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**miR-143 and miR-145 inhibit the growth of colon
cancer cells by targeting multiple oncogenic
activities**

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Introduction

Cancer is a genetic disease caused by uncontrolled proliferation and the inappropriate survival of damaged cells. Cells have developed several safeguards to ensure that cell division, differentiation and death occur correctly and in a coordinated fashion, both during development and in the adult body. Many factors are involved in the regulation of genes that direct cellular proliferation and differentiation. Damage to these genes, which are referred to as tumour-suppressor genes and oncogenes, is selected for in tumorigenesis. Most tumour suppressor genes and oncogenes belong to the wide category of protein coding genes, however, recent evidence indicates that small non coding RNA molecules, called microRNAs (miRNAs), might also function as tumour suppressors and oncogenes (Esquela-Kerscher et al., 2006).

1. MicroRNAs

MicroRNAs are single-stranded RNAs of 19–25 nucleotides in length that are generated from endogenous hairpin-shaped transcripts (Ambros, V. et al. 2003; Bartel, 2004; Cullen et al., 2004). miRNAs function as guide molecules in post-transcriptional gene silencing by base pairing with target mRNAs, which leads to mRNA cleavage or translational repression. With more than 700 members per species in higher eukaryotes, miRNAs are one of the largest gene families, accounting for ~1% of the genome (Bartel, 2004). Recent studies have revealed that miRNAs have key roles in several regulatory pathways, including control of developmental timing, haematopoietic cell differentiation, apoptosis, cell proliferation and organ development. miRNAs and their targets seem to form complex regulatory networks. For example, a single miRNA can bind to and regulate many different mRNA targets and, conversely, several different miRNAs can bind to and cooperatively control a single mRNA target (Lewis et al., 2003). Recent work by the Bartel and Burge laboratories predicted that over one third of all human genes are targeted by miRNAs (Lewis et al, 2005). Consequently, the unique combination of

miRNAs that are expressed in each cell type might affect or ‘dampen’ the utilization of thousands of mRNAs (Bartel et al., 2004). Although the complexity of this regulatory circuitry is currently overwhelming, one of the first key steps towards dissecting the network would be to understand how miRNA genes themselves are regulated.

1.1 The discovery of miRNAs

MicroRNAs were identified for the first time in the 1993 from Victor Ambros, Rosalind Lee and Rhonda Feinbaum. They discovered that *lin-4*, a gene known to control the timing of *C. elegans* larval development, does not code for a protein but instead produces a pair of small RNAs (Lee et al., 1993). One RNA is approximately 22 nt in length, and the other is approximately 61 nt; the longer one was predicted to fold into a stem loop proposed to be the precursor of the shorter one. The Ambros and Ruvkun labs then noticed that these *lin-4* RNAs had antisense complementarity to multiple sites in the 3’ UTR of the *lin-14* (Lee et al., 1993; Wightman et al., 1993). This complementarity fell in a region of the 3’ UTR previously proposed to mediate the repression of *lin-14* by the *lin-4* gene product (Wightman et al., 1991). The Ruvkun lab went on to demonstrate the importance of these complementary sites for regulation of *lin-14* by *lin-4*, showing also that this regulation substantially reduces the amount of LIN-14 protein without noticeable change in levels of *lin-14* mRNA. Together, these discoveries supported a model in which the *lin-4* RNAs pair to the *lin-14* 3’ UTR to specify translational repression of the *lin-14* message as part of the regulatory pathway that triggers the transition from cell divisions of the first larval stage to those of the second (Fig. 1) (Lee et al., 1993; Wightman et al., 1993).

For seven years after the discovery of the *lin-4* RNA, the genomics of this type of tiny regulatory RNA appeared simple: there was no evidence for *lin-4*-like RNAs beyond nematodes and no sign of any similar noncoding RNAs within nematodes. This all changed upon the discovery that *let-7*, another gene in the *C. elegans* heterochronic pathway, encoded a second ≈ 22 nt regulatory RNA. The *let-7* RNA acts to promote the transition from late-larval to adult cell fates in the same way that

the *lin-4* RNA acts earlier in development to promote the progression from the first larval stage to the second (Reinhart et al., 2000; Slack et al., 2000). Similar to *lin-4*, *let-7* performs its function by binding to the 3' UTR of *lin-41* and *hbl-1* (*lin-57*), and inhibiting their translation (Reinhart et al., 2000; Lin et al., 2003; Abrante et al., 2003; Slack et al., 2000; Vella et al., 2004). The identification of *let-7* not only provided another vivid example of developmental regulation by small RNAs, but also raised the possibility that such RNAs might be present in species other than nematodes. Both *let-7* and *lin-41* are evolutionarily conserved throughout metazoans, with homologues that were readily detected in molluscs, sea urchins, flies, mice and humans (Pasquinelli et al., 2000). This extensive conservation strongly indicated a more general role of small RNAs in developmental regulation, as supported by the recent characterization of miRNA functions in many metazoan organisms.

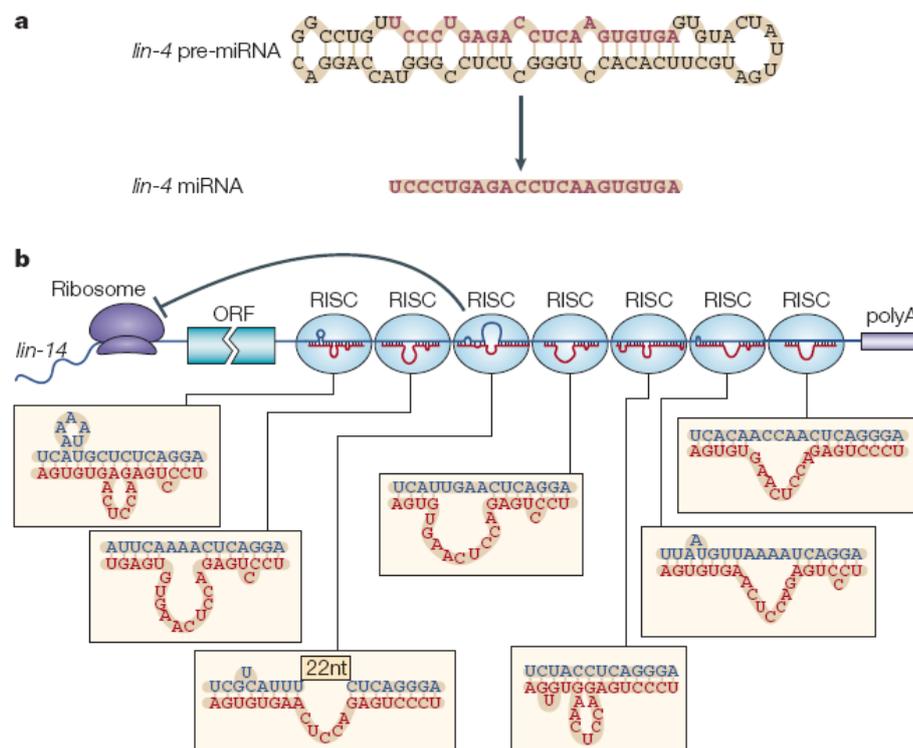


Figure 1. The molecular hallmarks of *lin-4*, the founding member of the microRNA family.

a) The precursor structure and mature microRNA (miRNA) sequence of *lin-4*. **b)** Sequence complementarity between *lin-4* (red) and the 3'-untranslated region (UTR) of *lin-14* mRNA (blue). *lin-4* is partially complementary to 7 sites in the *lin-14* 3' UTR. RISC, RNA-induced silencing complex. (Lin et al., 2004).

1.2 miRNAs and siRNAs

Almost coincident with the discovery of the second miRNA, *let-7*, small RNAs were also characterized as components of a seemingly separate biological process, RNA interference (RNAi). RNAi is an evolutionarily conserved, sequence-specific gene-silencing mechanism that is induced by exposure to dsRNA (Hannon, 2002). In many systems, including worms, plants and flies, the stimulus that was used to initiate RNAi was the introduction of a dsRNA (the trigger) of ~500 bp. The trigger is ultimately processed *in vivo* into small dsRNAs of ~21–25 bp in length, designated as small interfering RNAs (siRNAs) (Elbaschir et al., 2001). It is now clear that one strand of the siRNA duplex is selectively incorporated into an effector complex (the RNA-induced silencing complex; RISC). The RISC directs the cleavage of complementary mRNA targets, a process that is also known as post-transcriptional gene silencing (PTGS) (Fig. 2) (Zamore et al., 2000). The evolutionarily conserved RNAi response to exogenous dsRNA might reflect an endogenous defense mechanism against virus infection or parasitic nucleic acids (Hannon, 2002). Indeed, mutations of the RNAi components greatly compromise virus resistance in plants, indicating that PTGS might normally mediate the destruction of the viral RNAs (Baulcombe, 1999). In addition, siRNAs can also regulate the expression of target transcripts at the transcriptional level, at least in some organisms. Not only can siRNAs induce sequence-specific promoter methylation in plants (Aufsatz et al. 2002; Mette et al., 2000) but they are also crucial for heterochromatin formation in fission yeast (Grewal et al, 2003; Volpe et al. 2002) and transposon silencing in worms (Ketting et al, 1999; Chen, 2004). Fundamentally, siRNAs and miRNAs are similar in terms of their molecular characteristics, biogenesis and effector functions. So, the current distinctions between these two species might be arbitrary, and might simply reflect the different paths through which they were originally discovered. miRNAs and siRNAs share a common RNase-III processing enzyme, Dicer, and closely related effector complexes, RISCs, for post-transcriptional repression (Fig. 2). In fact, much of our current knowledge of the biochemistry of miRNAs stems from what we know about siRNAs and the RNAi pathway. On the other hand, miRNAs differ from siRNAs in their molecular origins and, in many of the cases that have been characterized so far, in their mode of target recognition. miRNAs are

produced as a distinct species from a specific precursor that is encoded in the genome. The structure of the primary miRNA transcript and the recognition of this precursor by a nuclear processing machinery probably determines the sequence and structure of mature miRNAs (Bartel, 2004). Conversely, siRNAs are sampled more randomly from long dsRNAs that can be introduced exogenously or produced from bi-directionally transcribed endogenous RNAs that anneal to form dsRNA (Hannon, 2002). In many cases, miRNAs bind to the target 3' UTRs through imperfect complementarity at multiple sites, and therefore negatively regulate target expression at the translational level. Conversely, siRNAs often form a perfect duplex with their targets at only one site, and therefore direct the cleavage of the target mRNAs at the site of complementarity (Fig. 2).

