 min at room temperature in the dark. Directly before measurement, 400 μ l 1x binding buffer was added and the suspension was measured within 1 h by FACS.

3.2.8 Molecular biology methods

3.2.8.1 Isolation of microRNA

To isolate microRNA, the mirVanaTM miRNA isolation kit was used for purification of RNAs suitable for studies of both siRNA and miRNA in natural populations. This method employs an organic extraction followed by immobilization of RNA on glass-fiber filters to

purify either total RNA, or RNA enriched for small RNA species from cells or tissue samples.

Adherent cells were washed with PBS and trypsinized, then inactivated and pelleted. The pellets were washed with 10 ml PBS, centrifuged again and the supernatant was aspirated.

300–600 μ l of lysis-binding buffer was added (to lyse the cells). The cells were lysed immediately and vortexed vigorously to completely lyse the cells and to obtain a homogenous lysate. In case of tumor tissue, the lysis-binding buffer was added as 10 volumes to the weight of the tumor.

To the homogenous lysate, a 1/10 volume of miRNA Homogenate Additive was added and mixed well by vortexing, and the mixture was left on ice for 10 min.

A volume of acid-phenol chlorophorm that is equal to the lysate volume before addition of miRNA homogenate was added and vortexed for 30-60 sec.

The mixture was centrifuged for 5 min at maximum speed at room temperature to separate the aqueous and organic phases. The aqueous (upper) layer was carefully removed without disrupting the lower layer and transferred to a fresh tube (the removed volume was exactly measured).

One-third volume (1/3) 100% ethanol was added to the aqueous phase recovered in the previous step and was mixed by vortexing or inverting the tube several times.

For each sample, a filter cartridge was placed into one of the collection tubes. The aqueous phase-ethanol mixture was pipetted onto the filter (for volumes more than 700 μ l, the mixture was supplied in successive steps to the same filter).

The collection tubes were centrifuged for 15 sec at maximum speed to pass the mixture through the filters and the filtrate was collected for the next step.

Two-third (2/3) volume 100% ethanol was added to the filtrate, mixed well and pipetted onto a new filter cartridge, then centrifuged for 15 sec at maximum speed.

The filtrate was discarded and this step was repeated several times until all of the filtrate/ethanol mixture had passed the filter.

The previous collection tube was used in the following washing step where 700 μ l of miRNA wash solution 1 was applied to the filter and centrifuged for 15 sec at maximum speed, then followed by another two washing step by application of 500 μ l of miRNA wash solution 2/3 and centrifuged as indicted above.

The filter cartridge was transferred to a new collection tube and 100 μ l pre-heated (95°C) Elution Solution was added to the filter and centrifuged for one minute at maximum speed. The collection tubes containing the small RNA including microRNA was stored at -20°C.

3.2.8.2 Isolation of total RNA

The total RNA was extracted by Trizol Reagent (Sigma-Aldrich) according to the manufacturer's protocol. Tumor tissues were homogenized in 10 volumes of Trizol reagent using power homogenizer, while the adherent cells were lysed directly in 6-well plates. The homogenized samples were incubated for 5 min at room temperature to permit a complete dissociation of the nucleoprotein complex.

1ml lysates was transferred to a fresh tube, where 0.2 ml chloroform was added, vortexed thoroughly and incubated for 10 min at room temperature, for phase separation.

The samples were centrifuged at 12000g and 4°C for 15 min, which led to the separation of the mixture into three phases (upper colorless aqueous RNA phase, middle DNA phase and lower red protein phase).

The upper layer was carefully removed and transferred to a new tube, and 0.5 ml isopropanol was added and then mixed well. The mixture was allowed to stand at room temperature for 10 min, and then centrifuged at 12000g and 4°C for 20 min.

The supernatant was removed and the RNA pellet was washed two times with ice-cold 75% ethanol and then centrifuged at 7500g for 5 min at 4°C. All leftover ethanol was completely discarded, RNA pellets were dried (not completely) for 5 min in the heat block at 65°C, then dissolved in RNAase-free water and measured in a spectrophotometer to determine the concentration and purity of RNA.

2 µl sample was diluted in 98 µl of DEPC-water and 50 µl of dilution was transferred to a spectrophotometer tube then measured. The A_{260}/A_{280} should be above 1.7.

Later some samples were measured using a Nanodrop spectrophotometer where only 2 µl was added directly to the machine without the need to dilute and a RNAase free-water was used as blank.

3.2.8.3 Quantitative real time reverse transcription PCR

In order to determine the expression level of microRNA and messenger RNAs, a quantitative real time reverse transcription PCR was performed using the light cycler detection system (Roche Diagnostics) and the SYBR Green PCR master mix. The expression level of microRNA in each cell line was measured and normalized to RNU6, which was used as internal control, while β-actin was used in case of transcripts normalization.

To determine the expression level of mature microRNA, specific stem looped RT primer (Chen et al., 2005), while mirVana™ specific RT primer was used for determination of precursor microRNA. Random hexamer primers were used for reverse transcription of targeted transcripts.

Small RNA containing microRNA was isolated from cell lines and tumor xenografts by the mirVana™ isolation Kit while total RNA was isolated using the Trizol Reagent.

Reverse transcription was performed with 100 ng denatured RNA using recombinant Transcription Reverse Transcriptase as described by the manufacturer under the following conditions: 16°C for 30 min, 42°C for 60 min and 85°C for 5 min.

The cDNA product was diluted 1:10 in nuclease free water and used as a template for quantitative real time PCR under the following conditions: 95°C for 15 min followed by 55 cycles of 95°C for 10 sec, 55°C for 10 sec and 72°C for 10 sec.

Relative fold changes of microRNA and mRNAs were calculated by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) and the values were expressed as $2^{-\Delta\Delta C_t}$.

3.2.9 Identification of the possible target mRNA for miR-143 and miR-145

Target Scan databases such as microRNA.org, miRDB and TargetScan were all used to identify the predicted targets of miR-143 and miR-145.

3.2.10 *In vivo* analysis of tumor growth

For the animal experiments, athymic nude mice (6-8 weeks of age) were used and kept in tight cages with standard rodent chow and water available *ad libitum*, and a 12 h light/dark cycle.

The experiments were performed according to the national regulations and approved by the local animal experiments ethical committee. Subcutaneous LS174t as well as HCT-116 tumors were induced by inoculation of 1.5×10^6 LS174t or HCT116 cells respectively in both flanks of the mice. At a tumor volume of $\sim 0.5-1 \text{ cm}^3$. One group received PEI/miRNA complex (miR-145 complexed with PEI F25-LMW) while another received negative control siRNA and a third group set as non-treated wild type control (eight mice each).

The microRNA complex as well as the negative control RNA was injected intraperitoneally in case of LS174t xenografts or intratumorally in case of HCT-116 xenografts.

The complexes were prepared for one injection as follows:

- 1- 10 μ g miR-145 or anti-Luc-siRNA as negative control was diluted in 100 μ l HEPES/NaCl buffer.
- 2- 10 μ l PEI F25-LMW (mass ratio 5) was added to 90 μ l HEPES/NaCl buffer.
- 3- After 5 min both tubes were mixed together (PEI solution was added to microRNA solution, not reverse)
- 4- The complexes were incubated at room temperature for 45 min.
- 5- Finally, 150 μ l of complex was intraperitoneally injected.

In case of HCT-116 xenografts, the complexes were injected intratumorally, and the amount of injected complex was depended on the tumor volume (50-100 μ l).

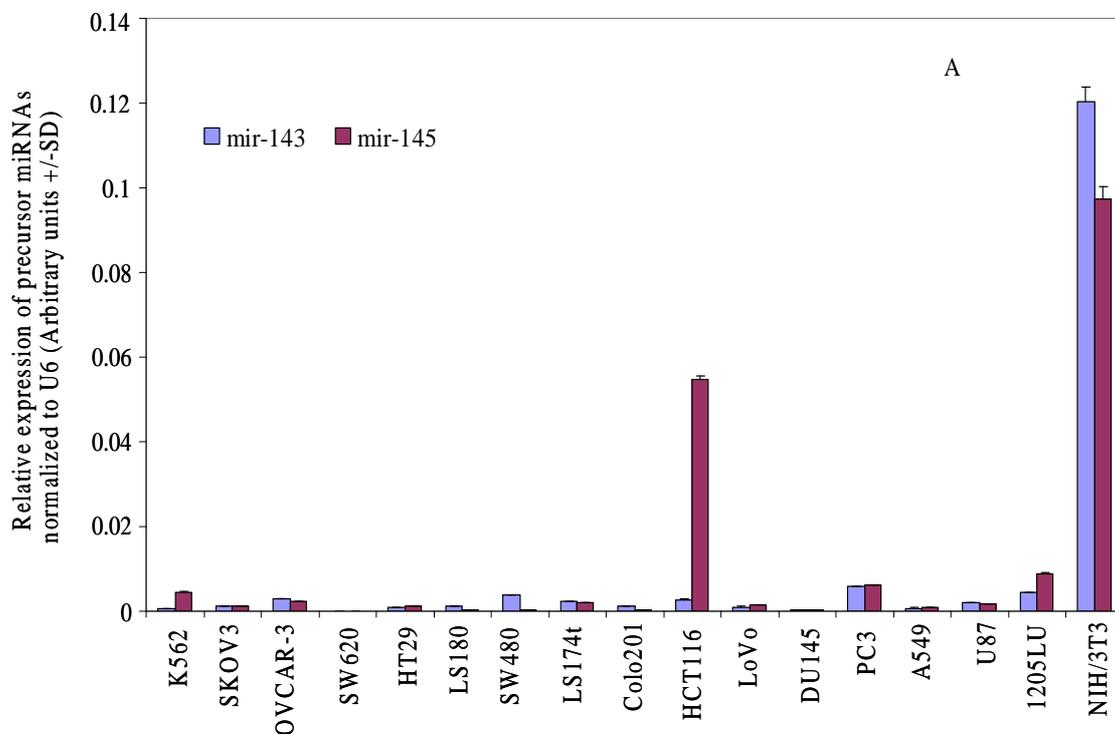
At the end of the experiment, the animals were sacrificed and tumor tissues and surrounding skin was excised and removed. The tumors were sectioned into three parts and snapped frozen immediately in liquid nitrogen for quantitative real time PCR and Western Blot experiments, while the third section was immediately fixed in 10% paraformaldehyde for paraffin embedding preparations.

4. Results

4.1 Reduced expression of miR-143 and -145 in different cell lines

Quantitative real time PCR was first performed to analyze expression levels of precursor and mature microRNAs in 16 cell lines of different origins and compared to the non-tumor fibroblast cell line NIH/3T3 (Figure.4.1 A and B). Two different types of primers were used to determine the expression levels of precursor microRNA as well as mature microRNA.

Precursor microRNAs, mir-143 and mir-145, were determined using linear specific primers, while mature microRNAs, miR-143 and miR-145, were analyzed using specific stem looped RT primers. Expression levels of Precursor and mature microRNA were normalized to RNU6 as internal control.



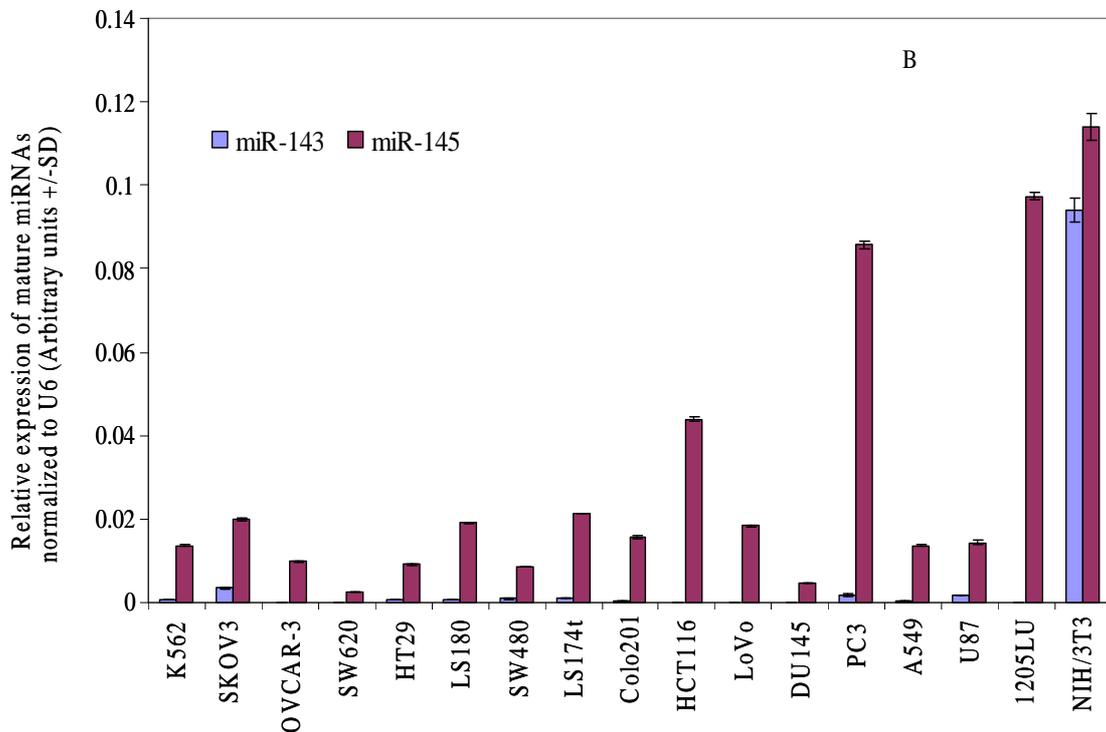


Figure 4.1: Relative expression level of **A)** precursor mir-143 and mir-145 and **B)** mature miR-143 and miR-145. All colon carcinoma cell lines showed low expression level of both precursor and mature miR-143 and miR-145 as compared to non-tumorous NIH3T3.

In comparison to the normal NIH 3T3 cells, the expression of precursor microRNAs as well as mature microRNA was downregulated in all cell lines, particularly in colon carcinoma cells. The results also demonstrated that the expression level of precursor and mature microRNAs was depending on the cell line. This is particularly true for prostate carcinoma cells with levels being very low in DU-145 cells, while PC3 cells showed high level of mature miR-145. Generally, the expression level of precursor miRNAs showed low level in all cell lines in comparison to the correspondent mature microRNAs. For subsequent experiments, the tumorigenic colon carcinoma cell lines LS174T and HCT-116 were selected

4.2 PEI-mediated delivery of mature microRNAs

To validate the efficiency of Jet-PEI to deliver the microRNAs, LS174t cells were transfected with 50 nM mature microRNA-PEI complex. Thereafter, the levels of both miR-143 and miR-145 were determined through RT-qPCR method and it was found that the level of mature microRNAs was upregulated or, in other words, was elevated after transfection of exogenous mature microRNAs using Jet-PEI as transfection reagent.

This experiment illustrated that Jet-PEI has the efficacy to complex mature microRNAs and to deliver them into the cells. As shown in Figure 4.2 A and B, the levels of miR-143 and miR-145 was markedly elevated after transfection with 50 nM miR-143 compared to non-treated wild type cells. In the same way, the level of both miRNAs were elevated after transfection with 50 nM miR-145, the level of miR-143 was not as the same as the first assessment.

As specific primers were designed for the detection of miRNAs, no-cross reactivity should have been observed. Therefore, To confirm these results, the same experiments were done on the RNA extract (after cell lysis), where the same concentrations of miRNAs or non-specific control RNA without a transfection reagent were added to the RNA extracts of LS174t cells.

The same results (data not shown) have been observed, where the level miR-143 was markedly increased in case of addition mature miR-143 but not miR-145. Likewise, the level of miR-145 was highly elevated in case of addition of mature miR-145 but not miR-143 (all results were compared to non-treated RNA extracts as negative control).

These results illustrated the specificity of the used primers for determining both miR-143 and miR-145.

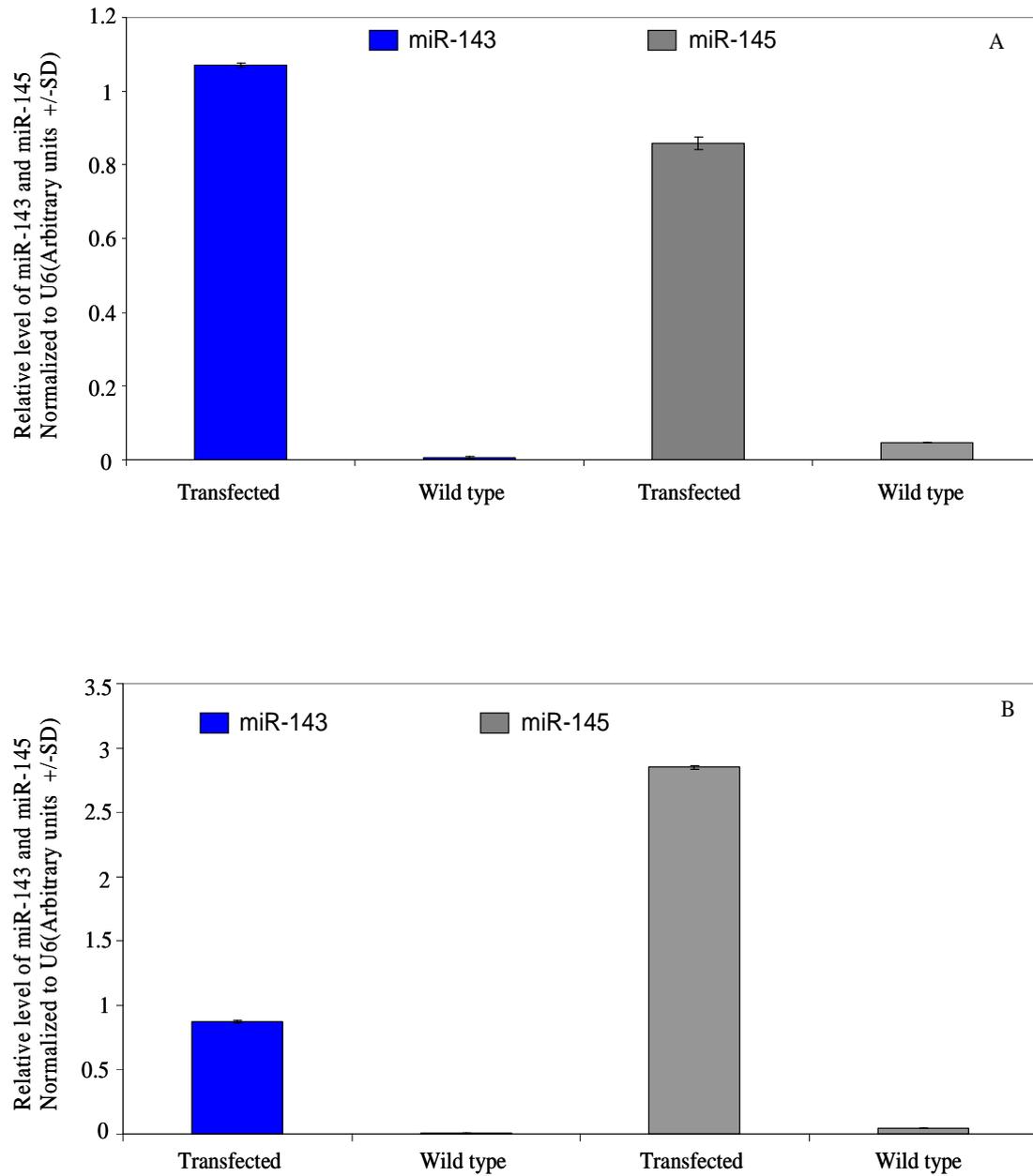


Figure 4.2: Relative level of miR-143 and miR-145 in LS174t cells after transfection with **A)** 50 nM miR-143, **B)** 50 nM miR-145 complexed with Jet-PEI as a transfection reagent.