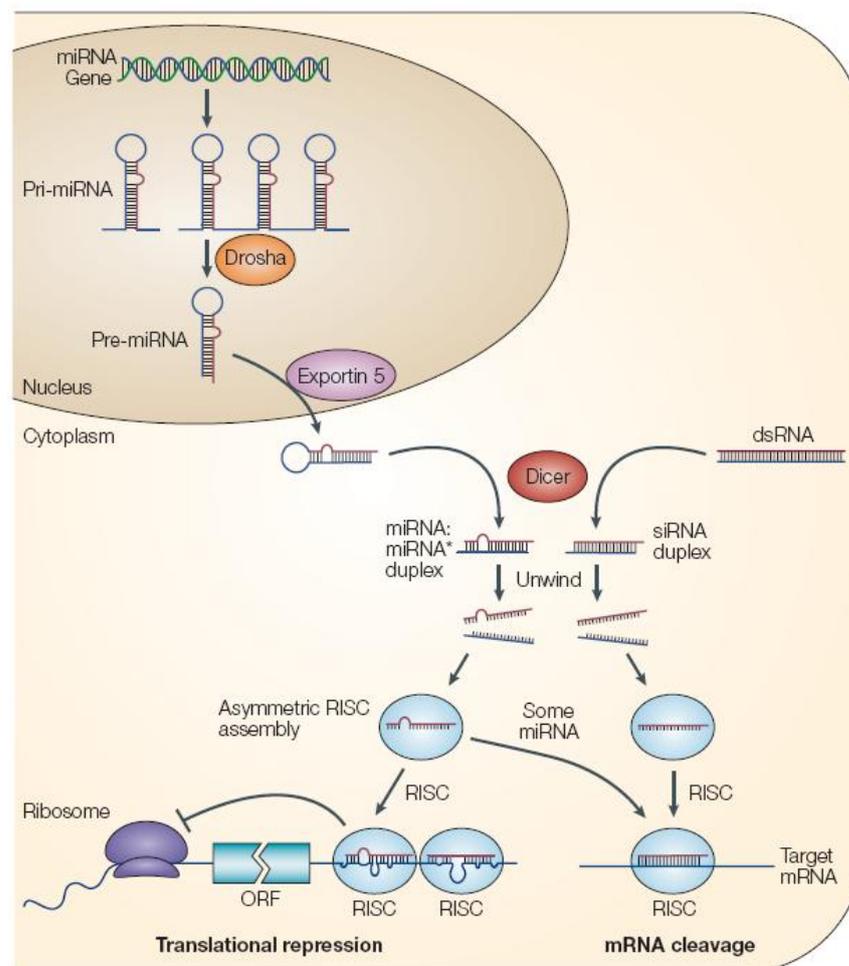


Figure 2. The current model for the biogenesis and post-transcriptional suppression of microRNAs and small interfering RNAs (He et al., 2004).

1.3 Biogenesis of microRNA

Early annotation of the genomic position of miRNAs indicated that most miRNAs are located in intergenic regions (>1 kb away from annotated/predicted genes), although a sizeable minority was found in the intronic regions of known genes in the sense or anti-sense orientation (Lau et al., 2001; Lagos-Quintana et al., 2001). This led to the postulation that most miRNA genes are transcribed as autonomous transcription units. Another interesting observation was that ~50% of known miRNAs are found in close proximity to other miRNAs (Lau et al., 2001; Lagos-Quintana et al., 2001; Mourelatos et al., 2002), which raised the possibility that these clustered miRNAs might be transcribed from a single *polycistronic transcription unit*. A detailed analysis of miRNA gene expression showed that miRNA genes can be transcribed from their own promoters (Lee et al., 2004; Cai et al., 2004), and that the clustered miRNAs are generated as polycistronic primary transcripts (pri-miRNAs) (Lee et al., 2002).

A recent analysis showed that miRNAs can be categorized into three groups according to their genomic locations relative to their positions in an exon or intron. First, exonic miRNA in non-coding transcription units; second, intronic miRNA in non-coding transcription units; and third, intronic miRNA in protein-coding transcription units. Mixed miRNA genes can be assigned to one of the above groups depending on the given splicing pattern (Rodriguez et al, 2004).

miRNA genes are transcribed by either RNA polymerase II or RNA polymerase III into primary miRNA transcripts (pri-miRNA) (Lee et al., 2004; Cai et al., 2004; Borchert et al., 2006).

This primary transcripts that encode miRNAs (pri-miRNAs) contain 5' cap structure as well as 3' poly(A) tails (Lee et al., 2004; Cai et al., 2004).

The sequential cleavages of miRNA maturation are catalysed by two RNase-III enzymes, Drosha and Dicer (Lee et al., 2003; Hutvagner et al., 2001). Both are dsRNA-specific endonucleases that generate 2-nucleotide-long 3' overhangs at the cleavage site.

Drosha form a nuclear microprocessor complex with DGCR8 protein (DiGeorge critical region 8) (also known as Pasha in *D. melanogaster* and *C. elegans*) (Lee et

al., 2003). This complex is predominantly localized in the nucleus and contains two tandem RNase-III domains, a dsRNA binding domain and an amino-terminal segment of unknown function (Lee et al., 2003).

Regardless of the diverse primary sequences and structures of pri-miRNAs, Drosha cleaves these into ~70-bp pre-miRNAs that consist of an imperfect stem-loop structure (Fig. 3) (Lee et al., 2003). Although the precise mechanisms that Drosha uses to discriminate miRNA precursors remain unknown, several studies have addressed the features of pri-miRNAs that contribute to Drosha cleavage both in vitro and in vivo (Lee et al., 2003; Zeng et al., 2003). The efficiency of Drosha processing depends on the terminal loop size, the stem structure and the flanking sequence of the Drosha cleavage site, because shortening of the terminal loop, disruption of complementarity within the stem and removal or mutation of sequences that flank the Drosha cleavage site significantly decrease, if not abolish, the Drosha processing of pri-miRNAs (Lee et al., 2003; Zeng et al., 2003).

Drosha-mediated processing of pri-miRNAs into pre-miRNAs is not obligatory. Intron-derived miRNAs are released from their host transcripts after splicing (Fig. 3). If the intron resulting from the action of the splicing machinery has the appropriate size to form a hairpin resembling a pre-miRNA, it bypasses Drosha cleavage and is further processed in the cytoplasm by Dicer. These miRNAs, called *mirtrons*, have been discovered in several species including mammals, *D. melanogaster* and *C. elegans* (Okamura et al., 2007; Ruby et al., 2007; Berezikov et al., 2007).

After the initial process, pre-miRNAs are exported from the nucleus into the cytoplasm by Exportin 5 (Exp5), a Ran-GTP dependent nucleo/cytoplasmic cargo transporter (Fig. 3) (Lund et al., 2004). Once inside the cytoplasm, these hairpin precursors are cleaved by Dicer into a small, imperfect dsRNA duplex (miRNA: miRNA*) that contains both the mature miRNA strand and its complementary strand (miRNA*) (Hutvagner et al., 2001; Grishok et al. 2001; Ketting et al., 2001). Dicer contains a putative helicase domain, a DUF283 domain, a PAZ (Piwi–Argonaute–Zwille) domain, two tandem RNase-III domains and a dsRNA-binding domain (dsRBD) (Bernstein et al., 2001).

Following their processing, miRNAs are assembled into ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes

(miRISCs). The assembly is a dynamic process, usually coupled with pre-miRNA processing by Dicer.

The target specificity, and probably also the functional efficiency, of a miRNA requires that the mature miRNA strand from the miRNA:miRNA* duplex is selectively incorporated into the RISC for target recognition (Schwarz et al., 2003; Khvorova et al., 2003). The miRNA* strand, on the other hand, is probably degraded rapidly on its exclusion from the RISC, as the recovery rate of miRNA*s from endogenous tissues is ~100-fold lower than that of miRNAs (Schwarz et al., 2003). As Dicer processes the pre-miRNA into the miRNA:miRNA* duplex, the stability of the 5' ends of the two arms of the miRNA:miRNA* duplex is usually different. Although mature miRNAs can reside on either strand of the hairpin stem, it is almost always derived from the strand with the less stable 5' end compared with the miRNA* strand (Schwarz et al., 2003; Khvorova et al., 2003). These findings indicate that the relative instability at the 5' end of the mature miRNA might facilitate its preferential incorporation into the RISC. The selective assembly of the mature miRNA into the RISC probably reflects the relative ease of unwinding from one end of the miRNA: miRNA* duplex (Fig. 3) (Schwarz et al., 2003; Khvorova et al., 2003). Therefore, the thermodynamic properties of the miRNA precursor determine the asymmetrical RISC assembly, and therefore, the target specificity for posttranscriptional inhibition. However, in rare cases in which miRNA and miRNA* have similar 5'-end stability, each arm of the miRNA precursor is predicted to be assembled into the RISC at similar frequencies. This prediction has been confirmed by similar recovery rates for such miRNAs and miRNA*s from endogenous tissues (Schwarz et al., 2003). This thermodynamic model also applies to the asymmetrical assembly of the siRNA duplex, in which the strand of siRNA with the less stable 5' end is preferentially assembled into the RISC complex to target mRNA cleavage (Schwarz et al., 2003; Khvorova et al., 2003).

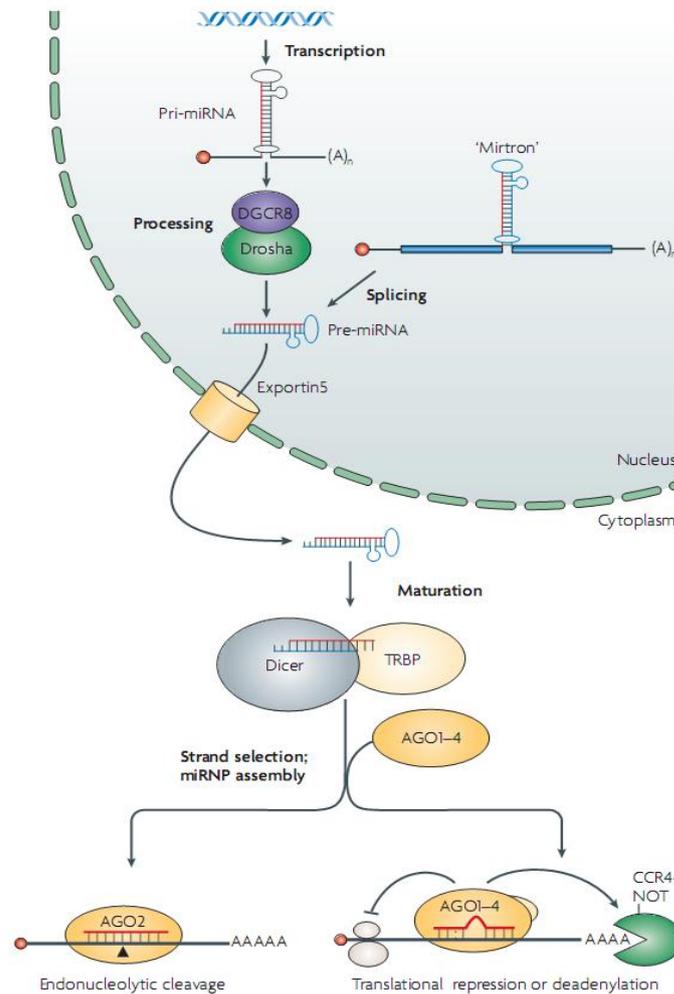


Figure 3. Biogenesis of miRNAs and their assembly into micro-ribonucleo-proteins complex (miRNPs) (Filipowicz et al. 2008).

1.4 Mechanism: post-transcriptional repression by miRNA

MicroRNAs function as negative gene regulators in association with ribonucleoprotein (RNP) complexes or RISCs (induced silencing complexes).

The most important and best-characterized components of miRNPs are proteins of the Argonaute family. Mammals contain four Argonaute (AGO) proteins, AGO1 to AGO4. AGO2 is the only AGO that functions in RNAi because it can cleave mRNA at the centre of the siRNA-mRNA duplex. Apart from the AGO proteins, miRNPs

often include other proteins, which probably function as miRNP assembly or regulatory factors, or as effectors mediating the repressive miRNP functions (Peters et al., 2007; Tolia et al., 2007).

Today, the molecular mechanism by which miRNP complexes function as repressors of protein synthesis are not clear. However, several recently published papers provide important mechanistic insights into the miRNP complex repression at the different mRNA translational steps (Filipowicz et al., 2008).

mRNA translation can be divided into three steps: initiation, elongation and termination.

Initiation starts with the recognition of the mRNA 5'-terminal cap structure (m^7GpppN in which N is any nucleotide) by the eIF4e subunit of the eukaryotic translation initiation factor (eIF) eIF4F, which also contains eIF4G, an important scaffold for the assembly of the ribosome initiation complex. Interaction of eIF4G with another initiation factor, eIF3, facilitates the recruitment of the 40S ribosomal subunit (Merrick et al. 2004; Kapp et al., 2004). eIF4G also interacts with the polyadenylate-binding protein 1 (PABP1). The ability of eIF4G to interact simultaneously with eIF4e and PABP1 brings the two ends of the mRNA in close proximity (Wells et al., 1998, Derry et al., 2006). This 'circularization' stimulates translation initiation by increasing the affinity of eIF4e for m^7GpppN , and might facilitate ribosome recycling (Derry et al., 2006). Some cellular and viral mRNAs initiate translation independently of the m^7G cap and eIF4e; in this case, 40S ribosomes are recruited to the mRNA through interaction with an internal ribosome entry site (IRES) (Jackson et al., 2005). Joining of the 60S subunit at the AUG codon precedes the elongation phase of translation.

The investigations revealed that the translation of m^7G -capped mRNAs, but not of mRNAs containing an IRES, is repressed by miRNAs (Pillai et al., 2005; Humphreys et al., 2005).

There is substantial evidence that factors bound at the 3' UTR exert their inhibitory effect on translational initiation by recruiting proteins that either interfere with the eIF4E–eIF4G interaction or bind directly to the cap but, unlike eIF4e, are unable to associate with eIF4G and promote assembly of the 40S initiation complex (Chekulaeva et al., 2006; Richter et al., 2005; Cho et al., 2005). Kiriakidou et al.

recently reported that the central domain of AGO proteins contains limited sequence homology to the cap-binding region of eIF4e. These data indicate that AGO2 and related proteins can compete with eIF4e for m⁷G binding and thus prevent translation of capped, but not IRES-containing, mRNAs (Kiriakidou et al., 2007).

An alternative mechanism of miRNA action was recently proposed by Chendrimada et al. The authors reported that eIF6 and 60S ribosomal subunit proteins co-immunoprecipitate with the mRNPs complex. eIF6 was first described as a protein that binds the 60S subunit to prevent its precocious interaction with the 40S subunit (Russell et al., 1978), and was thought to act as an initiation factor. However, it was shown later that eIF6 is not involved in translation in yeast, but rather has a crucial function (both in yeast and mammals) in the biogenesis of the 60S subunit in the nucleolus, and accompanies the 60S subunit to the cytoplasm (Sanvito et al., 1999; Si et al., 1999; Basu et al., 2001). Chendrimada et al. showed that partial depletion of eIF6 in either human cells or *C. elegans* rescues mRNA targets from miRNA inhibition, possibly by reducing eIF6-mediated impediment of 60S joining (Chendrimada et al., 2007).

Although initial studies suggested that the levels of miRNA-inhibited mRNAs remain mostly unchanged, more recent work has demonstrated that the repression of many miRNA targets is frequently associated with their destabilization (Wu et al., 2006; Bagga et al., 2005; Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu et al., 2005). In eukaryotes, mRNA degradation can follow two pathways, each of which is initiated by a gradual shortening of the mRNA poly(A) tail. The mRNA body can then be degraded by progressive 3'→5' decay, which is catalysed by the exosome, or by the removal of the cap followed by 5'→3' degradation, which is catalysed by the exonuclease. Levels of mRNA are controlled by mRNPs through the recruitment of decay machinery components, leading to mRNA deadenylation and decapping. The degradation, or at least its final steps, is thought to occur in P-bodies, cellular structures that are enriched in mRNA-catabolizing enzymes and translational repressors (Eulalio et al., 2007; Parker et al., 2007). The mechanism of miRNA-mediated mRNA destabilization is best understood in *D. melanogaster*. Studies in *D. melanogaster* S2 cells demonstrated that the P-body protein GW182 (protein that recruits the CCR4–NOT deadenylation complex to the miRNA-bound mRNA),

which interacts with the miRNP Argonaute1, is a key factor that marks mRNAs for decay (Behm-Ansmant et al., 2006).

On the basis of many lines of evidence, it is widely believed that miRNAs suppress protein synthesis by different mechanism.

1.5 Target recognition: interaction miRNA-mRNA

MicroRNAs interact with their mRNA targets by base pairing. In plants, most miRNAs base pair to mRNAs with nearly perfect complementarity and induce mRNA degradation by an RNAi-like mechanism: the mRNA is cleaved endonucleolytically in the middle of the miRNA- mRNA duplex (Jones-Rhoades et al., 2006). By contrast, with few exceptions, mammalian miRNAs base pair with their targets imperfectly, following a set of rules that have been identified by experimental and bioinformatics analyses (Doench et al., 2004; Brennecke et al., 2005; Lewis et al., 2005; Grimson et al., 2007; Nielsen et al., 2007).

One rule for miRNA–target base pairing is perfect and contiguous base pairing of miRNA nucleotides 2 to 8, representing the ‘seed’ region (Fig. 4, shown in dark red and green), which nucleates the miRNA–mRNA association. G:U pairs or mismatches and bulges in the seed region greatly affect repression. However, an A residue across position 1 of the miRNA, and an A or U across position 9 (Fig. 4, shown in yellow), improve the site efficiency, although they do not need to base pair with miRNA nucleotides.

Another rule is that bulges or mismatches must be present in the central region of the miRNA–mRNA duplex, precluding the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA.

The third rule is that there must be reasonable complementarity to the miRNA 3’ half to stabilize the interaction. Mismatches and bulges are generally tolerated in this region, although good base pairing, particularly to residues 13–16 of the miRNA (Fig. 4, shown in orange), becomes important when matching in the seed region is suboptimal (Brennecke et al., 2005; Lewis et al., 2005; Grimson et al., 2007).

Other factors that can improve site efficacy include an AU-rich neighborhood and, for long 3’ UTRs, a position that is not too far away from the poly(A) tail or the termination codon; these factors can make the 3’ UTR regions less structured and

hence more accessible to miRNP recognition (Grimson et al., 2007; Nielsen et al., 2007; Gaidatzis et al., 2007). Indeed, accessibility of binding sites might have an important effect on miRNA-mediated repression (Kertesz et al., 2007). Some experimentally characterized sites deviate significantly from these rules and can, for example, even require a bulged nucleotide in the seed region pairing. In addition, combinations of sites can require a specific configuration (for example, separation by a stretch of nucleotides of specific sequence and length) for efficient repression (Velle et al., 2004). Usually, miRNA-binding sites in mammalian mRNAs lie in the 3' UTR and are present in multiple copies. Importantly, multiple sites for the same or different miRNAs are generally required for effective repression. When they are present close to each other (10–40 nucleotides apart) they tend to act cooperatively, that is, their effect exceeds that expected from the independent contributions of two single sites (Doench et al., 2004; Brennecke et al., 2005; Lewis et al., 2005; Grimson et al., 2007; Nielsen et al., 2007).