4.3 *In vitro* analysis of LS174t cells

4.3.1 Proliferation assay

4.3.1.1 Jet-PEI

The functions of microRNAs in regulating cell growth and proliferation have been well established in recent years because they can target genes involved in cell proliferation. Downregulation of miR-143 and miR-145 in different types of cancer suggests their role in controlling cell proliferation serving as a tumor suppressor. LS174t cells as colon carcinoma model were transfected with different concentrations of mature microRNAs using different transfection reagents, Jet-PEI, INTERFERin and PEI F25-LMW. The different transfected concentrations were depending on the used reagent. For example, in case of Jet-PEI, LS174t cells were transfected with 50 and 100 nM miR-143 and miR-145.

At both concentrations, miR-145 had a profound inhibitory effect on the proliferation rate more than that of miR-143. As shown in the representative figures, miR-143 and miR-145 had a marked inhibitory effect at 120 h of transfection and increased even for longer period until 168 h.

Non-specific activity of PEI at both concentrations was not observed, indicated by the non-specific control RNA, even for long period of incubation, while at 168 h (7 d), PEI-mediated delivery of miR-145 led to a marked inhibition of proliferation (> 70% and > 55% after transfection with 50 nM and 100 nM, respectively). While transfection of miR-143 led to inhibition of growth by 40% and 2% in case of 50 nM and 100 nM respectively.

In case of combination of both miR-143 and miR-145, each at half amounts, the proliferation rate was decreased by 36% and 8% after transfection with 50 nM and 100 nM respectively.

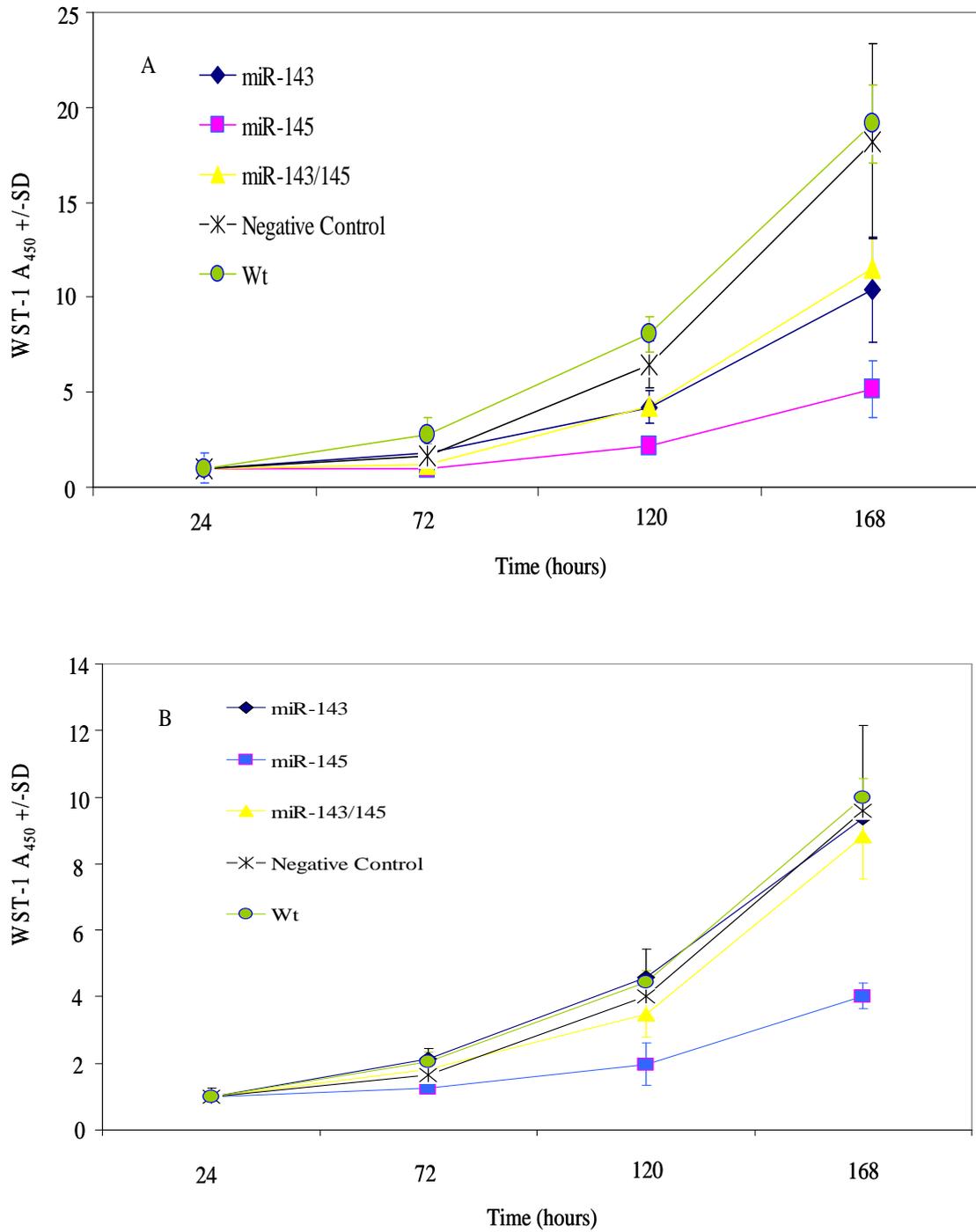


Figure 4.3.1.1: Proliferation Assay of LS174t cells after transfection with **A**) 50 nM and **B**) 100 nM miR-143, miR-145 or negative control RNA using Jet-PEI.

4.3.1.2 INTERFERin

In the case of INTERFERin, LS174t cells were transfected with 20 nM and 40 nM mature microRNAs and in this experiment, miR-145 and miR-143 had an inhibitory effect on the growth rate. Consistent with the previous results, miR-145 had a greater effect than miR-143 or even the combination between them (each half). After 168 h at 20 nM, miR-145 reduced the growth rate by 76% while miR-143 reduced the proliferation rate by 41%, but when combined of both microRNAs at half concentrations (10 nM miR-143 and 10 nM miR-145), a reduction by 45% was observed. All of these values were compared to the non-specific control RNA, where non-specific activity was not detected along the incubation time.

When LS174t cells were transfected with a higher concentration of microRNAs, 40 nM, both microRNAs had an inhibitory effect on the growth of these cells (Figure 4.3.1.2). More specifically, miR-145, miR-143 or the combination of them reduced the growth rate by 41%, 39% and 38%, respectively. Nevertheless, after 168 h, some of non-specific activity represented by the reduced proliferation of negative control RNA was observed. Therefore, 20 nM was used as the optimal concentration in the subsequent experiments on LS174t cells using INTRERERin as a transfection reagent.

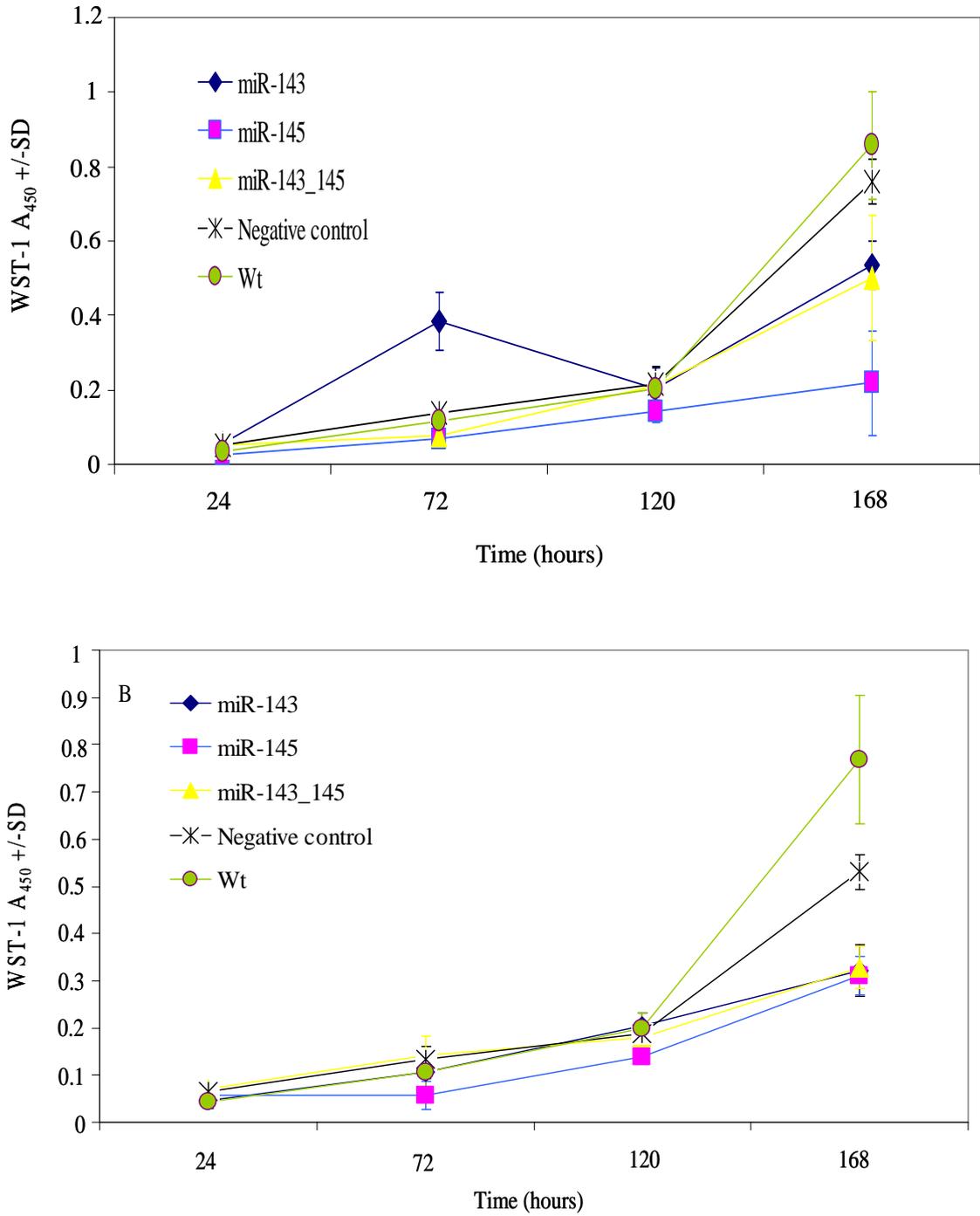
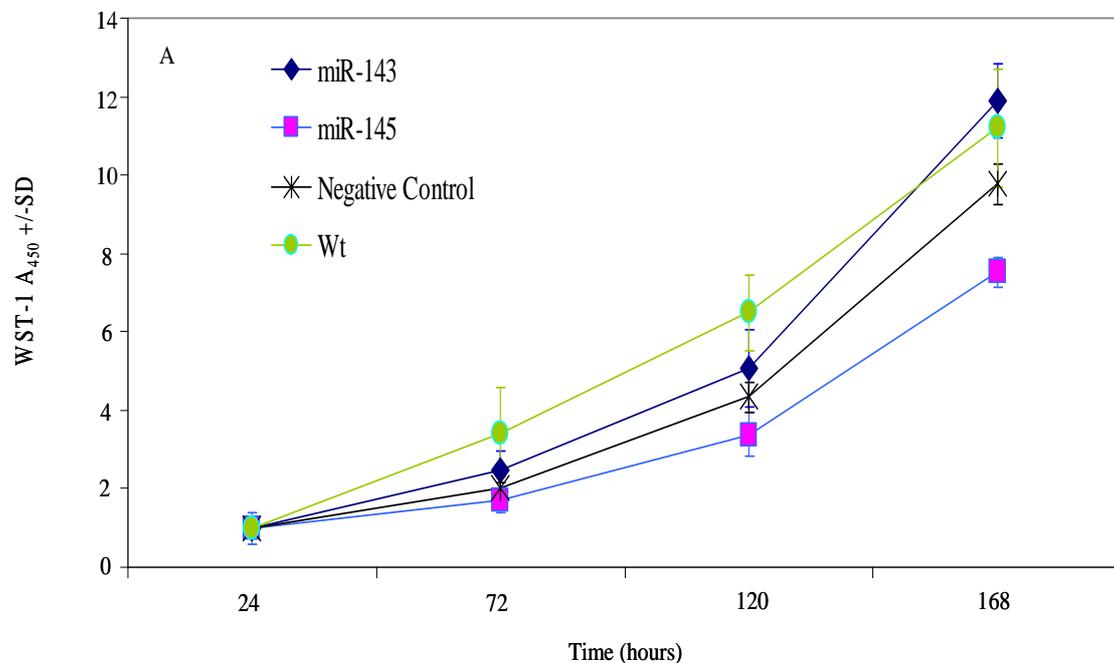


Figure 4.3.1.2: Proliferation activity of LS174t cells after transfection with A) 20 nM and B) 40 nM miRNAs using INTERFERin.

4.3.1.3 PEI F25-LMW

LS174t cells were transfected with 5, 10 and 30 nM mature microRNAs complexed with PEI F25-LMW (N/P ratio 33). In these cells, miR-143 and miR-145 exerted antiproliferative effect that was a dose dependent. In case of transfection with 5 nM, miR-145 inhibited the growth after 168 h by 23%, while miR-143 had no inhibitory effect and the non-specific activity of PEI F25-LMW has not been detected as represented by the negative control RNA.

LS174t cells were further transfected by 10 nM and 30 nM and the results as shown in Figure 4.3.1.3 B and C, both miR-143 and miR-145 affect markedly the proliferation of LS174t cells in comparison to wild type cells, while the results show that there is some non-specific activity of PEI as indicated by negative control RNA treated cells. In case of transfection of LS174t cells with 30 nM mature miRNAs, both miR-143 and miR-145 exerted antiproliferative effect as same as transfection with 10 nM, but the non-specific activity of PEI has been increased as represented by negative control RNA treated cells.



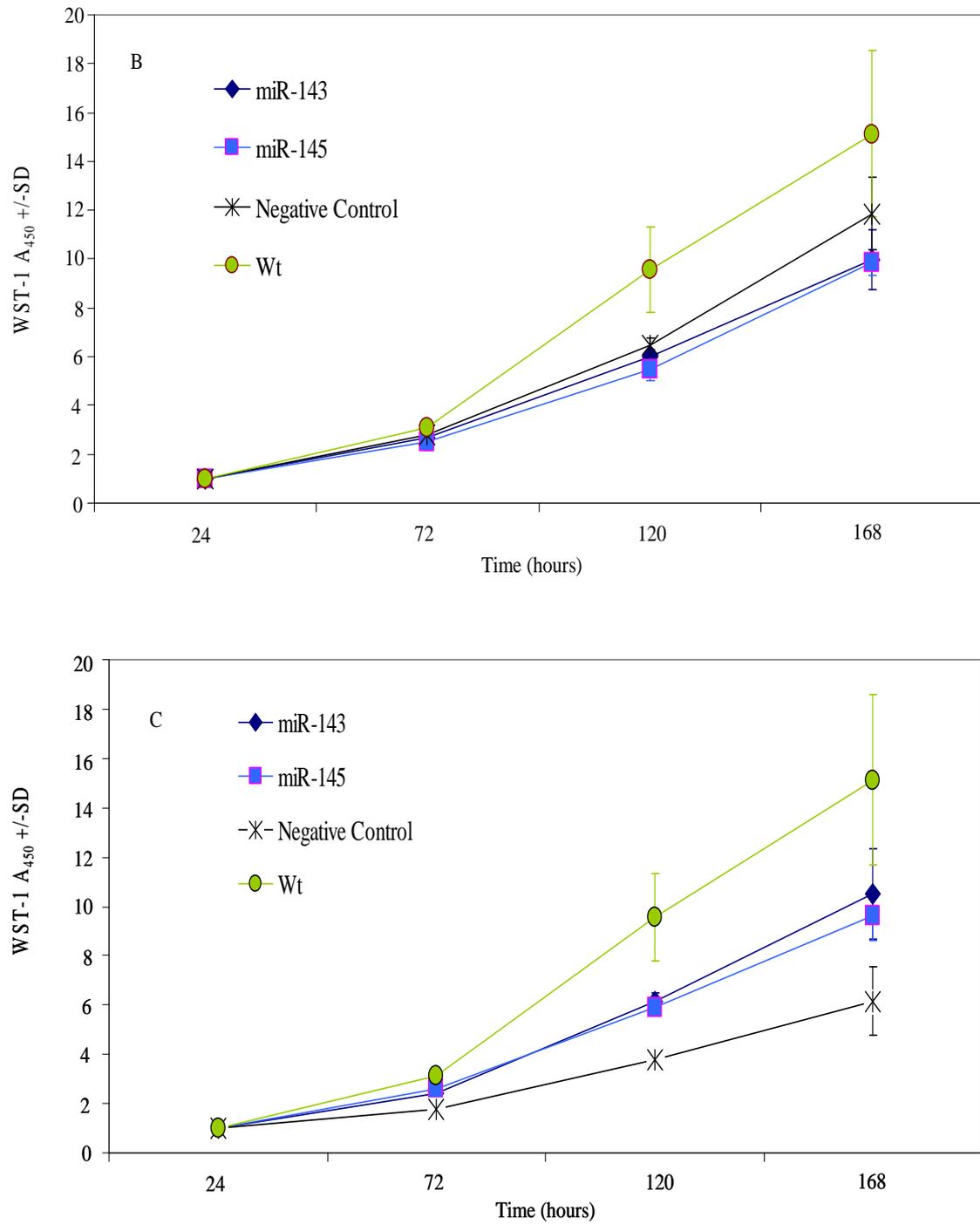


Figure 4.3.1.3: Proliferation activity of LS174t cells after transfection with **A)** 5 nM and **B)** 10 nM **C)** 30 nM miRNAs using PEI F25-LMW.

4.3.2 Soft agar analysis

The soft agar assay for colony formation is an anchorage independent assay, which is believed to be a reasonably good predictor of *in vivo* activity. The assay was done in 6-well plate in triplicates with manual counting by two unbiased counters. Figure 4.3.2 shows the efficacy of miR-145 and miR-143 to inhibit the formation of colonies after three weeks of incubation, when LS174t cells were transfected with 100 nM mature microRNAs or non-specific control RNA for 24 h before seeding in agar plates.

Number and diameter of colonies were reduced in plates seeded with cells, which were previously transfected with mature microRNAs, in comparison to non-specific control and wild type cells. The number of colonies was reduced by 37% and 45% for miR-145 and miR-143, respectively, as compared to the negative control RNA that indicated the absence of non-specific effects of PEI.