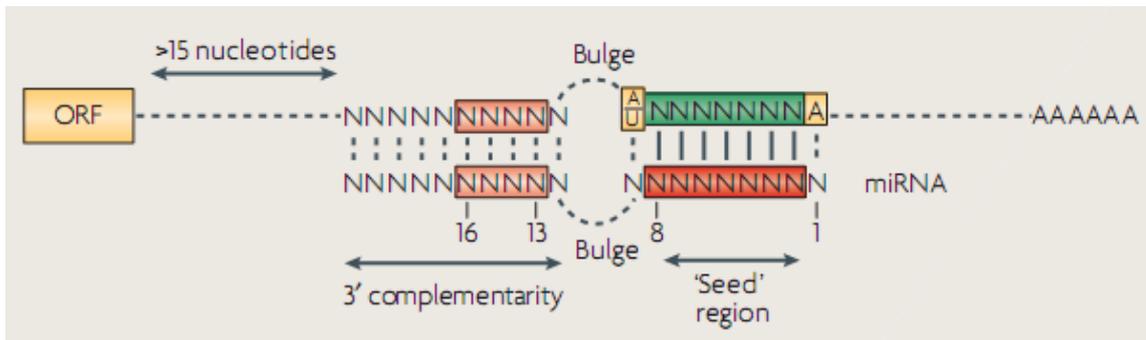


Figure 4. Principles of microRNA–mRNA interactions (Filipowicz et al. 2008).

2. MicroRNAs and Cancer

The 'classic' view of molecular oncology indicates that cancer is a genetic disease involving tumor suppressor and oncogenic proteins. However, in the recent years, it has been demonstrated that miRNAs are involved in human tumorigenesis, thus revealing a new layer in the molecular architecture of human cancer. Gene expression studies revealed that hundreds of miRNAs are deregulated in cancer cells and functional studies clarified that miRNAs are involved in all the molecular and biological processes that drive tumorigenesis (Negrini et al, 2009).

The mechanisms by which miRNA expression is altered in cancer are multifaceted. The function of miRNAs in cancer seems to be disrupted by the same mechanisms as those that affect the expression of protein-encoding genes (amplifications, translocations, deletions, and point mutations of the pri-miRNA-encoding DNA sequence) and by epigenetic disruption of miRNA transcription (Calin et al., 2006).

Abnormal miRNA function in cancer can be explained by different mechanisms: mutation in miRNAs, epigenetic regulation of miRNA expression, miRNAs localization at translocation breakpoint, change in the miRNAs processing apparatus, sequence variation in the miRNA-mRNA binding sites.

A genome-wide study proved that half of the annotated human miRNAs are located in cancer-associated-genomic-regions (CAGRS). Such regions include minimal regions of LOH (loss-of-heterozygosity), which are thought to harbour tumour-suppressor genes, minimal regions of amplification, which might contain oncogenes, common breakpoint regions in or near possible oncogenes or tumour-suppressor genes and fragile sites (FRA). FRA are preferential sites of sister-chromatid exchange, translocation, deletion, amplification or integration of plasmid DNA and tumour-associated viruses such as human papilloma virus (HPV) (Calin et al., 2004).

An example of miRNAs that are located in chromosomal regions showing LOH in cancer, are miR-15 and -16 in CLL. A germ-line mutation was observed in the miR-16-1-miR-15a pri-miRNA, and the presence of this mutation correlated with low miRNA expression and deletion of the second allele. The exact mechanism whereby this is mediated is unclear; however, it was suggested that the mutations affect the miRNA hairpin formation. Many (73%) of the patients with mutant miRNAs had a

family history of CLL or other cancers, this led to the hypothesis that miRNA gene mutations might play a major role in cancer (Diederichs et al., 2006).

DNA hypomethylation, CpG island hypermethylation and histone-modification losses represent epigenetic markers of malignant transformation (Fraga et al., 2005). Several groups have investigated whether such events also affect miRNA expression. Furthermore, Saito *et al.* found that the combined treatment of human bladder cancer cells with 5-aza-2'-deoxycytidine (5-Aza-CdR) and the histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (PBA) has a significant effect on the expression of miRNAs (Saito et al., 2006). Seventeen miRNAs (out of 313 screened by a microarray assay) were upregulated more than threefold, and miR-127 was the most differentially expressed. This miRNA is located in a CpG island at chromosome 14q32, a region that is involved in several types of translocations identified in haematological cancers and deleted by LOH in solid tumours (Calin et al., 2004). Furthermore, the combined treatment was accompanied by a decrease in DNA methylation and an increase in active histone markers around the transcription start site of miR-127. The investigators showed that miR-127 can repress the transcription of the zinc-finger repressor BCL6. BCL6 is a proto-oncogene upregulated in a large proportion of B-cell lymphomas, where it acts as a transcriptional suppressor of TP53 and downstream effectors of TP53. The induction of miR-127 by 5-Aza-CdR and PBA treatment in cancer cells might have an anticancer effect by down-regulating anti-apoptotic factors, such as BCL6, and inducing apoptosis (Saito et al., 2006). In another study a number of miRNAs are upregulated in the DNMT1/DNMT3b (DNA methyltransferase 1 and 3b) double knockout (DKO) cells. The miR-124a was selected from this panel, and further studies showed that it was inactivated by promoter methylation in a variety of tumors, including colon-, breast-, lung-, and hematopoietic cancers. One oncogenic target of this miRNA is the cell cycle regulator cyclin-dependent kinase 6 (CDK6), which accelerates cell cycle progression through the G1/S checkpoint by phosphorylating Rb. Indeed, hypermethylation of miR-124a correlated with CDK6 activation and Rb phosphorylation in this study (Lujambio et al., 2007).

Several observations document the presence of miRNAs at chromosomal breakpoints, suggesting their role as translocation partners. The classical example is

the translocation of the miR-142 to the MYC oncogene in the t(8;17). In analogy to the translocations of MYC to the immunoglobulin gene locus, this translocation brings MYC under the control of the miR-142 gene promoter, which leads to its upregulation in aggressive B-cell lymphoma (Lagos-Quintana et al., 2002). Other indications of a role for miRNAs in translocations include the observation that the pri-miR-122a gene is located at chromosome 18 near the MALT1 gene, which is involved in translocations of the majority of mucosa-associated lymphoid tissue (Calin et al., 2004). Furthermore, insertion of the miR-125b into the rearranged immunoglobulin heavy (IgH) chain gene locus, has been demonstrated in a case of precursor B-cell acute lymphoblastic leukemia. Since most translocations in B-cell tumors involve the IgH-locus, this observation may further support a role for miRNAs at translocation breakpoints (Sonoki T et al., 2005).

The protein machinery that is involved in the biogenesis of miRNAs is complex, alterations of these proteins should have dramatic effects on miRNA expression. In fact, expression of Dicer has been shown to be down-regulated in lung cancer. Karube et al. examined RNA expression levels of DICER and DROSHA in 67 non-small-cell lung cancer samples. They found that reduced expression of DICER correlates with shortened post-operative survival, which indicates that Dicer might be able to prevent the transformation of lung tissue. As Dicer has been implicated in heterochromatin maintenance and centromeric silencing, reduced protein levels might directly result in genomic instability and lead to tumour formation (Karube et al, 2005; Fukagawa et al., 2004).

Finally, inappropriate base pairing due to variations in the 3'-UTR sequence of the target mRNAs or in the mature miRNA sequence may be another mechanism of miRNA functional disruption in cancer. Tumors may evade growth inhibition by tumor-suppressor-miRNAs if they do not bind properly to their target oncogenic mRNA sequence, or alternatively, tumor growth may be enhanced by sequence variations that promote the binding properties of oncomiRs to tumor suppressor mRNAs. Loss of the KIT protein in thyroid cancers has been associated with high expression of the miRs -221, -222, and -146b, and polymorphic changes in 3'-UTR of the KIT-mRNA were demonstrated in half of these cases (He et al., 2005). Owing to the high incidence of familial thyroid cancer, it was speculated whether these

polymorphisms might predispose to this disease. Polymorphic changes in the mature miRNA sequences have also been observed. For example, a sequence variation that may alter the target selection has been identified in the mature miR-30c-2, which is overexpressed in many solid tumors (Iwai et al., 2005).

2.1 Onco-miRNA and tumor-suppressor-miRNA.

A number of miRNAs have been shown to promote cell proliferation and survival, while others diminish cell proliferation and survival. These two classes of miRNAs may play a central role in cancer development as novel oncogenes and tumor suppressors, respectively. In normal tissues, some of these miRNAs have been documented to inhibit the translation of proto-oncogenes by targeting the 3'UTR of their mRNAs. Such miRNAs are therefore considered as “tumor suppressor miRNAs” since their normal function is to control the expression of an oncogene. Conversely, certain miRNAs seem to be upregulated in cancer and may act as “oncomiRs”, as they can enable the downregulation of a tumor suppressor. Since miRNAs have several potential targets that may be the mRNAs of both oncogenes and tumor suppressors, the actual function of a particular miRNA as either tumor suppressor miRNA or onco-miR may depend on the cellular context (Esquela-Kerscher et al., 2006).

The first indication that miRNAs could function as tumour suppressors came from a report by Calin et al. that showed that patients who were diagnosed with a common form of adult leukaemia, B-cell chronic lymphocytic leukemia (CLL), often have deletions or downregulation of two clustered miRNA genes, mir-15a and mir-16-1 (Calin et al., 2002). Deletions within the 13q14 locus occur in more than 65% of CLL cases, as well as in 50% of mantle cell lymphomas, 16–40% of multiple myelomas and 60% of prostate cancers. Therefore, it was predicted that a tumour-suppressor gene must reside in this 30-kb region. Interestingly, mir-15a and mir-16-1 map within the intron of a non-protein-coding RNA gene of unknown function, which is called LEU2. Subsequently, a report by Cimmino et al. showed that miR-15a and miR-16-1 negatively regulate BCL2, an anti-apoptotic gene that is often overexpressed in many types of human cancers, including leukaemias and

lymphomas (Cimmino et al., 2005). Therefore, it is thought that the deletion or downregulation of mir-15a and mir-16-1 results in increased expression of BCL2, promoting leukaemogenesis and lymphomagenesis in haematopoietic cells. The tumour-suppressor role of miR-16 was further supported by the finding that two patients with CLL carried a germline C to T mutation 7 base-pairs downstream of the mir-16-1 precursor, resulting in decreased miR-16-1 expression levels (Calin et al., 2005).

Another early and well-documented finding was the down-regulation of oncogenic Ras by the let-7 family members of miRNAs in lung cancer (Johnson et al., 2005). Ras proteins are membrane-associated GTPase signalling proteins that regulate cellular growth and differentiation. About 15–30% of human tumours possess mutations in Ras genes and activating mutations that result in the increased expression of Ras cause cellular transformation. The miRNAs that are encoded by the let-7 family, which include 12 human homologues, were implicated as tumour suppressors because they map to fragile sites associated with lung, breast, urothelial and cervical cancers (Calin et al., 2004). More direct evidence, presented by Takamizawa et al., showed that transcripts of certain let-7 homologues were significantly downregulated in human lung cancer and that this correlated with a poor prognosis (Takamizawa et al., 2004). These studies further indicate that let-7 could be used diagnostically, as patients with non-small-cell lung carcinomas who expressed lower levels of let-7 had a poorer prognosis and shortened post-operative survival. Successive study found that let-7 negatively regulates Ras in human cells. In fact, overexpression of let-7 in human cancer cell lines results in decreased levels of Ras compared with untreated cells. Conversely, the depletion of RNA levels of let-7 in human cancer cell lines that normally express high levels leads to a marked increase in Ras protein expression. let-7 directly controls Ras expression through 3'-UTR mediated repression in human cells (Johnson et al., 2005; Esquela-Kerscher et al., 2008).

Several studies have implicated other miRNAs in tumorigenesis. miR-143 and miR-145 have been shown to be constantly down-regulated in colorectal tumors (Michael et al., 2003) and breast cancer (Iorio et al., 2005). Moreover, the location of these miRNAs is known to be in a genomic locus frequently

deleted in myelodysplastic syndrome (Calin et al, 2004). Some studies have ascribed the down-regulation of miR-143 and miR-145 in colon cancer to a block in Dicer processing (Michael et al., 2003).

In contrast to tumor-suppressor-miRNAs, oncomiRs are frequently upregulated in cancers and show proliferative and anti-apoptotic activity. One of the first oncomiRs to be identified was miR-155, which is co-expressed with the non-protein-coding gene BIC. This gene was initially identified as a common integration site for the avian leukosis virus and was found to be overexpressed in B-cell lymphomas (Clurman et al. 1989; Tam et al., 1997). For years, researchers were puzzled as to how an RNA that was presumably not protein-coding, and was poorly conserved between avian, mouse and human genomes, could promote lymphomas in cells that also overexpressed MYC. Metzler et al. showed that miR-155 expression is upregulated 100-fold in pediatric Burkitt lymphoma, and other studies showed increased miR-155 levels in Hodgkin lymphoma and in primary mediastinal and diffuse large-B-cell lymphomas with activated rather than germinal-centre phenotypes (Metzler et al., 2004; Eis et al., 2005). Therefore, it seems that mir-155 can function as an oncogene in cooperation with MYC, whereas it normally functions during B-cell selection and possibly targets genes that antagonize the MYC pathway. The members of the miR-17-92 cluster represent another intensely studied group of potential oncomiRs that are frequently upregulated in lymphomas. This cluster consists of seven individual miRNAs: miR-17-5p, 17-3p, -18, -19a,-19b1, -20, and 92, which are all encoded from a frequently amplified locus at 13q31.3 (Hayashita et al., 2005; Ota et al., 2004). It was shown in MYC transgenic mice that the miR-17-92 cluster overexpression, but not the individual miRNAs, could enhance tumorigenesis by inhibiting apoptosis, as compared with the latent malignancies that arose from increased MYC expression levels alone. (He et al, 2005). Further studies in human cell lines showed that transcription of the miR-17-92 cluster was directly regulated by c-Myc, and that the individual miRs -17-5p and -20 regulate the translation of E2F1, a transcription factor with both pro-apoptotic and pro-proliferative activity. Thus, co-expression of c-Myc and miR-17 is believed to fine tune E2F1 activity so that proliferation is enhanced and apoptosis is inhibited (O'Donnell et al., 2005).

Anti-apoptotic activity has also been documented to be a feature of miR-21, one of the most frequently upregulated miRNAs in solid tumors (glioblastoma, breast, colon, lung, pancreas, prostate and stomach) (Chan et al., 2005; Volinia et al., 2006). As recently shown, miRNA-21 also participates in RAS oncogenic signaling. miR-21 is transcriptionally induced by AP1 downstream of RAS and exerts its oncogenic effect by keeping in check PTEN and PDCD4 (Si et al. 2007; Talotta et al., 2009). Finally, various studies have reported that miRNAs are also aberrantly expressed in brain tumors. During the analysis of a global expression level of 245 miRNAs in glioblastoma multiforme, Ciafre et al. (2005) observed that miR-221 was highly upregulated. In a recent work, Gillies and Lorimer (2007) have reported that miR-221 and miR-222 directly target p27^{Kip1}, a key negative regulator of the cell cycle, in glioblastoma.

2.2 miRNA profiling in cancer

Multiple lines of evidence indicating that miRNAs are differentially expressed in normal and in tumor samples suggesting that miRNAs could serve as useful tumor profiling tools. Indeed, profiling studies have shown that each cancer type possesses a distinct miRNA expression signature, and this signature can provide useful information about the malignancy. The adaptation of high-throughput technologies have facilitated the study of the expression of multiple miRNAs in a given sample, making it possible to profile substantial sample numbers with relative ease. Among the most important methods for miRNA profiling is the use of oligonucleotide miRNA microarray analysis, which allows the researcher to simultaneously determine the expression levels of hundreds of miRNAs in a given sample (Kim et al., 2006; Liu et al., 2004; Hammond et al., 2006). Another method to determine miRNA expression levels involves the use of a bead-based flow-cytometric technique (Lu et al., 2005). Other developments include quantitative real-time PCR for precursor miRNAs (Schmittgen et al. 2004) or active miRNAs (Chen et al. 2005; Raymond et al., 2005), miRAGE (a genome-wide miRNA analysis with serial analysis of gene expression SAGE) (Cummins et al. 2006) or the high-throughput array-based Klenow enzyme (RAKE) assay (Nelson et al., 2004). Taking advantage

of these tools, several groups have studied the miRNA expression of several types of cancer.

Using a bead-based miRNA microarray assay, Lu et al. conducted the expression analysis of 217 miRNAs in a panel of 334 samples that included primary tumors, tumor-derived cell lines and normal tissues. The investigators found that miRNA profiles can distinguish between normal and cancer tissues, separate different cancer types, stratify the cancer differentiation state and cluster sample groups according to their embryonic lineage. Moreover, miRNA profiles were more accurate than mRNA profiles in classifying tumor types and were able to categorize 17 poorly differentiated tumors where histological appearance was not diagnostic into their specific tissue lineages (Lu et al., 2005).

Studying colorectal cancer, Michael et al. were the first to recognize aberrant miRNA expression in solid tumors as the investigators identified 28 different miRNAs in colonic adenocarcinoma and normal mucosa, and found that miR-143 and miR-145 were consistently downregulated in cancer (Michael et al., 2003). Bloomston et al. showed that a signature of 21 upregulated and 4 downregulated miRNAs correctly differentiated pancreatic cancer from benign pancreatic tissue in 90% of samples. In the profile, two commonly malignancy associated miRNAs, miR-21 and miR-155, were uniquely overexpressed in pancreatic cancer compared to normal pancreas (Bloomston et al., 2007).

Of significant importance is a study performed by Rosenfeld et al., which needed only 48 miRNA markers to identify 22 tissue origins with an accuracy of 90% (Rosenfeld et al., 2008). This work holds promise for the 3–5% of patients from all new cancer cases diagnosed with metastatic cancer of unknown primary origin (Pimiento et al., 2007). Enabling oncologists to identify the tissue of origin will markedly improve treatment decision and prognosis.

Recently, a very new and exciting venue of potential cancer diagnosis has emerged through the detection of miRNAs in serum. In one of the first reports of this method, Lawrie et al. showed that sera levels of miR-21 were associated with relapse-free survival in patients with diffuse large B-cell lymphoma (Lawrie et al., 2008). In another study, Mitchell et al. showed that miRNAs can enter the circulation even when they originate from an epithelial cancer cell type. The investigators found that

serum levels of miR-141 can distinguish patients with prostate cancer from healthy subjects (Mitchell et al., 2008). Other two groups were able to identify functional miRNAs in circulating tumor exosomes with specific miRNA signature discerning patients with ovarian and lung cancers from healthy controls (Taylor and Gercel-Taylor, 2008; Rabinowits et al., 2009).