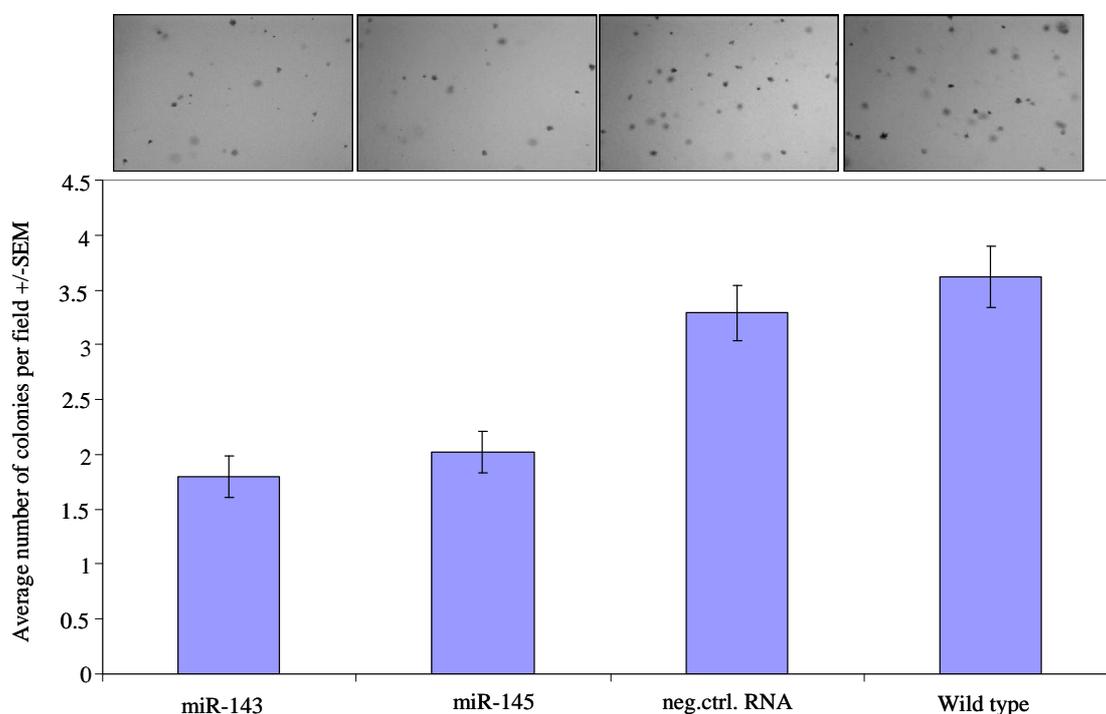


Figure 4.3.2: Soft agar assay of LS174t cells transfected with 100 nM mature microRNAs. Upper panel: Representative images of soft agar assay.

4.3.3 Apoptosis assay

4.3.3.1 Increased apoptosis upon PEI-miRNA delivery

Apoptosis is a programmed, physiological mode of cell death, which plays an important role during embryonic development, the maintenance of tissue homeostasis and the removal of aberrant cells. The caspases consist of a group of aspartic acid-specific cysteine proteases. These unique proteases, which are synthesized as zymogens, are involved in the initiation and execution of apoptosis once activated by proteolytic cleavage.

Mammalian caspases may be grouped by function: cytokine activation includes caspases 1, 4, 5, 13; apoptosis initiation includes caspases 2, 8, 9, 10; and apoptosis execution utilizes caspases 3, 6, 7.

In this experiment, the LS174t cells were transfected with PEI/miRNA complex and apoptosis was measured after 24, 48 and 72 h using the Caspase Glo3/7 assay. While no change was observed at the first two time points, a slight induction was detected at 72 h after transfection with miR-145.

These results strongly indicated that the effectors caspases participate in the execution of cell death induced by miR-143 and miR-145, where apoptotic cells were increased by 20% to 50% for miR-143 and miR-145 respectively in comparison to wild type cells. On comparison to the non-specific control RNA, apoptosis has been increased by 26% and 5% for miR-145 and miR-143 respectively. This indicates that both miRNAs particularly miR-145 induces or initiates cell death through caspase cascades.

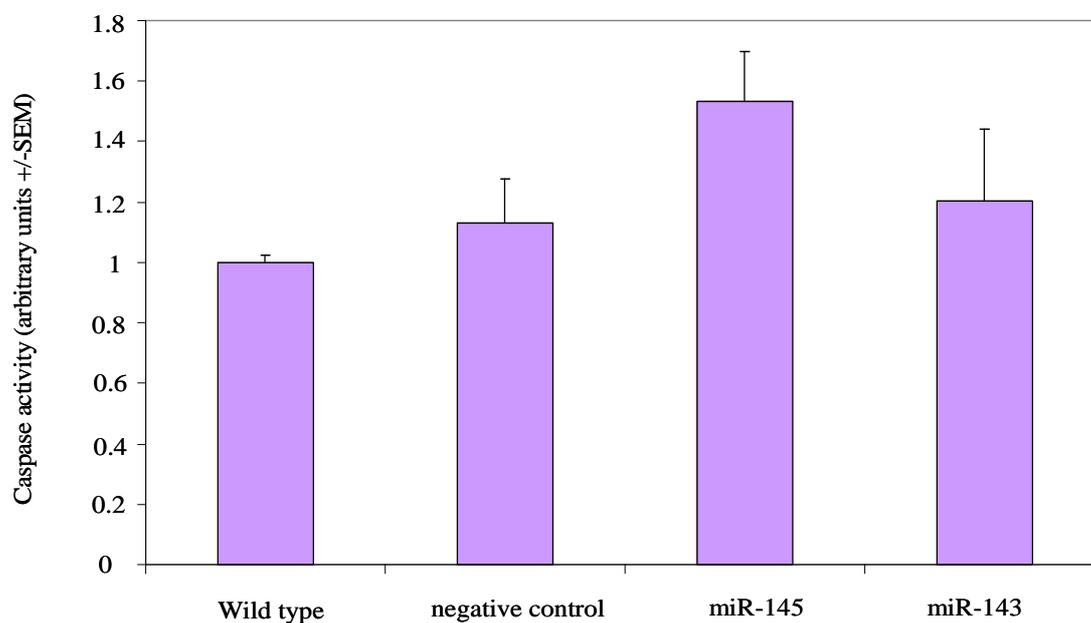


Figure 4.3.3.1: Activity of Caspase 3/7 in LS174t transfected with 100 nM mature miRNAs using Jet-PEI.

4.3.3.2 FITC-Annexin assay

Another assay to confirm the role of microRNAs in apoptosis has been conducted, FITC-annexin assay. Under normal conditions, a cell maintains a strictly asymmetric distribution of phospholipids in the two leaflets of the cellular membranes with phosphatidylserine (PS) facing the cytosolic side; however, during early apoptosis this membrane asymmetry is rapidly lost without concomitant loss of membrane integrity. This in turn results in the exposure of PS at the outer leaflet of the plasma membrane. This phenomenon can be detected by annexin V, which is a 35 kDa phospholipid-binding protein and has a high affinity for PS residues.

At the end stage of apoptosis, where no clear distinction between classical apoptosis and necrosis, propidium iodide was used to detect dead cells (end stage). LS174t cells were transfected with 100 nM mature microRNAs using Jet-PEI and after 120 h; the apoptosis

activity was measured by FITC-Annexin assay using flow cytometry. It was found that It was found that In a FACS-based FITC-annexin assay, a > 2-fold increase in early stage and late stage apoptotic cells was observed upon PEI/miR-145 treatment as compared to negative controls.

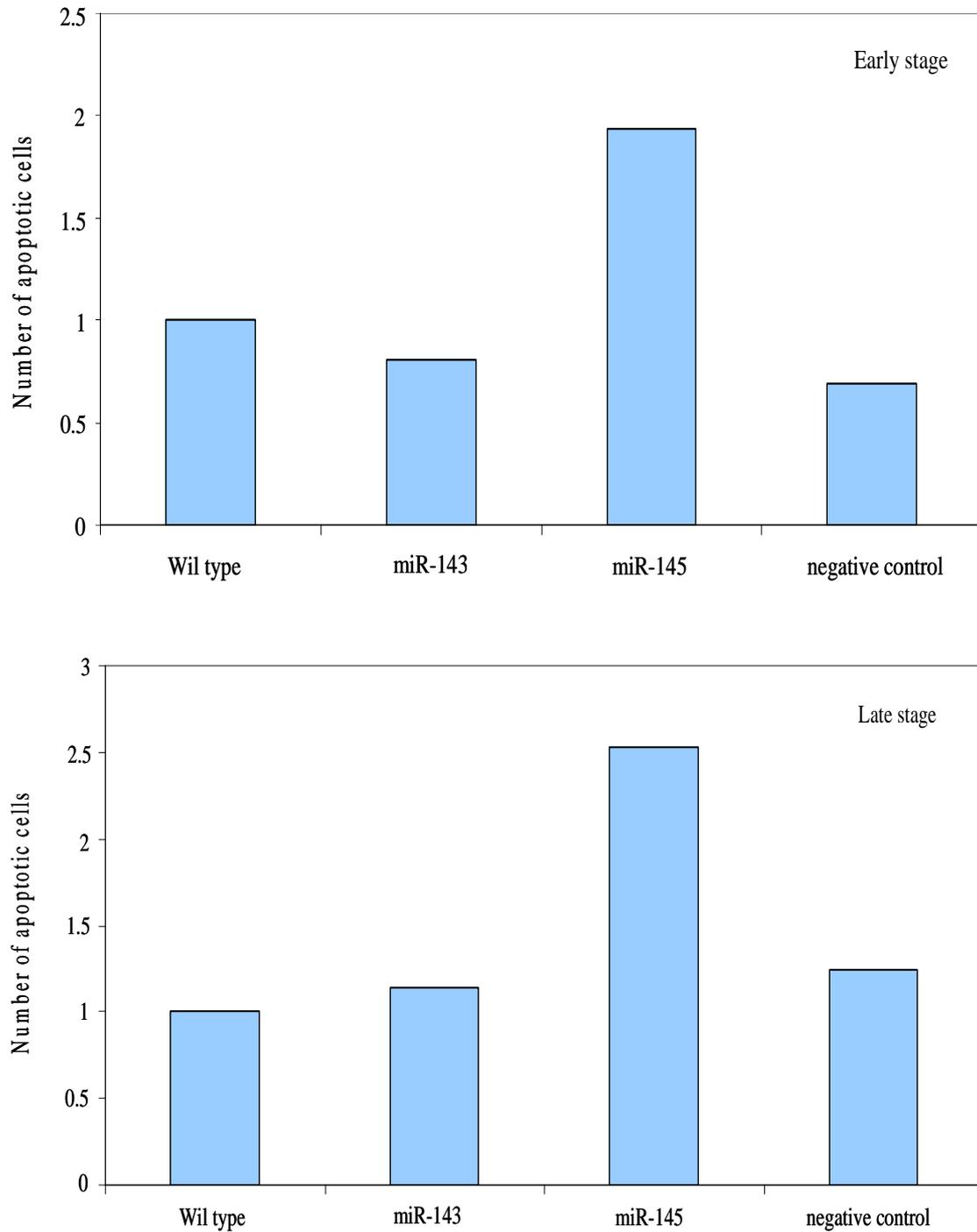


Figure 4.3.3.2: FITC-Annexin analysis of LS174t cells transfected with 100 nM mature miRNAs showed the early stage of apoptosis (upper graph) and the late stage of apoptosis (lower graph).

4.3.4 Relative expression level of ERK5

ERK5 is a member of mitogen activated protein kinase and it was reported as a direct target for miR-143 but not miR-145 (according to surveyed databases).

In this experiment, a quantitative analysis of ERK5 transcripts was measured by Real time PCR after transfection of LS174t cells with 100 nM mature microRNAs using jet-PEI and 20 nM using INTERFERin as transfection reagents.

LS174t cells were transfected with mature microRNAs or non-specific control RNA and incubated for 5 d, and then total RNA was extracted as described in chapter 2. Figure 4.3.4 shows that neither miR-145 nor miR-143 has an inhibitory effect on expression level of ERK5 transcripts.

Nevertheless, at the posttranscriptional level, miR-143 and mir-145 decreased the level of ERK5 in dose dependent manner, where at concentration 50 nM, miR-143 and miR-145 decreased the total ERK5 pool by 44% and 25.5%, respectively, while at concentration 100 nM the rate of reduction was 67.8% and 46.5% for miR-143 and miR-145, respectively. These results were compared to non-specific control RNA, which showed absence of non-specific activity at 50 nM as well as at 100 nM either at the transcriptional or translational level.

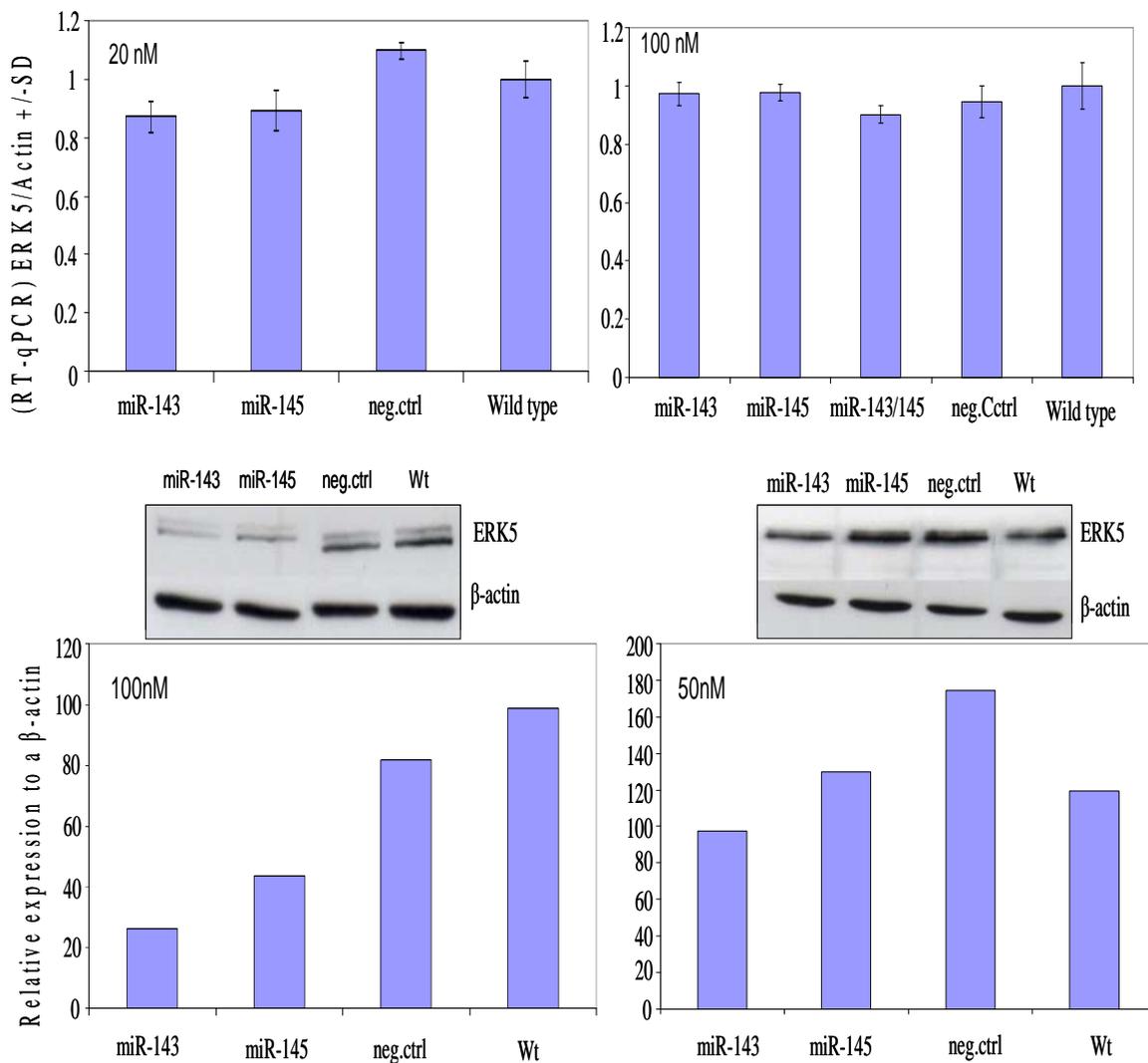


Figure 4.3.4: Relative expression levels of ERK5 in LS174t normalized to actin. The upper right graph shows the expression level of ERK5 mRNA in LS174t cells transfected with 100 nM miRNAs complexed with Jet-PEI and the upper left graph shows 20 nM miRNAs complexed with INTERFERin. The lower Panel shows the expression level of ERK5 proteins in LS174t cells transfected with 50 nM mature miRNAs (right) and 100 nM mature miRNAs (left) complexed with Jet-PEI.

4.3.5 Relative expression level of c-Myc

The correlation between microRNAs and c-Myc was further examined by evaluating the expression of c-Myc in human colorectal carcinoma LS174t cells after transfection of miR-143 and miR-145. LS174t cells were transfected with 100 nM mature microRNAs/Jet-PEI complex or 20 nM miRNA complexed with INTERFERin in comparison to control cells transfected with non-specific control RNA and wild type cells (not transfected).

As shown in Figure 4.3.5, when the cells were transfected with 100 nM mature miRNA, the expression of c-Myc transcripts were reduced by 33% and 17% after transfection with miR-145 and mir-143 respectively. In contrast, in cells transfected with mature microRNA complexed with INTERFERin, no significant change of the expression level was detected.

The level of c-Myc protein was measured using the Western Blot analysis after transfection of LS174t cells with 100 nM miRNAs/Jet-PEI complex. The results showed that miR-145, to some extent, decreased the expression level by 7%. All results were compared to non-specific control RNA, which showed the absence of the non-specific activity during the assessment of either transcript level or protein level.

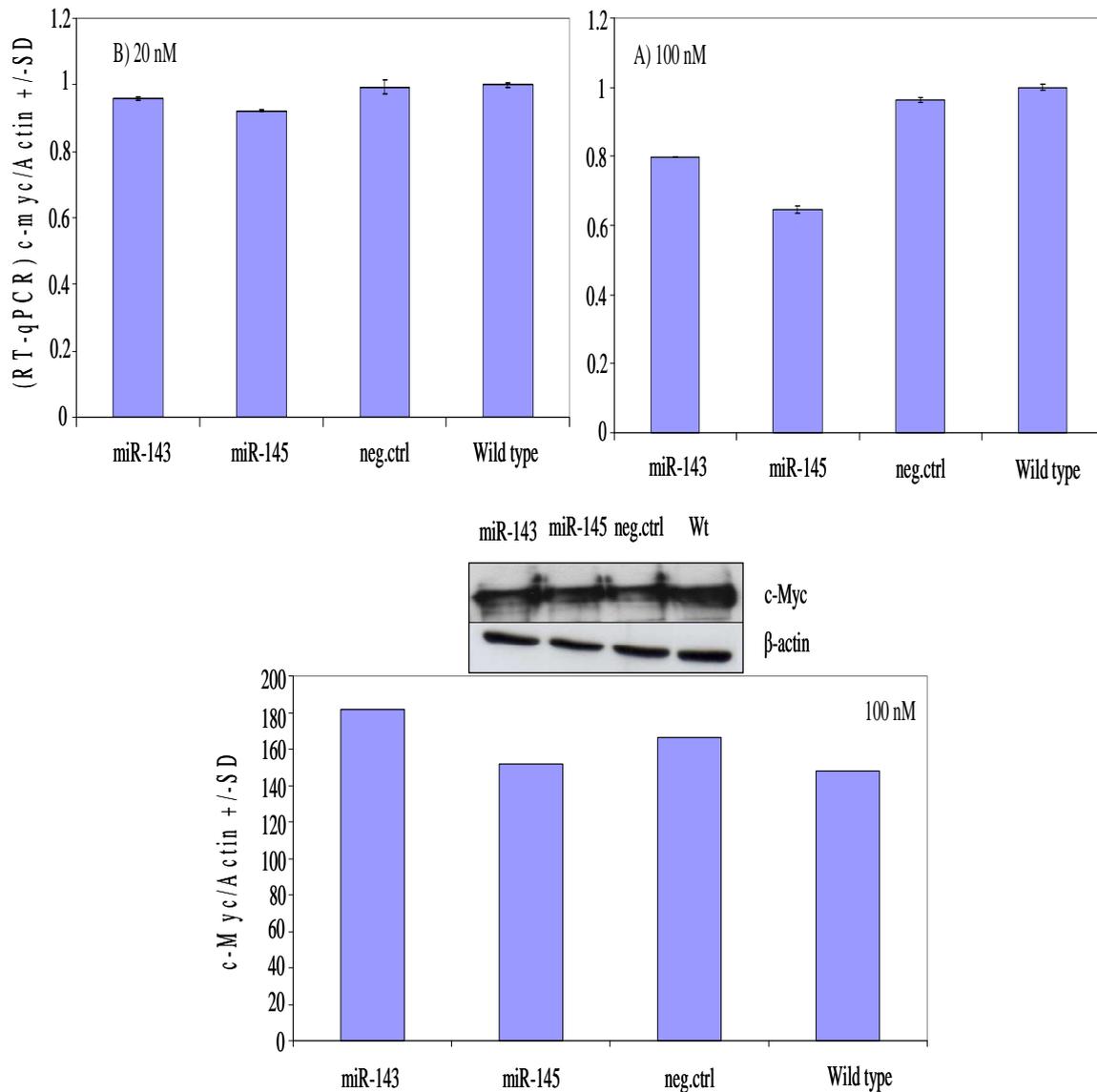


Figure 4.3.5: Relative expression level of c-Myc in LS174t normalized to actin. **A)** 100 nM with Jet-PEI and **B)** 20 nM with INTERFERin. The upper Panel shows the expression level of c-Myc mRNA in LS17t cells transfected with 20 nM miRNAs complexed with INTERFERin and 100nM miRNAs complexed with JET-PEI. The lower Panel shows the expression level of c-Myc proteins in LS174t cells transfected with 100 nM miRNAs complexed with Jet-PEI.