One continuing concern for oncologists is giving patients the right prognosis and being able to predict the outcome for certain types of cancer. Several studies profiling the different levels of Dicer and Drosha and miRNAs between normal and tumor tissues were able to shed some light on this problem. A recent study, showed that levels of the miRNA-processing enzymes Drosha and Dicer were able to predict survival in ovarian cancer, with low level of Dicer associated with advanced tumor stage, reduced response to chemotherapy and poor clinical outcome. Low levels of Drosha are associated with suboptimal surgical cytoreduction, a poor prognostic factor. High levels of Dicer and Drosha are associated with increased median survival (Merritt et al., 2008). In a lung cancer outcome study, expression levels of DICER and DROSHA in 67 non-small-cell lung cancer (NSCLC) samples were examined and reduced expression of DICER were correlated with shortened postoperative survival (Karube et al., 2005).

2.3 miRNA in cancer therapy

The revealing role of miRNAs functioning as potential oncogenes and tumor suppressors in tumorigenesis has generated great interest in using them as targets for cancer therapies. In general therapeutic strategies consist of an antisense-mediated inhibition of oncogenic miRNAs and miRNA replacement with miRNA mimetics or viral vector-encoded miRNAs.

Synthetic anti-miRNA oligonucleotides (AMOs) with 2'-O-methyl modification have been shown to be effective inhibitors of endogenous miRNAs in cell culture and xenograft mice models. Application of 2'-O-methyl AMOs targeting the onco-miR-21 potently inhibited glioblastoma and breast cancer cell growth in vitro and tumor growth in an MCF-7 breast cancer xenograft mice model (Chan et al., 2005; Si et al., 2007). As one of the first studies illustrating the utility of AMOs in vivo, modified AMOs conjugated with cholesterol termed 'antagomirs' were

systematically delivered through intravenous injection to target the liver-specific miR-122 (Krutzfeldt et al., 2005). Impressively, a single injection of 240 mg/kg body weight conferred specific miR-122 silencing for up to 23 days. Moreover, as miR-122 was predicted to affect cholesterol biosynthesis (Krutzfeldt et al., 2005; Esau et al., 2006), the serum cholesterol level was seen to decrease by 44% in antagomir-treated mice. As an alternative to 2'-hydroxyl-modified AMOs, lock nucleic acid (LNA)-based oligonucleotides (LNA-antimiR) have been shown to be more stable and less toxic in inhibiting endogenous miRNAs in vivo (Vester and Wengel, 2004; Elmen et al., 2008). In this study, systemic administration by intravenous injections at a dose of 3 or 10 mg/kg LNA-antimiR to African green monkeys with three intravenous infusions over 5 days resulted in the depletion of mature miR-122 and a dose-dependent and long-lasting lowering of plasma cholesterol for up to 3 months (Elmen et al., 2008).

In an alternative strategy to single anti-miRs, Ebert et al. recently developed miRNA inhibitors called 'miRNA sponges', which can be transiently expressed in cultured cells. These molecules are transcripts expressed from strong promoters, containing multiple tandem binding sites to specific miRNAs, and competitively inhibit them (Ebert et al., 2007). Similarly, Gentner et al. recently illustrated that the stable knockdown of miRNA in vivo can be achieved through the construction of lentiviral vectors carrying multiple complementary binding sites for the targeted miRNA acting as an anti-miRNA decoy (Gentner et al., 2009). In this study, the authors retransplanted hematopoietic stem cells carrying lentiviruses expressing the anti-miR-223 decoy into lethally irradiated mice, which resulted in the functional knockdown of miR-223 that phenocopied important aspects of the mouse miR-223 knockout (Johnnidis et al., 2008). One advantage of these systems is that one could construct sponges and anti-miRNA decoys with a combination of miRNA binding sites to potentially inhibit an entire miRNA family or an miRNA cluster.

With anti-miRNA strategies showing great therapeutic promise, the reverse approach of miRNA replacement may be equally attractive for anticancer therapy. To examine the therapeutic potential of let-7 miRNA replacement in NSCLC, Esquela-Kerscher et al., previously examined two murine models of human lung cancer, a xenograft model using human lung cancer cells and a well characterized autochthonous

NSCLC mouse model, LSL-Kras-G12D. In this mouse model, tumorigenesis is initiated by the activation of a gain-of-function Kras G12D gene through the inhalation of adenovirus expressing Cre recombinase (Jackson et al., 2001). Using these models, they showed that let-7 administration suppresses lung tumor initiation, including a 66% reduction of the tumor burden when comparing Kras G12D mice treated with adenovirus expressing let-7 miRNA with those that were treated with a control miRNA (Esquela-Kerscher et al., 2008). Similar findings were made by Jack's lab (Kumar et al., 2008). Since in both studies let-7 miRNA was delivered at the same time as tumorigenesis was initiated, these two studies showed that let-7 can be used as a preventive therapy against lung cancer in the LSL-Kras G12D mice.

Finally, in a very recent work, Mendell and colleagues showed that the systemic delivery of a single miRNA can cause tumors from a mice model of liver cancer to regress (Kota et al., 2009). In this study, Kota et al. delivered adeno-associated virus 8 (AAV8)-expressing miR-26a intravenously through the tail veins of MYC induced mice harboring preformed liver tumors. The authors showed that 3 weeks after AAV8 infection, they observed a significant regression of tumors in mice receiving the miR-26a treatment compared with mice to which the control treatment was given. The result from this study is exciting, as the ability to treat existing tumors closely reflects clinical scenarios. Furthermore, has been demonstrated that a single miRNA is powerful enough to have a dramatic suppression of tumor progression. The use of miRNA therapy to complement traditional anticancer treatments appears to have great potential, as it has been reported by several groups that miRNAs can enhance the response and suppress resistance to anticancer cytotoxic therapies. Meng et al. reported the use of anti-miR-21 and anti-miR-200b AMOs to increase the susceptibility of colangiocarcinoma cells to the chemotherapy drug gemcitabine (Meng et al., 2006). MiR-200c levels were reported to be high in well-differentiated endometrial, breast and ovarian cancer cell lines, but extremely low in poorly differentiated cancer cells and restoration of miR-200c in these cells increases their sensitivity to microtubule-targeting agents by 85% (Cochrane et al., 2009).

3. Colon cancer

Colorectal cancer is the third most common type of cancer and the second leading cause of cancer related death in the Western world, causing 655,000 deaths worldwide per year (Jemal et al, 2006). The incidence of colon cancer is usually increased in developed countries, being seemingly linked with a sedentary lifestyle and a high caloric intake. The occurrence of colon cancer is slightly higher in men than in women and increases with age. Most cases occur between the sixth and the seventh decade of life, while cases before age 50 are uncommon, unless a family history of early colon cancer is present. Colorectal cancer originates from epithelial cells lining the gastrointestinal tract, which undergo sequential mutations in specific DNA sequences that disrupt normal mechanisms of proliferation and self-renewal. Such mutations, which can derive by inborn genetic aberrations, tobacco smoking, environmental carcinogens and chronic inflammatory states, drive the transition from healthy colonic epithelia to colorectal cancer. (Ricci-Vitiani et al., 2008)

The disease begins as a benign adenomatous polyp, which develops into an advanced adenoma with high-grade dysplasia and then progresses to an invasive cancer (Kinzler et al., 2002). Invasive cancers that are confined within the wall of the colon (tumor–node–metastasis stages I and II) are curable, but if untreated, they spread to regional lymph nodes (stage III) and then metastasize to distant sites (stage IV). Stage I and II tumors are curable by surgical excision, and up to 73% of cases of stage III disease are curable by surgery combined with adjuvant chemotherapy. Recent advances in chemotherapy have improved survival, but stage IV disease is usually incurable (Libutti et al., 2008; Compton et al., 2008).

3.1 Colonic crypt organization.

The colon is organized into four histologically distinct layers. The epithelial layer, at the luminal surface, consists of a single sheet of columnar epithelial cells folded into finger-like invaginations that are supported by the lamina propria to form the functional unit of the intestine called crypts of Lieberkühn (Fig. 5a). Approximately 14,000 crypts per square centimeter are located in the adult human colon. Given a rate of 5 days for colonic epithelium renewal, it has been estimated that over 6×10^{14}

colonocytes are produced during the individual lifetime (Potten et al., 1992). The terminally differentiated cells, which are found in the top third of the crypt, are continually extruded into the lumen. They are derived from multipotent stem cells located at the bottom of the crypt. During asymmetric division, these cells undergo self-renewal and generate a population of transit-amplifying cells that, upon migration upward the crypt, proliferate and differentiate into one of the epithelial cell types of the intestinal wall. There are three major terminally differentiated epithelial lineages in the colon: the colonocytes, also termed absorptive enterocytes; the mucus-secreting goblet cells; and the less abundant enteroendocrine cells (Fig. 5b). Finally, Paneth cells, functionally similar to neutrophils, are scattered at the bottom of the crypt only in the small intestine epithelium and do not follow the downward migratory pathway. Transit-amplifying cells and stem cells occupy the lower two thirds of the crypt (Radtke et al., 2005). The maintenance of the stem cell compartment as well as the transition from proliferation to differentiation are finely regulated by Wnt signaling ligands that are thought to be produced by mesenchymal cells of the myofibroblast lineage, closely applied to the basal lamina that surrounds the crypt (Fevr et al., 2007). Other factors, such as the bone morphogenetic protein (BMP) antagonists gremlin 1 (GREM1) and gremlin 2 (GREM2) (Kosinski et al., 2007), Notch signaling pathways, ephrin-B1 (Eph-B1) and its receptors Eph-B2 and Eph-B3, contribute to stem cell behavior, migration, and differentiation (van Es et al., 2005; Batlle et al., 2002).