4.3.6 Anti-tumor effects of intraperitoneal PEI-mediated miR-145 delivery in s.c LS174t colon carcinoma xenograft mouse model

The purpose of the *in vivo* studies was to study the effect of miR-145 on the growth of solid tumors. To achieve this, athymic nude mice were injected subcutaneously with 1.5×10^6 LS174t cells in both flanks of the mice.

After the development of solid tumors, the mice were grouped into three groups (eight mice each), the mice were treated with miR-145/PEI complex or the non-specific control treated group or left untreated. The tumor volume was measured every 48 h using the formula (width * length * height) where, the length was the larger value, and at the same interval, the mice were injected intraperitoneally with 10 ug microRNA or non-specific control RNA.

A difference between the tumor volume in the microRNA treated group and the other groups was recognized after 10 d of treatment; i.e. after five injections. After about 23 d, a significant difference in the tumor growth between different groups was detected as shown in Figure 4.3.6. These results indicated that the PEI/miR-145 had the efficacy to inhibit the growth of the solid tumors through either an indirect effect or by direct acting on cancer related genes.

As illustrated in Figure 4.3.6, a non-specific activity of PEI was not observed as represented by the activity of the non-specific control RNA when compared to the wild type untreated xenografts.

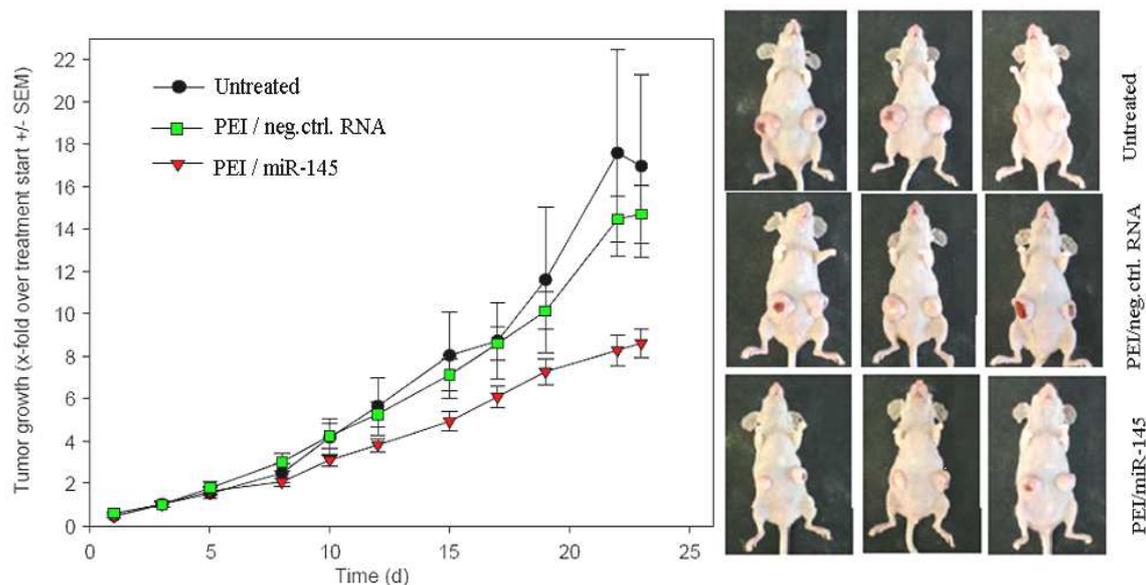


Figure 4.3.6: Anti-tumor effects of PEI/miR-145 treatment in s.c. LS174T colon carcinoma xenograft mouse models. (a) Systemic injection of PEI/miR-145 complexes results in reduced tumor growth (red) as compared to untreated (black) or PEI/RNA negative control treated mice (green)

4.3.6.1 Expression level of miR-145 targets in s.c LS174t xenografts

To evaluate the biological effect of miR-145 on colon carcinoma tumorigenesis, molecular and biochemical analyses on tumors after sacrificing the mice were performed.

The tumors were sectioned into three parts, frozen in liquid nitrogen and kept in -80°C , one part for quantitative analysis of target mRNAs, the second part for Western blot assay, while the third part was preserved in 10% paraformaldehyde for immunohistochemistry preparation.

To validate the efficiency of PEI mediated delivery of miR-145 *in vivo*, tumor sections from miR-145 treated mice, negative control group and the wild type untreated group were randomly selected. These tumor samples were exposed to Real time PCR after extraction of

small-enriched RNA (including the microRNAs fractions). As expected, a high level of miR-145 in treated samples (Figure 4.3.6.1A) has been observed in comparison to wild untreated samples and negative control RNA tumors.

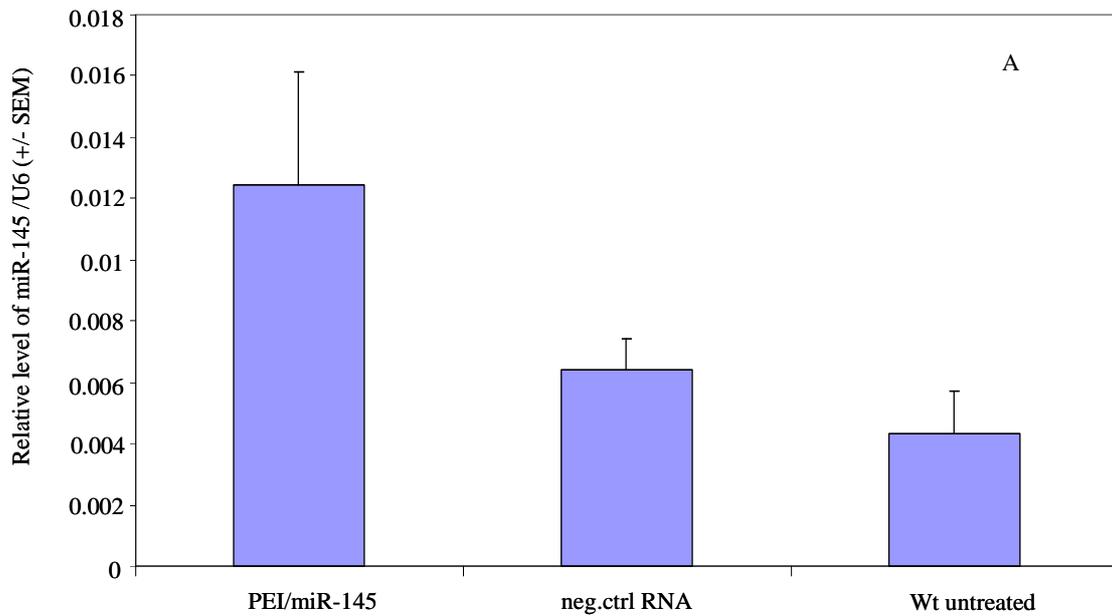


Figure 4.3.6.1A: miR-145 expression level in s.c LS174t colon carcinoma xenograft models.

Based on the *in vitro* data, ERK5 and c-Myc were the selected targets for both miR-143 and miR-145. To evaluate the silencing effect of mature miR-145 on these targets at the level of transcripts, a quantitative real time PCR was done.

Quantitative real time PCR assay *in vivo* indicated that ERK5 transcripts were non-significantly downregulated by miR-145 compared to the non-specific control RNA. The same results were obtained for the oncogen c-myc, a possible target for miR-145, where no marked effect of miR-145 on the expression level of c-myc were found.

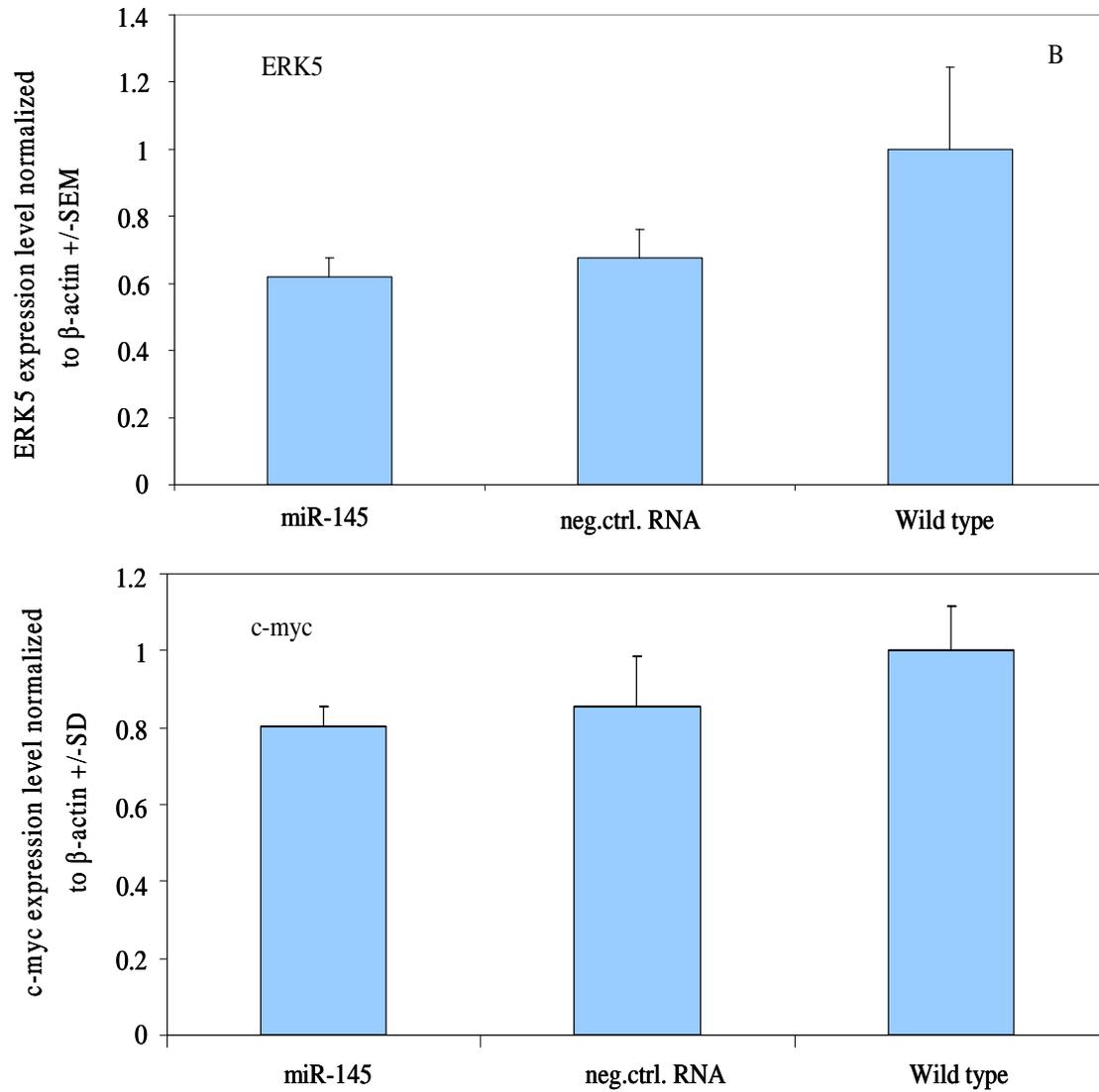
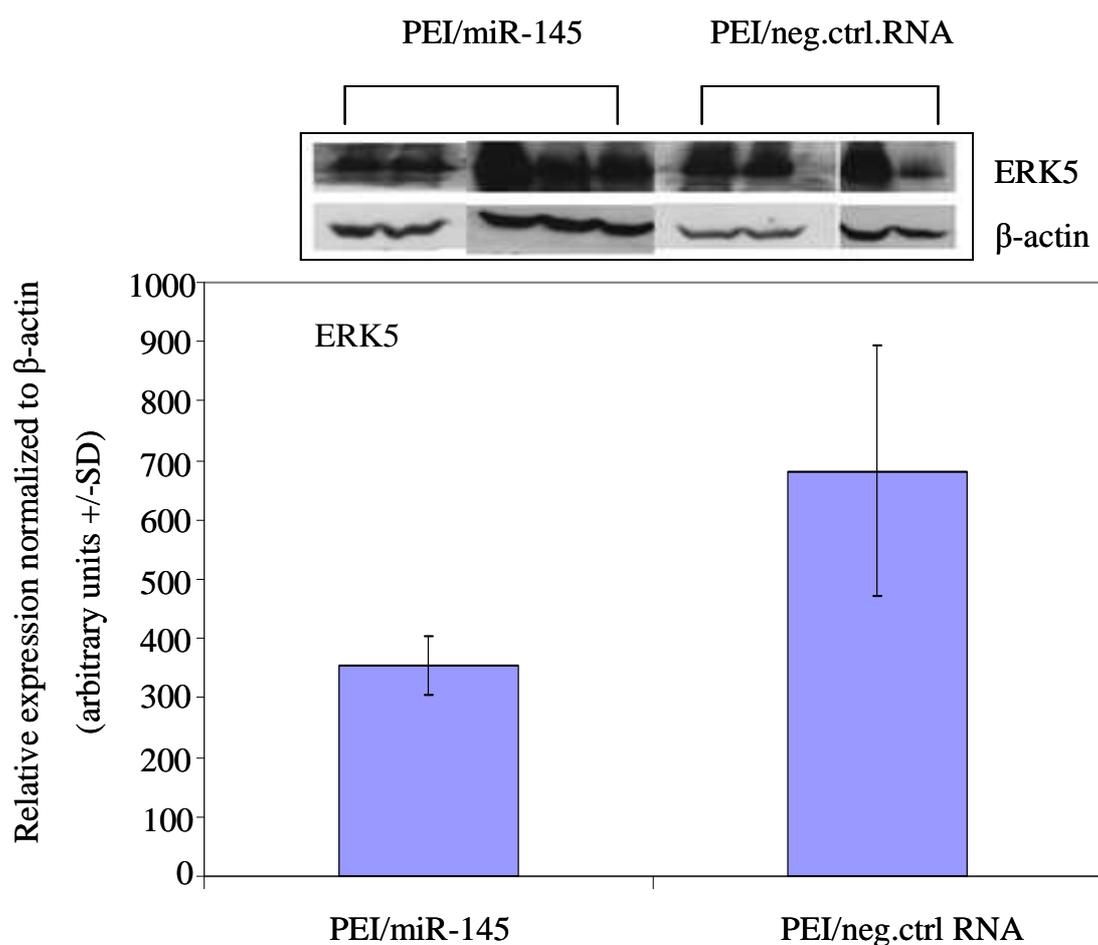


Figure 4.3.6.1 B: Quantitative real time PCR of ERK5 (upper graph), c-Myc (lower panel) and in s.c LS174t colon carcinoma xenograft models.

4.3.6.2 Expression of ERK5 and c-Myc at the post-transcriptional level

To determine the post-transcriptional effect of PEI/miR-145, the tumor sections were lysed and the protein was extracted and measured as described in chapter 2.

The expression level of ERK5 and c-Myc was measured by using specific antibodies. As shown in Figure 4.3.6.2, an inhibitory effect of mature microRNA on the expression level of both ERK5 and c-Myc was detected. The level of ERK5 was downregulated by 48% in miR-145 treated group in comparison to the non-specific control RNA. In case of c-Myc, the expression level was downregulated by more than 50% compared to the negative control RNA.



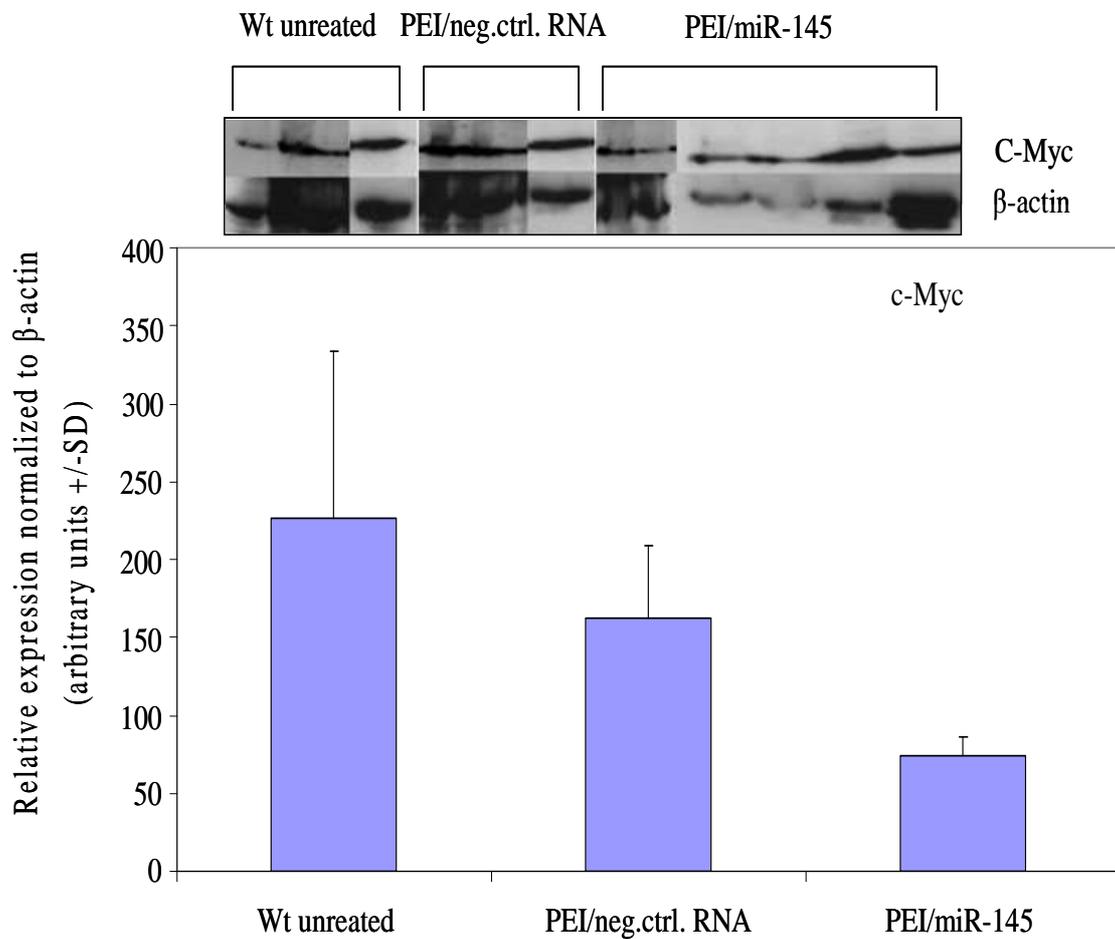


Figure 4.3.6.2: Western blot analyses of ERK5 (upper panel) and c-Myc (lower panel) in s.c LS174t colon carcinoma xenografts.

4.3.6.3 Reduced expression level of PCNA

The tumor sections were stained for evaluation of PCNA expression levels. The results of PCNA staining were analyzed according to the intensity of the stained nuclei, with the value 0 denoting no staining, 1 poor staining, 2 moderate staining and 3 intensive staining. As illustrated in Figure 4.3.6.3, it was found that the intraperitoneal injection of miR-145 led to a decrease in the proliferation rate of the tumors as indicated by the intensity of the stained

nuclei of PCNA in comparison to the negative control or the untreated wild type. The expression level of PCNA was reduced by 48% in comparison to the negative control RNA.

Until now, PCNA was not reported as a direct target for miR-145, despite this fact, a slight effect of miR-145 on the expression level of PCNA transcripts has been detected. However, this effect could be due to an indirect effect of miR-145.

The comparison between non-transfected and negative control transfected cells also demonstrated the absence of non-specific PEI effects on the expression level of PCNA.

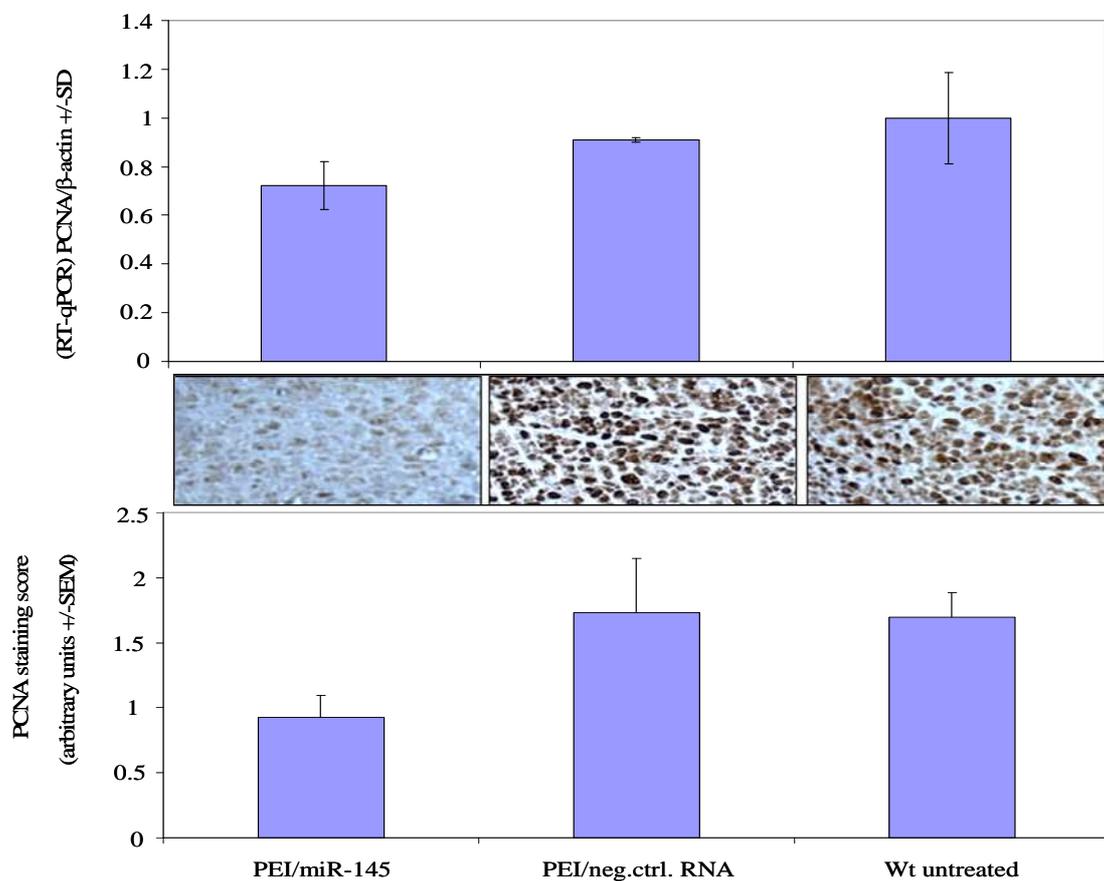


Figure 4.3.6.3 Expression level of PCNA in s.c LS174t xenografts. The upper panel shows the PCNA transcript level, lower panel shows immunohistochemistry staining with the representative images.

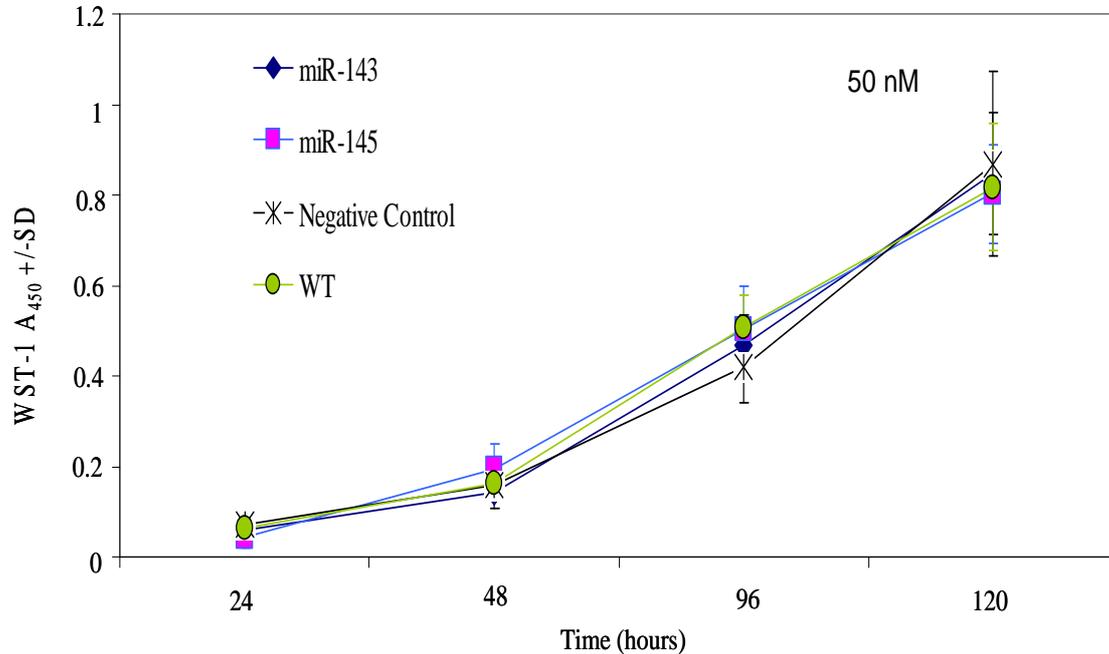
4.4 HCT-116 cells

4.4.1 Proliferation assay- Jet-PEI

HCT-116 cells were transfected with different concentrations of mature microRNAs or negative control RNA using Jet-PEI and INTERFERin as transfection reagents.

No inhibitory effect of both miR-143 and miR-145 was detected when HCT-116 cells were transfected with 50 nM using jet-PEI as a transfection reagent. Yet, when the cells were transfected with 100 nM, slight changes were observed, since miR-145 and miR-143 reduced the proliferation rate by 17% and 10%, respectively, compared to the negative control RNA.

Non- specific activity of PEI was not observed in all used concentrations, which indicated by the effect of negative control RNA on the proliferation rate as shown in Figure 4.4.1.



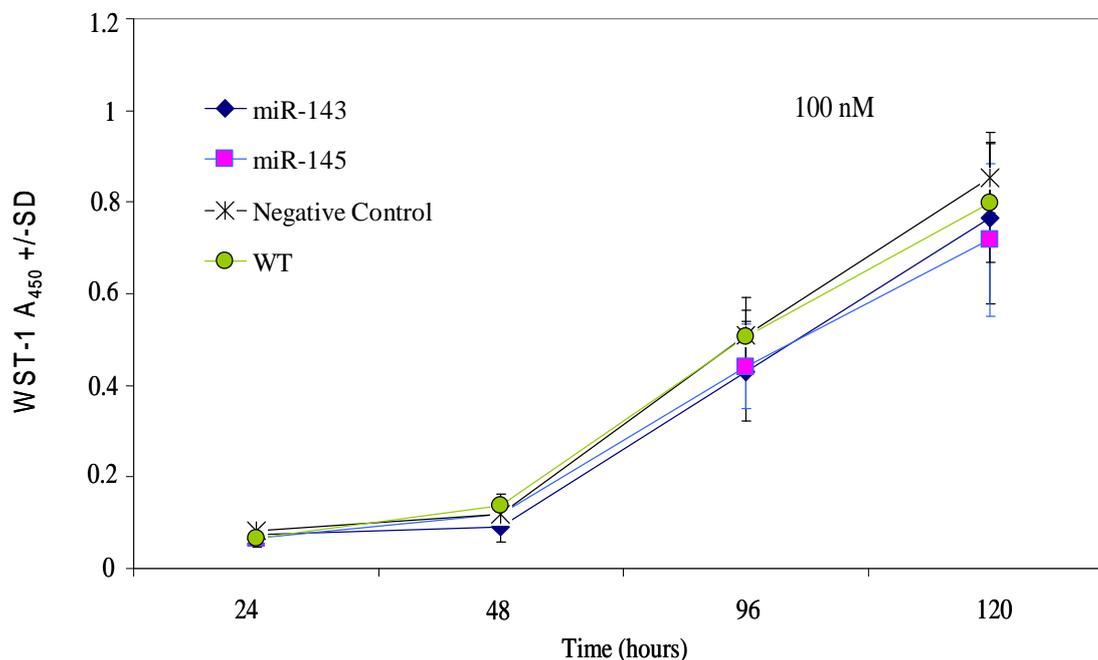


Figure 4.4.1: Proliferation of HCT-116 cells upon transfection with 50 nM and 100 nM miR-143, miR-145 or negative control RNA.

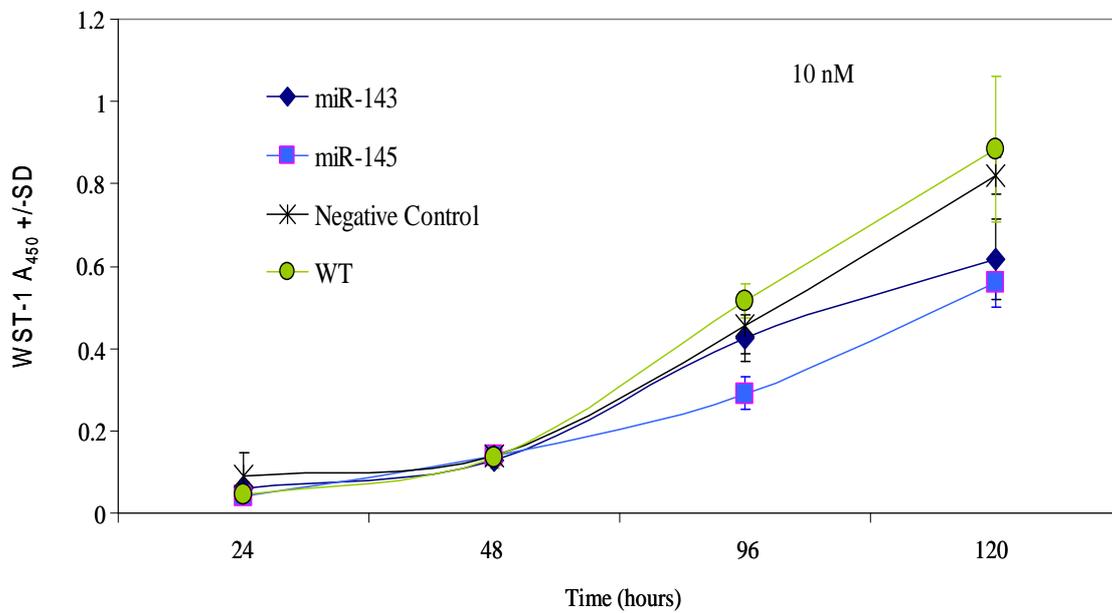
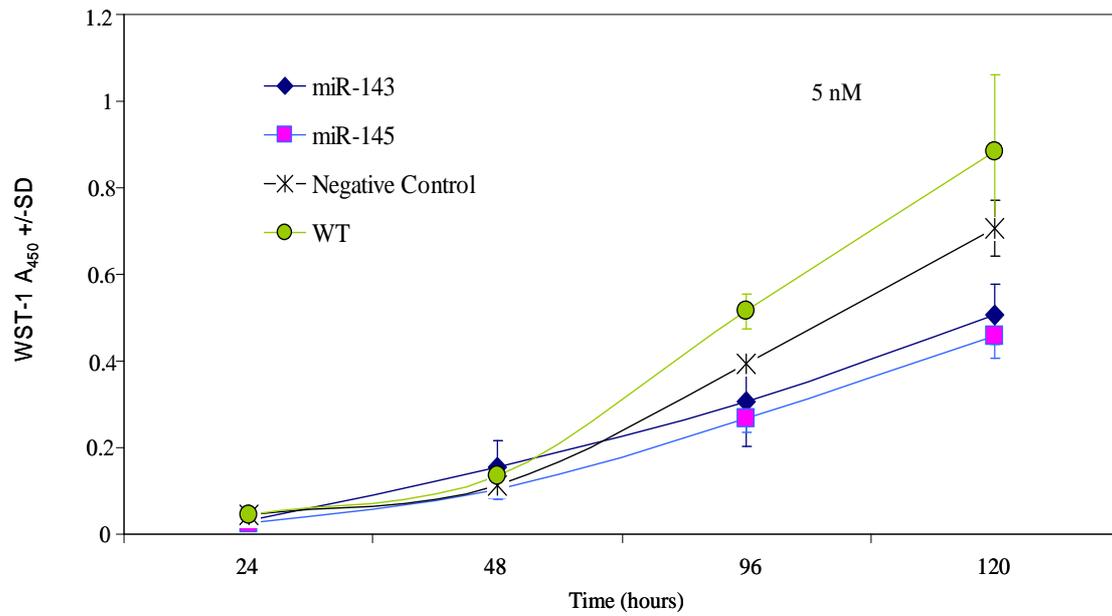
4.4.2 Proliferation assay- INTERFERin

INTERFERin was used as a transfection reagent to transfect HCT-116 cells with different concentrations of mature microRNAs, 5 nM, 10 nM and 20 nM. As shown in Figure 4.4.2, a profound inhibitory effect of miR-145 and miR-143 on the proliferation rate was observed after 120 h.

When the cells were transfected with 5 nM miRNAs or negative control, a marked inhibitory effect of both miR-145 and miR-143 was detected, where miR-145 and miR-143 reduced the cell proliferation by 35% and 28%, respectively, compared to the non-specific control RNA.

Increasing the concentration of microRNAs to 10 nM led to a reduction of the proliferation rate by 25% and 31% for miR-143 and miR-145, respectively, compared to the negative

control, while non-specific toxicity of INTERFERin has not been observed, but in case of transfection with 20 nM, a further reduction of the proliferation rate was detected by 41% and 17% for miR-145 and miR-143, respectively, compared to the negative control RNA.



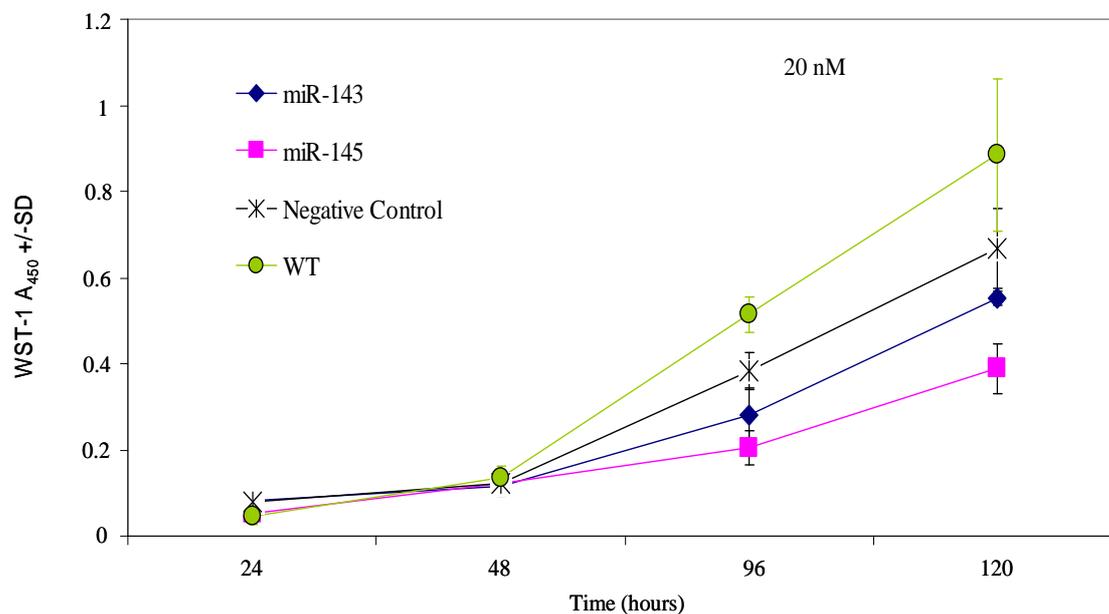


Figure 4.4.2: Proliferation activity of HCT-116 after transfection with 5 nM, 10 nM and 20 nM of miR-143, miR-145 or negative control RNA complexed with INTERFERin.

4.4.3 Soft agar assay of HCT-116 cells

HCT-116 cells were transfected with 20 nM miR-143, miR-145 or the non-specific control RNA complexed with INTERFERin. The cells were seeded onto agar plates and incubated for 21 days. Two unbiased investigators counted the formed colonies and it was shown that miR-143 and miR-145 reduced the proliferation rate, number and size, by 44% and 43% respectively.

As illustrated in Figure 4.4.3 and the representing images, the comparison between the negative control RNA transfected cells and wild type untreated cells showed the absence of non-specific activity of INTERFERin, which is consistent with the previous results at the level of anchorage dependent assay.