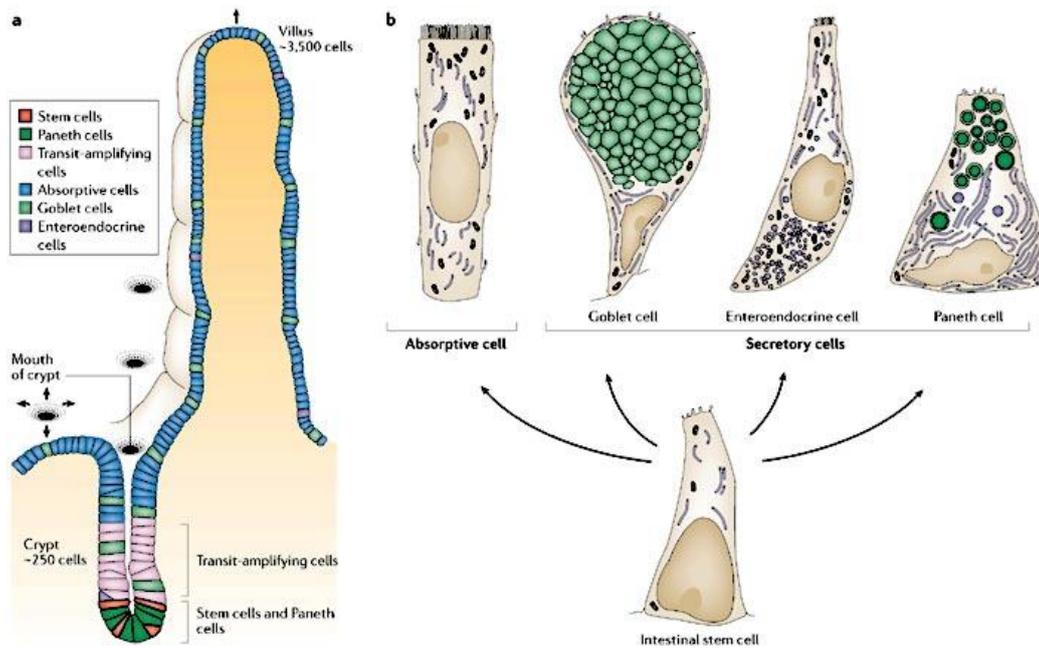


Figure 5. a) Morphological colon crypt organization unit. b) Schematic description of the different cell types in colon epithelium (Crosnier et al., 2006).

3.2 Molecular pathways in colon development

The homeostatic self-renewal of the intestine depends on a complex network of interplay involving many cellular processes, including proliferation, differentiation, migration, and cell death. All these phenomena are finely regulated by a relative small number of evolutionary highly conserved signaling pathways, among which the Wnt signaling cascade plays a major role (Fig. 6). In the so-called canonical Wnt pathway, the central player is the cytoplasmic protein β -catenin. In the absence of the Wnt ligand, free cytoplasmic β -catenin is targeted to degradation by recruitment to a multiprotein degradation complex containing the scaffold protein Axin, the tumor suppressor gene product adenomatous polyposis coli (APC), as well as casein kinase I (CKI) and glycogen synthase kinase 3 β (GSK3 β). Upon sequential phosphorylation of highly conserved serine and threonine residues at the N terminus domain, β -catenin is ubiquitinated and subsequently degraded by the proteasome machinery. In the presence of secreted Wnt ligand, signal occurs via interaction with a serpentine receptor of the frizzled (Fz) family and a member of the low-density lipid receptor family (Lpr5 or Lpr6). Following binding, Axin is recruited to the plasma membrane

through direct interaction with Lpr5/6 or indirectly through an interaction with Fz via disheveled (Dsh). Membrane docking results in Axin degradation and/or dissociation of the “destruction complex.” GSK3 β also might be displaced from this complex through Dsh action. Consequently, β -catenin accumulates in the cytoplasm in a stabilized non-phosphorylated form, enters the nucleus, and binds to a member of the Tcf/Lef family converting the Tcf/Lef transcription factors from transcriptional repressors to activators (Gregorieff et al., 2005). Several genetic studies have indicated the dominant role of the Wnt signaling pathway in the pathophysiology of the intestine. Tcf4 or β -catenin knockout mice fail to develop colonic crypts (Korinek et al., 1998; Pinto et al., 2003), as do transgenic mice for the expression of Wnt-specific secreted inhibitor Dkk-1 in the intestine (Kuhnert et al., 2004).

The molecular description of morphogenetic pathways in the intestine received a great impulse by studies aimed at defining the genetic background of familial syndromes associated with a high risk of colorectal carcinoma, such as Familial Adenomatous Polyposis (FAP), Juvenile Polyposis Syndrome and Cowden Syndrome. FAP results in the formation of multiple bowel adenomas in the second and third decades of life, which almost inevitably progress to colon carcinoma if patients are not subjected to prophylactic colectomy (Lynch et al., 2003). In FAP, approximately 80% of patients display truncating mutations of the APC (Adenomatous Polyposis Coli) gene (Gregorieff et al., 2005).

The complex processes that contribute to intestinal development and are deregulated in tumorigenesis involve the contribution of other pathways acting at the stem cell level to control colon homeostasis. The Wnt cascade interplays with the Notch pathway to maintain undifferentiated, proliferative cells in normal crypts and adenomas (van Es et al., 2005). Another way through which Wnt proteins regulate intestinal cell proliferation is by promoting the expression of B subclass ephrins and their tyrosine kinase receptors. The transition from adenoma to colon carcinoma is associated with loss of ephrin-B receptor expression, and EphB receptors have been recently shown to coordinate migration and proliferation in the intestinal stem cell niche (Holmberg et al., 2006). The study of Juvenile Polyposis Syndrome (JPS) has disclosed another crucial link between molecular genetics and developmental biology, revealing the importance of the SMAD/BMP (Bone Morphogenetic Protein)

pathway in shaping intestinal architecture. JPS is a condition that predisposes to hamartomatous gastrointestinal polyp formation, which can turn into malignant lesions in approximately 20% of cases (Haramis et al, 2004). JPS is due to germline mutations in the SMAD4 gene in 15-20% of cases, and to mutations in the gene encoding bone morphogenic protein receptor 1A (BMPR1A) in 25-40% of cases. Bone morphogenetic protein family ligands are expressed by the villus mesenchyme while epithelial cells display nuclear phosphorylated SMADs, implying these cells as terminal recipients of the signal. By ectopic expression of Noggin, a negative modulator of BMP signalling, it is possible to direct the unregulated formation of new crypts in the intestine, leading to lesions reminiscent of the hamartomatous polyps seen in JPS. This has led to the definition of “landscaper” function for BMPs in the intestine (Fearon et al., 1990) and highlights the concept that alterations in intestinal stem cell microenvironment might be as influential as cell-intrinsic events in normal development and tumorigenicity.

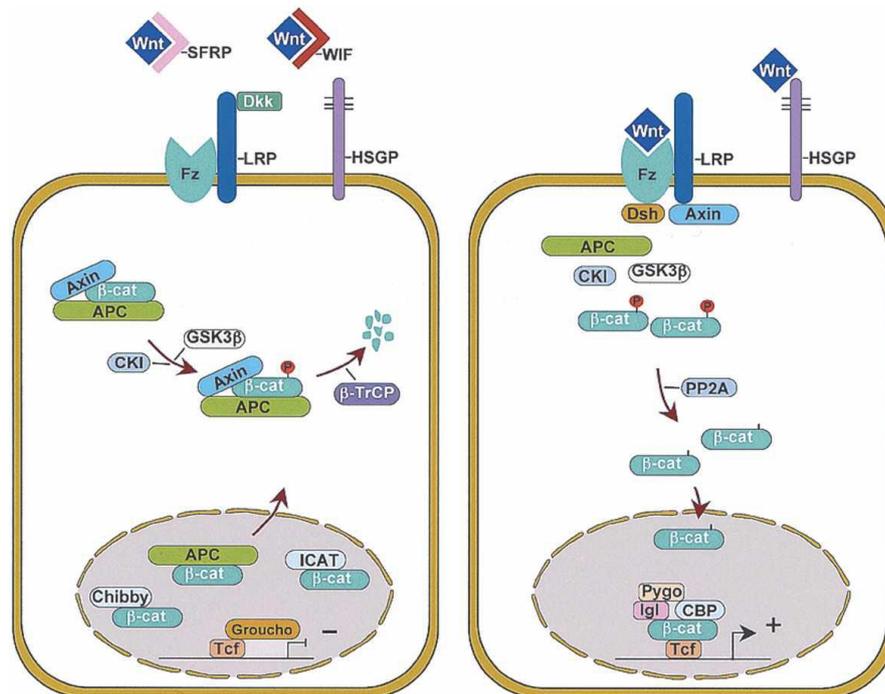


Figure 6. The Wnt canonical pathway. (Left) In the absence of Wnt stimulation, β -catenin levels are kept at a minimum through the destruction complex composed of APC, Axin, GSK3 β , and CKI. In the nucleus, Tcf factors associate with transcriptional repressors to block target gene activation. (Right) In the presence of Wnt stimulation, the destruction complex is destabilized, and β -catenin accumulates in the nucleus to activate transcription of Tcf target genes (Gregorieff et al., 2005).

3.3 Colorectal carcinogenesis

By a molecular point of view, colorectal carcinoma is one of the best-characterized cancer, mainly due to the studies performed on hereditary cases, which account for about 15% of colorectal carcinomas, that have significantly contributed to the understanding of many biological aspects of this neoplasm. The observation that the accumulation of mutations involving oncogenes and tumor suppressor genes follows the progression of the disease along the adenoma–carcinoma sequence induced Fearon and Vogelstein to formulate in the 1990s the “adenoma–carcinoma model” in which the neoplastic process, initiated by APC or β -catenin mutations and tumor progression, results from

the sequential mutation of other genes, such as K-Ras and p53, in the context of a growing genomic instability (Fig. 7) (Fearon et al., 1990). This model has been further refined and the studies performed on relatively rare inherited cases led to the identification of genetic alterations that play a major role in the development of sporadic CRC (Ricci-Vitiani et al., 2009).