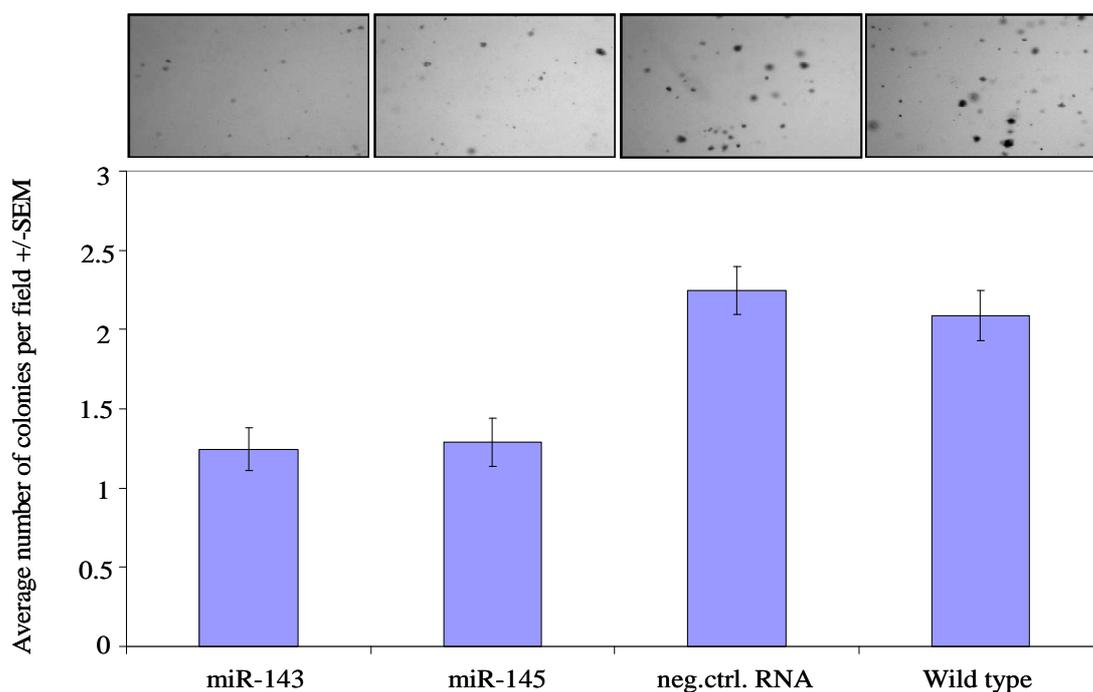


Figure 4.4.3: Anchorage independent assay of HCT-116 cells transfected with 20 nM miRNAs complexes formed with INTERFERin.

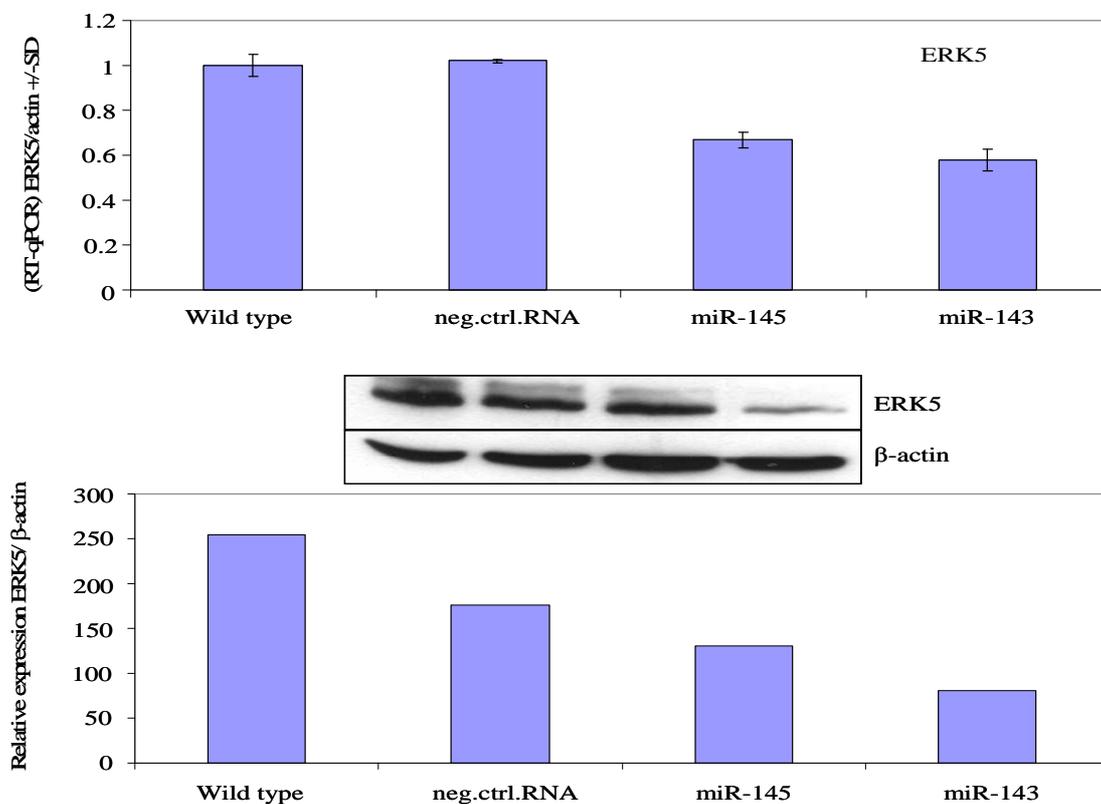
4.4.4 Relative expression levels of ERK5 and c-myc in HCT-116 cells

The expression levels of ERK5 and c-Myc were determined in HCT-116 cells transfected with 20 nM miRNAs complexed with INTERFERin as transfection reagent. At the transcriptional level, ERK5 was downregulated by 43% and 34% when the cells were transfected with miR-143 and miR-145, respectively, compared to the non-specific control RNA, which showed absence of a non-specific effect.

Likewise, at the post-transcriptional level, a downregulation of ERK5 protein was detected, where both miR-143 and miR-145 reduced the expression level by 41% and 23% for miR-143 and miR-145 respectively compared to the non-specific control RNA.

The proto-oncogene *c-myc* was also evaluated as an important tumorigenic target for both miR-143 and miR-145. At the transcriptional level, *c-myc* transcripts were slightly downregulated by 3% and 10% for miR-143 and miR-145, respectively, compared to the negative control, which showed absence of non-specific effect on the expression level.

At the post-transcriptional level, c-Myc protein was downregulated by 44% and 15% for miR-143 and miR-145, respectively, in comparison to the non-specific control RNA.



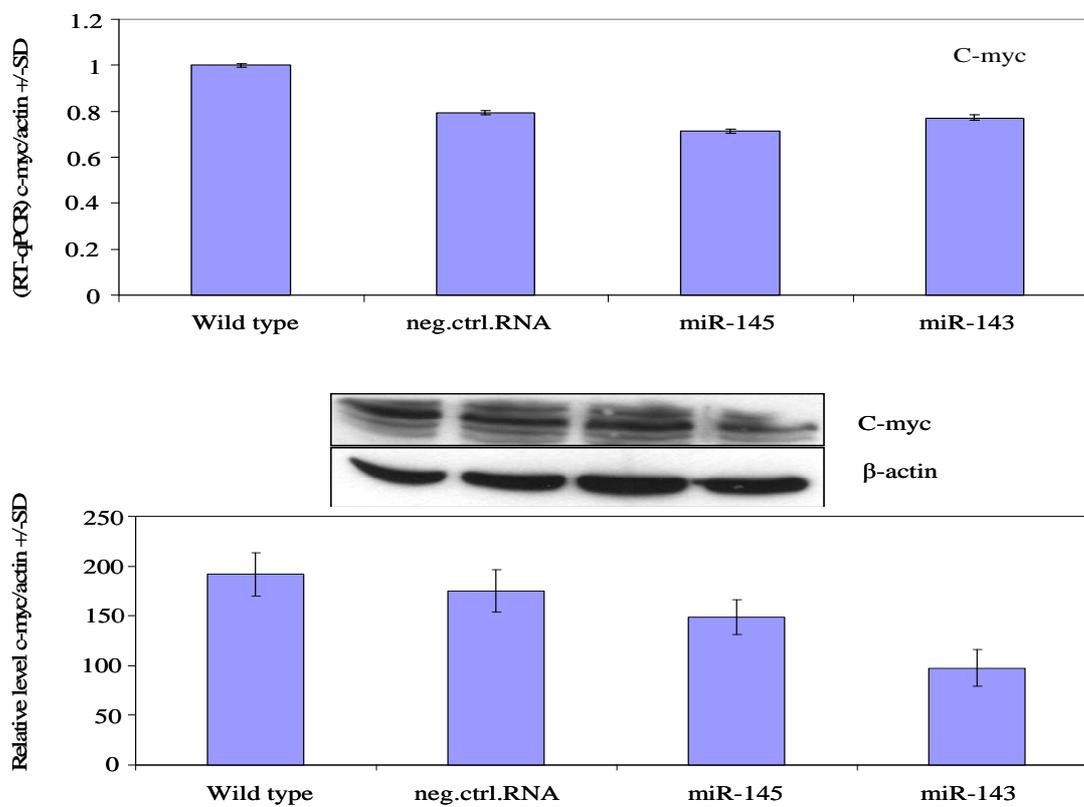


Figure 4.4.4: Relative expression of ERK5 (upper panel), c-Myc (lower panel) at the transcriptional and post-transcriptional level in HCT-116 cells transfected with 20 nM microRNAs using INTERFERin.

4.4.5 Anti-tumor effect of intratumorally PEI-mediated delivery of miR-145 in s.c HCT-116 colon carcinoma xenograft mouse model

To further explore the therapeutic applicability of PEI/miR-145 complexes, a local treatment regimen was tested next. In these experiments, the particularly tumorigenic colon carcinoma cell line HCT-116 was selected. Again, the mice were grouped into three groups, eight mice each. One group was treated by intratumoral injection of PEI-complexed miR-145, the second group was injected with the PEI-complexed non-specific control RNA and the third group served as wild type untreated controls. The treatment was three times per

week and was started after the establishment of tumor xenografts; however, since PEI complexes were applied locally, amounts were depending on the tumor size (50-100 μ l).

Untreated tumors showed a rapid growth with a \sim 8-fold increase over 2.5 weeks (Figure 4.4.5). Reduced tumor growth was observed in the PEI/negative control RNA group, indicating some non-specific effects of the PEI complexes upon local injection. Notably, the intratumoral application of PEI-complexed miR-145 resulted in a very profound tumor growth inhibition, with tumor volumes in the treatment group being \sim 40% or \sim 60% of the untreated or negative control treated tumors, respectively. Based on the previous results, a therapeutic potential of PEI/miR-145 to reduce the tumor growth *in vivo* by targeting some proliferative related gene either directly or indirectly was established.

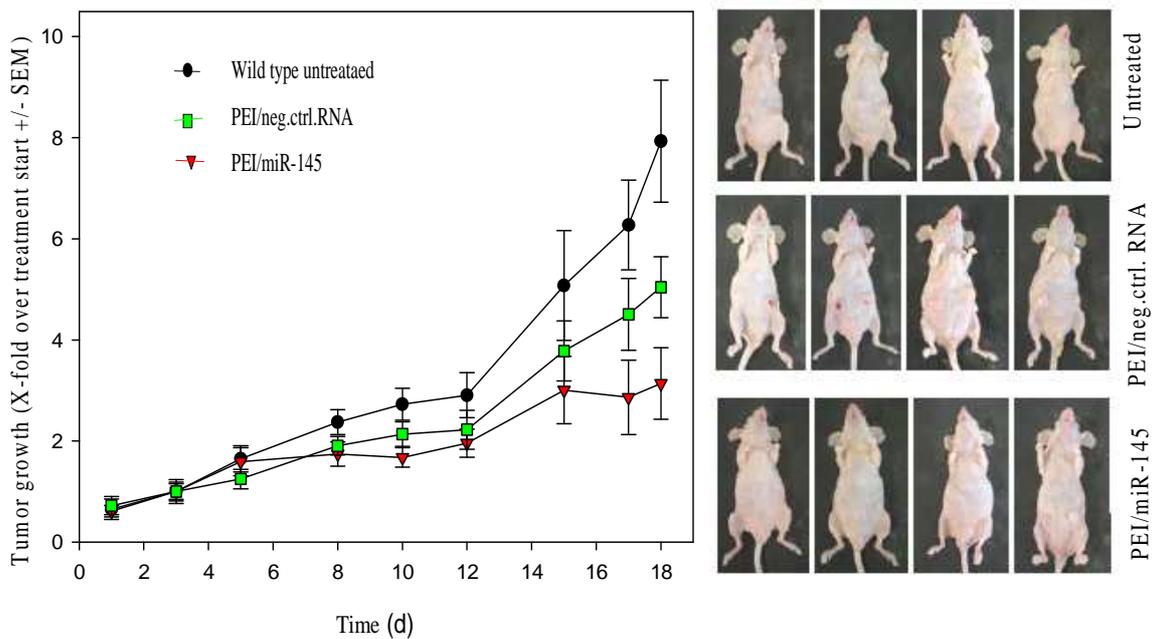


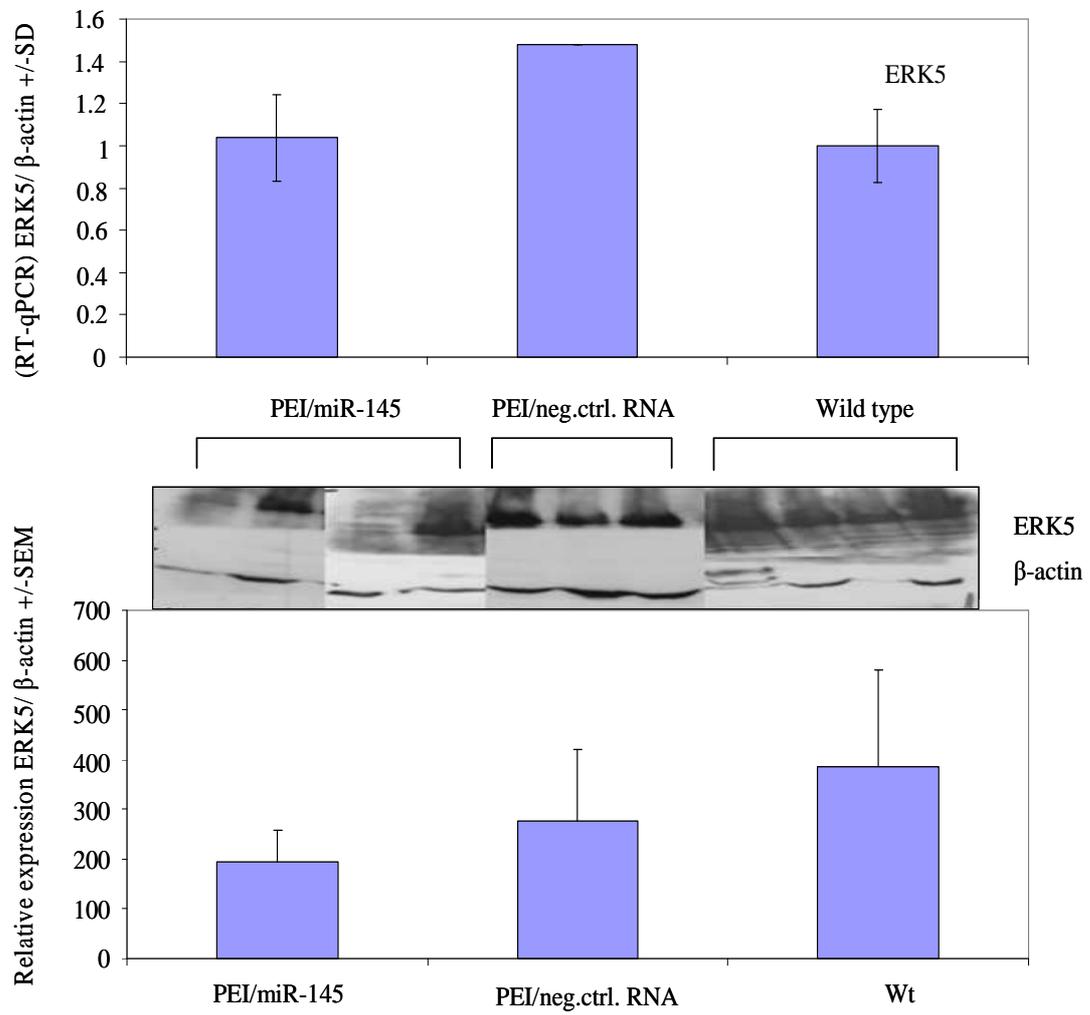
Figure 4.4.5: Tumor-inhibitory effects of PEI/miR-145 complexes in s.c. HCT-116 colon carcinoma xenografts upon intratumoral injection. The local administration leads to some non-specific effects of PEI/negative control RNA complexes (green) as compared to untreated controls (black); more profound effects, however, are observed in the PEI/miR-145 treatment group (red)

4.4.5.1 Expression levels of ERK5 and c-myc in s.c HCT-116 xenografts

HCT-116 xenografts were exposed to quantitative real time PCR analysis, where the expression levels of ERK5, c-myc mRNA level were determined. As shown in Figure 4.4.5.1, it was found that the expression level of these targets was not significantly changed in comparison to the non-specific control RNA xenografts. In case of ERK5, the expression of transcripts was downregulated by 30% compared to the non-specific control RNA, which indicated also the absence of the non-specific activity of PEI.

At the protein level, no significant reduction was observed. While the expression level was downregulated by 29% in PEI/miRNA complex treated xenografts compared to the non-specific control RNA, large standard deviations prevented more solid conclusions.

In case of c-myc, no effect of PEI/miR-145 was detected at neither the transcriptional level nor post-transcriptional level. Based on the previous results, a general conclusion has been established that the therapeutic effect of PEI-miR-145 complex is depending on the tumorigenic cell type as well as the method of treatment.



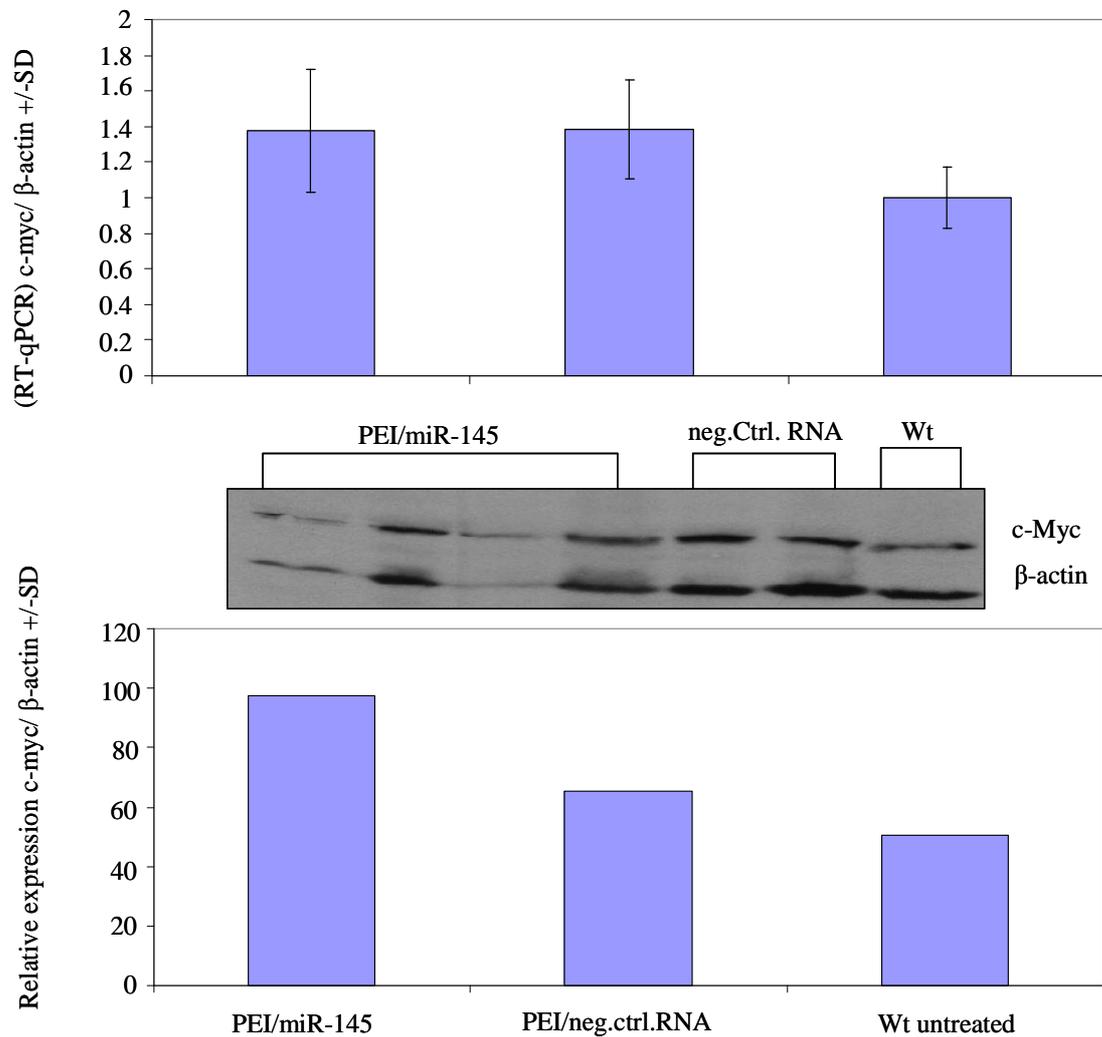


Figure 4.4.5.1: Relative expression level of ERK5 (upper graph), c-Myc (lower graph) in s.c HCT-116 colon carcinoma mouse xenografts.

4.4.5.2 Expression levels of PCNA in s.c HCT-116 xenografts

Immunohistochemistry assay was conducted to examine the expression status of PCNA in the HCT-116 induced xenografts. The evaluation of PCNA staining was semi-quantitative, when evaluation criteria were used which depended on the intensity of stained nuclei from

value 0 to 3 (0 for no staining, 1 for poor staining, 2 for moderate staining and 3 for strong staining). The slides were evaluated by unbiased counters.

Intensity of PCNA staining was reduced by 49% in tumors, which had been treated with PEI/miR-145, compared to the negative control RNA. This result is almost consistent with data found in LS174t-induced tumors, which indicates the antiproliferative role of miR-145 in colon cancer.

At the transcriptional level, PCNA was downregulated by 18% in miR-145 treated tumors compared to the non-specific control RNA, where non-specific effects of PEI were not observed.

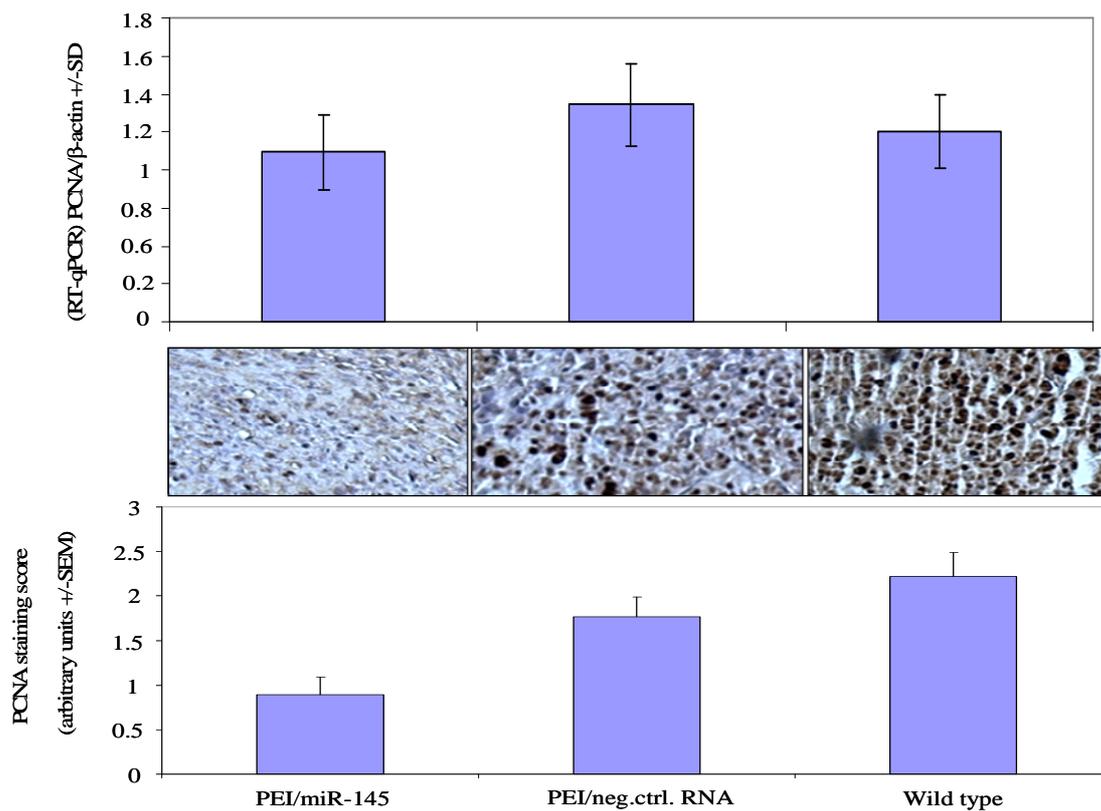


Figure 4.4.5.2 Expression levels of PCNA in s.c HCT-116 induced xenografts. The upper panel shows the PCNA transcript levels. The lower panel shows immunohistochemistry stainings with the representative images.

5. Discussion

The general feature of cancer is the loss of cell identity and aberrant proliferation. Characteristically, oncogenesis is the accumulation of mutations in protein encoding genes or tumor suppressors (McManus, 2003). A new class of non-protein coding, endogenous, small RNAs has been discovered, these molecules were named microRNAs, and were found to be important regulatory molecules in animals and plants (Zhang et al., 2007).

MicroRNAs are important post-transcriptional regulators of gene expression that control diverse physiological and pathological processes. This regulation allows for fine tuning of cellular processes, including regulation of proliferation, differentiation and apoptosis (Winter et al., 2009). MicroRNAs and their targets seem to form complex regulatory networks. For example, a single microRNA can bind to and regulate many different messenger RNA targets and, conversely, several different miRNAs can bind to and cooperatively control a single mRNA target (Lewis et al., 2003). It was reported that more than one third of all human genes are regulated by miRNAs (Lewis *et al.*, 2005).

Since the discovery of a functional RNA interference (RNAi) strategy in mammals, significant efforts have been undertaken to develop therapeutics that utilize this pathway (de Fougères *et al.*, 2007). While progress has been made toward the design and delivery of short interfering and short hairpin RNAs for therapeutic gene silencing, accumulating evidence indicates that the modulation of miRNA activity also represents an attractive strategy (Baek et al., 2008; Selbach et al., 2008).

The therapeutic application of miRNAs involves two strategies. One strategy is directed against a gain of function and aims to inhibit oncogenic miRNAs, whereas the second strategy, microRNA replacement, involves the reintroduction of a tumor suppressor miRNA to restore a loss of function (Johnson *et al.*, 2007; Esquela-Kerscher *et al.*, 2008; Trang *et al.*, 2009).

Many strategies of siRNA delivery have been developed from viruses as natural vehicles to liposomes, nanoparticles or bacteria (transkingdom RNAi) (Li, 2006; Aigner, 2009; Kruhn *et al.*, 2009; Nguyen and Fruehauf, 2009).

In this work, for the first time, one of the most widely examined synthetic cationic polymers, polyethylenimine, was used for microRNA delivery *in vitro* and *in vivo*.

Two types of polyethylenimine were used, linear Jet-PEI and branched low molecular weight, 4-10 kDa PEI, which was prepared from commercially available 25 kDa PEI. Variations in their transfection efficiency as well as their cytotoxicity were found, which were depending on the used N/P ratio, the type of transfected cell lines and the period of transfection time.

The expression levels of both precursor and mature miR-143 and miR-145 were determined by quantitative real time PCR in 16 different cell lines representing different types of cancers such as colon cancer, prostate, bone marrow, ovary, lung, skin.

The quantification of the mature miRNAs by PCR presented a challenge because of the critical size, 19-22 nt, to be detected by standard PCR primers. For this reason, a new real-time PCR assay to quantify the mature miRNA was employed, stem-loop RT-PCR (Chen *et al.*, 2005).

One of the benefits of the analysis of microRNA expression is to use them in prognosis of cancer in the first stages as biomarkers and/or use them to monitor the responsiveness to the clinical treatment (Akao *et al.*, 2006; Osaki *et al.*, 2008).

Both miR-143 and miR-145 were found to be downregulated in all colon carcinoma cell lines. In agreement with these results, these microRNAs were significantly downregulated in colorectal carcinoma (Michael *et al.*, 2003; Akao *et al.*, 2006; Slaby *et al.*, 2007; Takagi *et al.*, 2009), in prostate cancer (Porkka *et al.*, 2007; Ozen *et al.*, 2008), in bladder cancer (Ichimi *et al.*, 2009), in ovarian cancer (Iorio *et al.*, 2007; Nam *et al.*, 2008).

The expression level of precursor microRNAs was far more downregulated than that of the corresponding mature; this may be due to the fast turnover of precursor microRNAs compared to mature microRNAs. In another study carried out by Michael and colleagues, they found that precursor microRNAs were accumulated in both normal and cancerous tissue and they attributed this result to reduced activity of the components of the microRNA processing machinery such as Dicer proteins (Michael *et al.*, 2003).

Reduced expression levels of these microRNAs can be attributed to the presence of the microRNA genes in fragile sites exposed to mutations, and in fact, it was previously found that p53 response elements, responsible for the activation of transcription, lie in the promoter region of the miR-145 gene. p53 is known to be inactivated in several cancers which in turn may inactivate transcription of miR-145 (Sachdeva *et al.*, 2009). Another reason which could explain the reduction of the expression level of microRNAs is the DNA hypermethylation (Ostenfeld *et al.*, 2010).

In order to evaluate the therapeutic abilities of microRNAs, two colon carcinoma cell lines, LS174t and HCT-116, were selected.

In this work, it was demonstrated that linear Jet-PEI has the ability to complex and to deliver microRNAs efficiently (when after transfecting the LS174t cells with miRNAs/Jet-PEI and incubated for 48 h, the levels of microRNAs were analyzed by quantitative real time PCR). Interestingly however, it was found that when the LS174t cells were transfected with miR-143 or miR-145, the level of both microRNAs, the transfected miRNA and the non-transfected miRNA, were elevated. These results could account for a mutual relationship between transcription of both miR-143 and miR-145.

Previously, it was reported that miR-143 and miR-145, which are localized at 5q32, are possibly transcribed as the same primary microRNA (pri-miRNA) (Takagi *et al.*, 2009). To confirm absence of cross reactivity of primers, miR-143 or miR-145 was added directly to the vials of RNA extracts. It was found that the level of miR-143 or miR-145 was separately elevated i.e., in case of addition of miR-143, a high level of miR-143 was

observed but not miR-145. Moreover, when miR-145 was added, the level of miR-145 was high but not miR-143. This indicates the specificity of both primers and no-cross reactivity was detected.

This establishes the efficiency of Jet-PEI to deliver microRNAs into the cells. Consistently, the efficient transfection of mature miR-145 *in vitro* was confirmed after 12, 24 and 48 h by transfection with Lipofectamine as a delivery reagent (Ostenfeld *et al.*, 2010).

Upon transfection of LS174t with PEI/miR-143 or PEI/miR-145, the rate of proliferation was markedly reduced. In case of Jet-PEI complexed with 50 nM or 100 nM mature microRNAs, the proliferation rate was reduced over a long time, 120h. Earlier studies have shown the ability of miR-145 to reduce the growth rate in colon and cervical cancer cells 96 h post transfection (Shi *et al.*, 2007; Schepeler *et al.*, 2008; Wang *et al.*, 2008).

Previously, it was found that both microRNAs reduced the proliferation of human gastric cancer MKN-1 cells by 55-60% in comparison with control cells (Takagi *et al.*, 2009). Furthermore, the function of both miRNAs in cell growth was evaluated, when two colon carcinoma DLD-1 and SW480 cells were transfected with 20-80 nM precursor miRNAs (Akao *et al.*, 2006, 2007b). By transfection of three different colon carcinoma cell lines DLD-1, LS174t and HCT-116 with miR-145, the growth rate was markedly reduced (Schepeler *et al.*, 2008).

However, in case of transfection of HCT-116 cells using Jet-PEI, only little effects of both microRNAs were detected. The HCT-116 cells were transfected with 50 nM and 100 nM miR-143, miR-145 or negative control RNA. This could be due to the cell type and its cytogenetic profile.

Upon transfection of LS174t cells with INTERFERin at concentrations 20 nM or 40 nM mature miRNAs, a profound inhibitory effect of miR-145 was already observed at 20 nM. MiR-143 had a lower activity than miR-145. Increasing the concentration to 40 nM, the

inhibitory effect of miR-143, miR-145, or of the combination of both increased, however a slight toxic effect of the negative control was detected in comparison to the wild type cells.

Upon transfection of HCT-116 cells with three different concentrations of mature microRNAs (5 nM, 10 nM and 20 nM) complexed with INTERFERin, the proliferation rate was fluctuating depending on the concentration and number of seeded cells. In these experiments, several parameters had to be optimized to set reliable results. Among others these included the number of seeded cells, which was found to be optimal at 200-300 cells per well.

Transfection of LS174t cells with different concentrations of microRNAs ranging from 5 nM to 30 nM using another type of transfection reagent, branched PEI F25-LMW, also led to an inhibition of growth. The proliferation was effectively reduced upon transfection with miR-145 and miR-143. A toxicity or non-specific activity of PEI was detected at 30 nM.

Compared to branched low molecular weight polyethylenimine, linear PEI had high transfection efficiency *in vitro* studies. This may be attributed to the large size of polyplexes containing linear-PEI in salt containing buffer compared to those prepared with its branched form (Wightman *et al.*, 2001; Kunath *et al.*, 2003). Larger particles sediment onto seeded cells more quickly than smaller ones, which may result in an increase of particle uptake *in vitro* (Boussif *et al.*, 1996; Ogris *et al.*, 1998). Furthermore, it has been postulated that large particles show a higher intrinsic endolysosomal activity, which may enhance their escape from the acidic compartment (Ogris *et al.*, 1998).

The anti-proliferative activity of miR-145 was more profound than that of miR-143 after transfection with the three previously mentioned concentrations. The anti-proliferative results confirmed that the inhibitory effect of microRNAs is depending on the cell line, the used transfection reagent as well as the concentration of introduced microRNA.

Some genes associated with proliferation and oncogenic activities had been previously found to be targeted by miR-145. These genes are responsible for cellular transformation such as insulin Receptor Substrate-1 (IRS-1) (Ostenfeld *et al.*, 2010), and YES and STAT1 in colon cancer cells DLD1 and HCT-116 (Gregersen *et al.*, 2010).

This thesis could also demonstrate that PEI F25-LMW mediated miR-145 delivery *in vivo*. Consequently, s.c tumor growth was reduced. The *in vivo* studies were conducted by injection of athymic nude mice subcutaneously with cell lines LS174t or HCT-116. In the *in vivo* experiments, the PEI F25-LMW/miR-145 complex was introduced intraperitoneally in case of LS174t tumors or intratumorally in case of HCT-116 tumors in comparison to a non-specific LUC3 siRNA negative control and the wild type untreated groups.

Both experiments showed a profound suppressor effect of miR-145 on the growth of tumors, which indicated the promising therapeutic ability of microRNAs in regulating and/or suppressing of tumors either as single therapeutic agents or in synergistic combination with other strategies.

Compared to the intratumoral application of PEI-complexed miR-145, it was found that the intraperitoneal injection was more efficient. Over 25 d, a non-specific activity was not observed in case of intraperitoneally-injected PEI/miR-145 complex as indicated by the negative control RNA treated tumors. In contrast, intratumoral injection of PEI/miR-145 showed some non-specific activity at the 18th day.

Soft agar assay set a standard method to validate the effects expected in the *in vivo* colon carcinoma models. The transfection of miR-143 and miR-145 affected tumor cell growth, number and size, as indicated by anchorage-independent growth of colon cancer cells LS174t and HCT-116 in the soft agar layer. These results provided strong evidence that miR-143 and miR-145 have a role in suppressing tumor growth. In LS174t cells and HCT-116 cells, the miR-143 and miR-145 showed a profound inhibitory effect on colony formations as illustrated in chapter 4.

The introduction of miR-143 had been shown previously to affect not only the proliferation rate but also the growth rate and colony formation of colon cancer cell lines 228 and SW480 in soft agar plates (Ng et al., 2009). Based on the results of this thesis, however, in combination with the proliferative assays, miR-145 as promising candidate was chosen to proceed with *in vivo* studies.

It was found that there is a relation between expression levels of microRNA and the fate of the cancer cells. For this reason, apoptosis assay on LS174t cells after transfection with 100 nM miRNAs were performed. Two different types of apoptosis assays, caspase Glo 3/7 assay and FITC-annexin assay were performed. In the first assay, miR-145 and miR-143 induced high levels of caspase 3/7 in comparison to the negative control RNA treated cells. After normalizing the results to the wild type (untreated cells), apoptosis levels were higher in negative control cells as compared to the wild type untransfected cells, which may reflect a non-specific transfection effect. Still, apoptosis was further increased upon transfection with miR-145 or miR-143. Previously, it was also proved that miR-145 is involved in caspase-dependent and independent apoptosis in bladder tumors and inhibits colon cancer proliferation (Shi *et al.*, 2007).

At the beginning of apoptosis, the early phase, the phospholipid phosphatidylserin (PS) is translocated to the outer surface of plasma membrane, which can effectively bind to and be detected with annexin. During the end stage of apoptosis, the cells lose their membrane integrity and there is no sharp distinction between apoptotic cells or necrotic cells. In this case, another dye, propidium iodide, was used. This dye detects only the dead cells but not viable ones. So by using double staining, FITC annexin-propidium iodide, a differentiation between the early and the end phase can be made.

Apoptosis of LS74t was induced after transfection with miR-143 and miR-145, where miR-145 induced both early and end phase of apoptosis higher than that of miR-143. This indicates the involvement of microRNAs in the initiation of apoptosis either directly or in combination with other molecular effectors. ERK5 is a pro-survival kinase during mitosis (Girio et al., 2007). When ERK5 is downregulated by an siRNA strategy, apoptosis is

initiated. Therefore, all of these data would tell us that there is a relationship between the expression level of microRNAs, levels of ERK5 and the apoptosis process.

It was found previously that miR-143 induced the Fas-dependent apoptosis by targeting the ERK5 which leads to caspase activation (Akao et al., 2009). Some genes were found to be involved in the apoptosis network and at the same time targeted by miR-145.

Zhang and coworkers reported that miR-145 targets the DNA fragmentation factor-45 (DFF45) protein, the caspase-3 and caspase-7 substrate, (Zhang et al., 2010). This protein forms a heterodimer with another protein DFF40, a protein responsible for generation of double-stranded breaks in inter nucleosomal chromatin regions as well as the chromatin condensation of the DNA during the apoptosis. DFF45 must be cleaved to release the DFF40 before the DNA fragmentation process (Widlak et al., 2000).

ERK5 may be a direct or indirect target of miR-143 (Esau et al., 2004), while (Clape et al., 2009) showed that ERK5 is a direct target of miR-143 in prostate cancer, as evidenced by the presence of a seed sequence of miR-143 in the 3'UTR of ERK5 mRNA. This fact was also reported by (Akao et al., 2007a), who reported that the microRNAs miR-143 and miR-145 were downregulated in chronic lymphocytic leukemia and that ERK5 is a direct target of miR-143. For the first time, this work reports that ERK5 was also influenced by miR-145, directly or indirectly.

Over-expression and activation of mitogen activated protein kinase receptors has been detected in colorectal cancer and has an important role in colorectal cancer progression (Fang and Richardson, 2005). Furthermore, ERK5 has been shown to be involved and to have a potential role in breast cancer initiation and progression (Montero et al., 2009). *In vivo* studies using animals in which ERK5 expression can be regulated, have demonstrated that ERK5 is important for sustaining tumor growth, probably due to its supportive role in vasculogenesis and blood vessel homeostasis (Hayashi *et al.*, 2004; Hayashi *et al.*, 2005).

In this work, the level of ERK5 (transcript and protein) was determined in lysates of LS174t cells and HCT-116 cells, which were transfected with different concentrations of mature microRNAs using Jet-PEI or INTERFERin as transfection reagents. Upon transfection of the LS174t cells with 100 nM miRNAs/Jet-PEI complex or with 20 nM miRNAs/INTERFERin complex, no significant change of ERK5 at the transcriptional level was observed.

HCT-116 cells were transfected with only 20 nM miRNAs/INTERFERin complex (depending on the proliferation results). ERK5 expression at both transcriptional and posttranscriptional level was downregulated in HCT-116 cell lysate after transfection with miR-143 or miR-145.

The expression level of ERK5 at the post-transcriptional level was evaluated. After transfection of LS174t cells with 50 nM and 100 nM miRNA complexed with Jet-PEI, the expression of ERK5 was markedly downregulated in a dose-dependent manner. At both concentrations, miR-143 had the higher activity than that of miR-145 in comparison to the negative control RNA. Nevertheless, miR-145 had a downregulatory effect on ERK5. Moreover, ERK5 protein was downregulated after transfection with 20 nM miRNAs complexed with INTERFERin.

It has been found previously that the proto-oncogene *c-myc* plays an important role in gastric carcinogenesis and it is estimated to be involved in 20% of all human cancers (Dang, 1999). However, *c-myc* expression was also evaluated at transcriptional and posttranscriptional level in both LS174t cells and HCT-116 cells.

In LS174t cells, the levels of *c-myc* transcripts was downregulated after transfection with 100 nM miRNAs/Jet-PEI complex, but no change in case of INTERFERin was detected. In contrast, the levels of *c-Myc* protein was not significantly downregulated in LS174t cells in case of transfection with miR-145, and no change at all was observed in case of miR-143. In HCT-116 cells, the levels of *c-myc* transcripts was not changed. Nevertheless, at the posttranscriptional level, *c-Myc* was downregulated by both miR-143 and miR-145.

Comparing the results, which emerged from the analysis of either transcript levels or protein levels, the microRNAs exerted a post-transcriptional regulatory role in the cells. Moreover, the results indicate that the expression level of targets, ERK5 or c-myc depends on the intracellular level of microRNAs, cell line or the transfection reagent. By this mechanism, microRNAs regulate about 30% of human genome (Lewis *et al.*, 2005).

Furthermore, the expression of ERK5, c-myc and PCNA in the s.c tumors at both transcriptional and post-transcriptional level were evaluated. In LS174t-induced xenografts, the expression level of ERK5 and c-myc transcripts were not changed, while levels of PCNA were downregulated in miR-145/PEI treated mice in comparison to the negative control RNA tumors.

At the protein level, c-Myc and ERK5 were downregulated in LS174t-induced xenografts in comparison to the negative control, which indicates that miR-145 interfering the translation of these targets at translational level. As it was before indicated that, c-Myc is a direct target of miR-145 but ERK5 has not proved as a direct target yet. Nevertheless, ERK5 is downregulated by miR-145 and this may be due to an either direct or indirect effect.

In HCT-116-induced xenografts, ERK5 and PCNA transcripts were downregulated in miR-145 treated tumors in comparison to the negative control, while no change was not observed at the transcriptional level of c-myc.

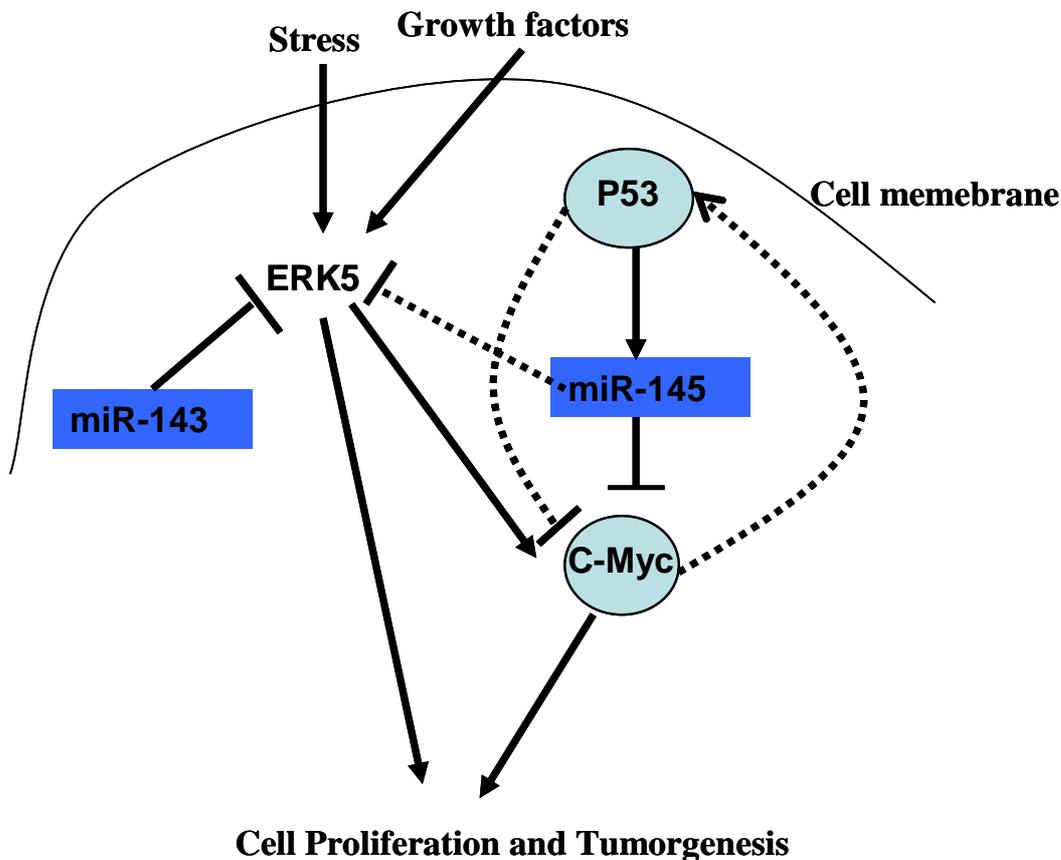


Figure 5: Schematic representation showing effects of miR-145 and miR-143 on the proliferation and survival of cancer cells.

The expression level of PCNA in both LS174t and HCT-116 xenografts was demonstrated by immunohistochemistry using a mouse monoclonal anti-PCNA antibody. The proliferating cell nuclear antigen is an essential regulator of the cell cycle, which is highly conserved between species (Bravo, 1987). The results showed a decrease in the intensity of stained nuclei. This result was in accordance with the tumor growth profile, which indicated the efficiency of using the PCNA as a reliable biomarker of the proliferation process. Moreover, this finding establishes *in vivo* the anti-proliferative effect of PEI-mediated miR-145 delivery.

6. Summary

MicroRNAs (miRNAs) are small RNA molecules with perfect or imperfect homology to their target mRNA. They have been shown to specifically interfere with the expression of their target protein(s). Moreover, microRNAs play an important role in the pathogenesis of cancer, where some microRNAs act as tumor suppressors by targeting lethal oncogenes, and others act as oncogenes by targeting suppressor genes. Thus, microRNAs can be involved directly or indirectly in important cellular processes such as proliferation, differentiation and apoptosis. Understanding the functions and importance of microRNAs could lead to the discovery of new strategies for controlling of cancer.

This thesis was aiming at the possibility of using non-viral delivery systems for microRNAs into mammalian cell lines, and examined their efficacies. To this end, three different transfection reagents, linear Jet-PEI, branched PEI F25-LMW and INTERFERin, were used *in vitro*, either commercially available or prepared in our lab. The work was concentrating on colon cancer as a tumor model, and two representative cell lines, LS174t and HCT-116, were selected as a platform to conduct the experiments. The studies focused on miR-143 and miR-145, which had been implicated previously with cancer development and progression.

It was found in miRNA transfection experiments that miR-143 and miR-145, which are downregulated in colon cancer, have the ability to inhibit the progression of the cancer through different regulatory mechanisms, proliferation and apoptosis.

Some important genes, which play important roles in tumorigenesis and tumor progression, were found to be negatively regulated by both miR-143 and miR-145. More specifically, ERK5 (Mitogen Activated Protein Kinase) which plays an essential role in the proliferation of colon cancer was suppressed by miR-143 and miR-145. Likewise, c-Myc was negatively regulated by miR-145, but these results were only at the protein level, i.e., no significant effects were determined on c-Myc transcription. Furthermore, it was found that the

inhibitory effects were depending on several factors such as cell line, transfection reagent, time of incubation and concentration of miRNA complex.

Thus, the cellular delivery of microRNAs miR-143 and miR-145 showed profound inhibitory effects on the proliferation and soft agar colony formation of LS174t and HCT-116 cells, and induced apoptosis in LS174t cells.

Moreover, miR-145 was examined for its tumor inhibitory effect *in vivo*. To this end, athymic nude mice bearing s.c. colon carcinoma xenografts were treated with PEI-complexed miR-145 by systemic or local injection. Profound anti-tumor effects upon PEI-mediated miR-145 delivery were found which were again dependent on the downregulation of specific target genes.

Taken together, microRNA replacement therapy may represent a promising approach in tumor treatment, and this thesis establishes the PEI-mediated therapeutic delivery of miR-145 in colon carcinoma.

7. Zusammenfassung

MicroRNAs (miRNAs) sind kleine RNA-Moleküle mit vollständiger oder unvollständiger Homologie zu ihrer Ziel-mRNA. Damit sind sie in der Lage, spezifisch die Expression einzelner Proteine zu beeinflussen. MicroRNAs spielen eine wichtige Rolle in der Krebs-Pathogenese, indem einige miRNAs gegen Onkogene gerichtet sind und somit als Tumor-Suppressoren fungieren, während andere miRNAs Onkogene darstellen und gegen Tumor-Suppressoren gerichtet sind. MiRNAs können somit direkt oder indirekt an wichtigen zellulären Prozessen wie Proliferation, Differenzierung und Apoptose beteiligt sein. Das Verständnis ihrer Funktionen und ihrer Relevanz könnte zur Entwicklung neuer Strategien in der Kontrolle von Tumoren führen.

Diese Arbeit untersuchte die Möglichkeit und Effizienz nicht-viraler Einschleusungssysteme für miRNAs in Tumor-Zellen. Hierzu wurden zunächst *in vitro* drei verschiedene Transfektionsreagenzien, lineares Jet-PEI, verzweigtes PEI F25-LMW und INTERFERin untersucht, die entweder kommerziell erhältlich oder im Labor hergestellt worden waren. Die Experimente konzentrierten sich auf das Kolonkarzinom als Tumormodell, und zwei repräsentative Zelllinien, LS174t und HCT-116, wurden für die Experimente ausgewählt. Als miRNAs wurden miR-143 und miR-145 eingesetzt, auf deren Rolle in Tumorentstehung und in Tumorprogression es aus Vorstudien bereits Hinweise gab.

Es wurde in miRNA-Transfektionsexperimenten gefunden, dass miR-143 und miR-145, deren Level im Kolonkarzinom vermindert sind, die Tumorprogression durch verschiedene regulatorische Mechanismen wie Proliferation und Apoptose steuern können.

Wichtige Gene mit funktioneller Relevanz in Tumorigenese und Tumor-Progression wurden durch miR-143 und miR-145 negativ reguliert. So wurde ERK5 (mitogene activated protein kinase), das eine wichtige Rolle in der Proliferation von Kolonkarzinom-Zellen spielt, durch miR-143 und miR-145 supprimiert. Gleichfalls wurde eine negative Regulation von c-Myc durch miR-145 ermittelt, jedoch nur auf Proteinlevel und nicht auf der Ebene der Transkription. Es wurde ferner gefunden, dass die inhibierenden Effekte

abhängig waren von verschiedenen Einflüssen und Parametern wie Zelllinie, Transfektionsreagenz, Inkubationszeit und Konzentration des miRNA-Komplexes.

Die zelluläre Einschleusung der microRNAs miR-143 und miR-145 zeigte folglich deutliche inhibierende Effekte auf die Proliferation und die Soft-Agar Kolonienbildung von LS174t und HCT-116-Zellen, sowie eine Apoptose-Induktion in LS174t-Zellen.

Weiterhin wurden die Tumor-inhibierenden Effekte von miR-145 *in vivo* untersucht. Hierzu wurden athymische Nacktmäuse mit s.c. Tumor-Xenotransplantaten mit PEI-komplexierter miR-145 durch systemische oder lokale Injektion behandelt. Es wurden nach der PEI-vermittelten miRNA-Einschleusung deutliche Antitumor-Effekte beobachtet, die wiederum auf der Herunterregulation von spezifischen tumorrelevanten Zielgenen basierten.

Zusammenfassend kann die 'miRNA replacement therapy' somit einen viel versprechenden neuen Ansatz in der Tumorbehandlung darstellen, und diese Arbeit etabliert die PEI-vermittelte therapeutische Einschleusung von miR-145 *in vivo* in Kolonkarzinom-Xenotransplantaten.

8. Abbreviations

APS	Ammonium Peroxodisulfate
ABC	Avidin-Biotin complex
APC	Adenomatous polyposis coli
ATCC	American Type Collection Center
B-CLL	B-cell chronic lymphocytic leukemia
BMK1	Big Mitogen Kinase1
BSA	Bovine serum albumin
Caspase	cysteine aspartic acid-specific protease
cDNA	complementary DNA
c-Myc	v-myelocytomatosis viral oncogene homolog
DAB	3,3'-Diaminobenzidinetetrahydrochloride
DEPC	Diethylpyrocarbonate
DFF40	DNA Fragmentation Factor 40
DFF45	DNA Fragmentation Factor 45
DMSO	Dimethylsulphoxide
dsRNA	Double strand DNA
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular Signal Regulated Kinase1/2
ERK5	Extracellular Signal Regulated Kinase5
FCS	Fetal calf serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IMDM	Iscove's Modified Eagle Medium
IRS-1	Insulin-Receptor Substrate1
kDa	Kilo dalton
MAPK	Mitogen Activated Protein Kinase
miRNA	microRNA

mRNA	messenger RNA
NP40	Nonyl Phenoxypolyethoxylethanol
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase chain reaction
PCNA	Proliferating Cell Nuclear Antigen
PEI	polyethylenimine
PMSF	PhenylMethylSulfonylFluoride
pre.miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PS	Phosphatidylserine
PVDF	Polyvinylidene Fluoride
RdRb	RNA dependent RNA Polymerase
RISC	RNA interference induced silencing complex
RNAi	RNA interference
RSK	Ribosomal Synthase Kinase
RT-PCR	Reverse transcription PCR
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamid Gel Electrophoresis
SGK	Serum and Glucocorticoid induced Kinase
shRNA	Short hairpin RNA
siRNA	Small interferring RNA
TBST	Tris Buffered saline tween
TCF4	T-cell factor 4
TEMED	N, N, N', N'-Tetramethylethylenediamine
WST-1	Water soluble Tetrazolium salt

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11. Declaration

I hereby declare that the submitted dissertation was completed by myself and none other and I have not used any sources or materials other than those enclosed. Moreover, I declare that the following dissertation has not been submitted further in this form and has not been used for obtaining any other equivalent qualification in any other organization. Additionally, other than this degree I have not applied or will not attempt to apply for any other degree, title or qualification in relation to this work.

Marburg, 14.01.2011

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12. Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „**Functional analysis of non-viral miRNA replacement therapy in colon carcinoma *in vitro* and *in vivo***“ im Pharmakologischen Institut der Philipps-Universität Marburg unter Leitung von Prof. Dr. Frank Czubayko / Prof. Dr. Achim Aigner ohne sonstige Hilfe selbst durchgeführt habe. Bei der Abfassung der Arbeit habe ich keine anderen als die in der Dissertation angeführten Quellen und Hilfsmittel benutzt und vollständig oder sinngemäss übernommene Zitate entsprechend gekennzeichnet. Ich habe bisher weder an einem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch eine andere Arbeit als Dissertation oder die vorliegende zu anderen Prüfungszwecken vorgelegt.

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- 1992-1995 Secondary School (Scientific Division)
- 1989-1992 Preparatory School
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Technical Experience

Microbiology: Preparation of media required for microbiological cultures, isolation and identification of bacteria and fungi from different sources.

Cell Biology: Culturing and maintenance of animal cell lines, Transfection of Cell lines, Fluorescence Activated Cell Sorting, Different Cell culture based assays like Cytotoxicity, Cell proliferation, Luciferase assays, Caspase assay etc.

Molecular Biology and Biochemistry Techniques: Isolation of RNA and small RNA molecules including microRNAs, PCR techniques, western Blot analysis, immunohistochemistry, Basic bioinformatics skills required for molecular biology work, for example gathering information from biological databases, analysis and manipulation of sequence data using different bioinformatics tools.

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PUBLICATIONS

- 1- **Ahmed Fawzy Ibrahim**, Ulrike Weirauch, Arnold Grünweller, Roland K. Hartmann, and Achim Aigner (2010). MiRNA replacement therapy through PEI-mediated *in vivo* delivery of miR-145 or miR-33a in colon carcinoma. Cancer Research (submitted).
- 2- Nahla Mansour, Ghada Awad, **Ahmed Fawzy**, Ahmed El Diwany (2009). Molecular Identification and Characterization of Lactobacilli Isolated from Egyptian Infants as Potential Probiotic Candidates. Arab Journal of Biotechnology. **12** :(2) 40- 46
- 3- **A.Fawzy Ibrahim** ;El-Daly,O;Sarhan.M;Farouk.M;Assad,A (2003) Antimicrobial Activity of Some Essential Oils Against Some Human Enteric Bacteria. Bulletin faculty of science, Zagazig University, 25(2) December, 1-12.

Poster

- 1- U. Weirauch , **A.Fawzy Ibrahim**, A. Grünweller, R.K. Hartmann and A.Aigner (2010). Non-viral delivery of miRNA-145 or miR-33 *in vivo* therapy of colon carcinoma, accepted for poster presentation at EMBL symposium, Heidelberg. The non-coding genome.

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