Studies on FAP and Hereditary non-polyposis colon cancer syndrome (HNPCC, also known as the Lynch syndrome) allowed the identification of two alternative pathogenic mechanisms for colon tumorigenesis. Work on FAP identified the "gatekeeper" tumour suppressor gene APC as the initial mutation involved in the adenoma-carcinoma progression. The early occurrence of APC mutations during colorectal tumorigenesis was confirmed by the sequence analysis of 41 colorectal tumors, revealing that most adenomas (63%) and carcinomas (60%) contained a mutated APC gene (Powell et al., 1992). Moreover, other studies indicated that more than 80% of tumors had at least one mutation in the APC gene, of which more than 60% had 2 mutations (Miyoshi et al., 1992). These observations strongly suggest that somatic mutations of the APC gene are associated with the development of the great majority of colorectal tumors (Nathke et al., 2004). APC mutations typically affect the central domain of the protein containing the binding site for β -catenin and, consequently, determine the increase of nuclear β -catenin and the transcriptional activation of specific target genes, such as the oncogene c-myc (Albuquerque et al., 2002; Sansom et al., 2007). Furthermore, approximately 10% of colorectal cancers carry activating mutations in the highly conserved serine/threonine residues of β -catenin, which are required for recognition and degradation of the protein

(Morin et al, 1997). Hereditary non-polyposis colon cancer syndrome is the most common form of hereditary CRC characterized by the absence of prior polyp formation (Lynch et al., 1996). 90% of HNPCC patients carry mutations in the “caretaker” mismatch repair genes, hMSH2 and MLH1 (Bronner et al., 1994; Leach et al., 1993). Consequent inactivation of the mismatch repair system leads to the instability of microsatellites, genomic region characterized by multiple repeats of short DNA sequences or single nucleotides, which represents the hallmark of tumors in HNPCC. The microsatellite instability typical of HNPCC creates a favorable state for accumulating mutations in genes that control cell survival, proliferation and self-renewal, likely fostering the generation of colon carcinomas. Not surprisingly, colorectal tumors with mismatch repair deficiency have been shown to bear an excess of frameshift mutations in the “gatekeeper” gene APC (Huang et al., 1996). However, it has been hypothesized that genomic instability alone is not sufficient for colon cancer development, but has to be combined to the selection of genetic alterations through the clonal expansion of an aberrant stem cell population. Taken together, the studies performed on FAP and HNPCC patients demonstrate the importance of both “gatekeeper” and “caretaker” gene function. FAP results from an increased rate of tumor initiation due to abrogation of the gatekeeper function of APC. In contrast, the mismatch repair defect in HNPCC results in an enhanced rate of mutation that greatly accelerates tumor initiation. HNPCC and FAP patients both develop cancer at a median age of 42 years, suggesting that both tumor initiation and tumor progression are rate limiting for colon cancer formation.

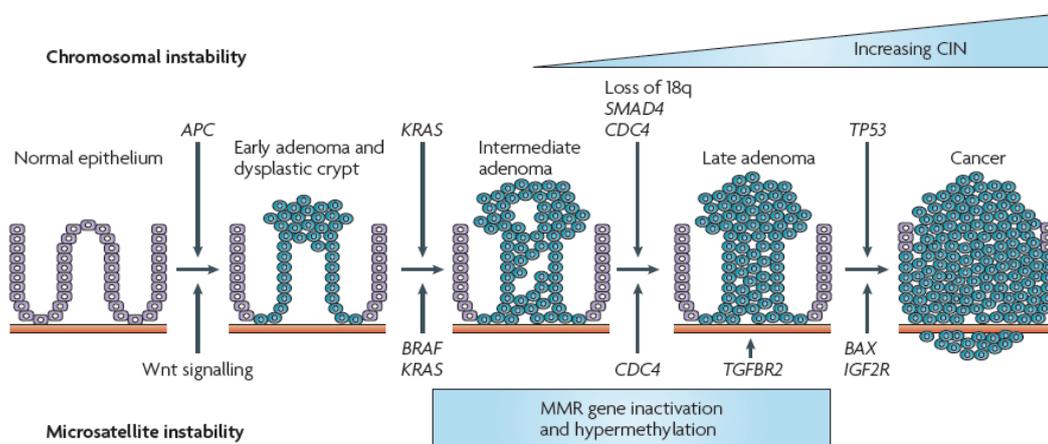


Figure 7. Adenoma–carcinoma sequence model. CIN, chromosomal instability; MMR, mismatch repair (Walther et al., 2009).

3.4 Colon cancer therapy

The prognosis of colorectal cancer is principally based on the stage of the disease at presentation and there is a significantly better survival outcome when detected in earlier stages. Adenomas and early cancers are often asymptomatic but potentially curable if found and removed as compared to symptomatic colorectal cancer that is more likely to be advanced in stage. Screening asymptomatic men and women over the age of 50 years for colorectal neoplasia has shown to detect cancers at an earlier stage compared to those not screened (Hardcastle et al. 1996; Kronborg et al. 1996; Mandel et al. 1993). A number of screening methods are now in use and under clinical evaluation, such as fecal occult blood test (FOBT), flexible sigmoidoscopy, colonoscopy, computed tomographic colonography or virtual colonoscopy.

The only curative strategy in the treatment of colorectal cancer is complete surgical resection. Surgical techniques and survival after surgery have improved over the past 15 years.

Surgery with wide resection margins is the only therapy needed for stage I and II disease, although some stage II patients receive chemotherapy (Boland et al. 2000). For stage III disease, adjuvant 5-FU-leucovorin or FOLFOX (5-FU, leucovorin, and oxaliplatin) for 6 months is standard and has been shown to improve survival (Goldberg et al. 2004; de Gramont et al. 2000; Moertel et al. 1995; Moertel et al. 1990; Laurie et al. 1989; Petrelli et al. 1989). For stage IV, surgery may be curative in highly selected patients with resectable bowel disease and resectable isolated hepatic or pulmonary metastases. In many patients with stage IV disease, surgery is often palliative to prevent bowel obstruction. Chemotherapy is offered but may not improve overall survival. Palliative chemotherapy regimens for stage IV colon cancer include 5-FU-leucovorin, FOLFOX, FOLFIRI (folic acid/5-FU/irinotecan), IFL (irinotecan/5-FU/leucovorin), although IFL is no longer used due to toxicity (Goldberg et al. 2004; de Gramont et al. 2000; Douillard et al. 2000; Saltz et al. 2000; Petrelli et al. 1989). Specific growth factor inhibitors, such as bevacizumab and cetuximab added to the 5-FU-based regimens improve tumor shrinkage and increase months to the survival of stage IV patients (Jonker et al. 2007; Hurwitz et al. 2004).

It is important to note that systemic therapeutic progress have improved survival of patients with metastatic colorectal cancer. Before the 1960s, patients with untreated metastatic colorectal cancer had a median survival of 4-6 months. With the introduction of 5-FU treatment, patient survival was extended to 11–12 months, and 5-FU continues to form the core for colorectal cancer therapy (Boland et al. 2000; Moertel et al. 1995; Moertel et al. 1990; Laurie et al. 1989). In the 1980s, biomodulators such as leucovorin were added to 5-FU, improving the survival by an additional 2–4 months (Petrelli et al. 1989). Irinotecan and later oxaliplatin were added to 5-FU as second-line treatments, and later were approved as first-line therapies for metastatic colorectal cancer, further improving overall survival to 15-17 months after diagnosis (Goldberg et al. 2004; de Gramont et al. 2000; Douillard et al. 2000; Saltz et al. 2000). Biological therapies such as cetuximab and bevacizumab bring average survival lengths close to 20 months after diagnosis, quadrupling the survival times from those observed three decades ago.

Systemic chemotherapeutic agents

5-Fluorouracil:

5-FU is a fluoropyrimidine that is incorporated into RNA (mRNA, rRNA, and tRNA), and is an inhibitor of thymidylate synthase (which catalyzes the rate limiting conversion of dUMP to dTMP) to inhibit DNA synthesis (Parker and Cheng, 1990). 5-FU is typically administered with the biomodulator leucovorin, a reduced form of folate that stabilizes the binding between 5-FU and thymidylate synthase (Petrelli et al. 1989). It can be given as active intravenous or pro-drug oral forms (capecitabine). Deficiency of dihydropyrimidine dehydrogenase (DPD), the exclusive enzymatic step for catabolism and deactivation of fluoropyrimidines, can lead to severe toxicities with 5-FU. In vitro analysis revealed that 5-FU selectively killed colon cancer cells with intact DNA MMR (mismatch repair), while cells without intact DNA MMR were 28-fold more resistant (Carethers et al. 1999). 5-FU-resistant cells in which DNA MMR was restored became sensitive to 5-FU, similar to other MMR-proficient cells (Arnold et al. 2003).

Irinotecan

Irinotecan, also known as CPT-11, inhibits topoisomerase I, an enzyme involved in the uncoiling of DNA prior to transcription and DNA replication. As a result of the uncoiling, topoisomerase I causes single-strand DNA breaks which are transient and easily repaired. Irinotecan, by blocking topoisomerase I, stabilizes the breaks within DNA, causing DNA fragmentation and ultimately cell death (Klein et al. 2002; Douillard et al. 2000; Saltz et al. 2000). Irinotecan is metabolized by hepatic carboxylesterases to its active compound, SN-38, and is inactivated through uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) (Klein et al. 2002).

Oxaliplatin

Oxaliplatin is a platinum derivative, and unlike its cousins cisplatin and carboplatin, has synergistic activity with 5-FU in treating colorectal cancer (Goldberg et al. 2004; de Gramont et al. 2000). Oxaliplatin forms bulky DNA adducts, and ultimately induces cell death, but may also down regulate thymidylate synthase, possibly potentiating the efficacy of 5-FU (Raymond et al. 2002; Boland et al. 2000). Oxaliplatin as single agent has limited efficacy, and is most efficacious clinically when combined with 5-FU-leucovorin in the FOLFOX regimen for patients with advanced colorectal cancer (Goldberg et al. 2004; de Gramont et al. 2000).

Targeted biologic therapies for colorectal cancer

Targeted therapies hold the promise of interrupting key cell pathways that are essential for the growth of the tumor, and involved in the cancer cell survival and metastasis. Some of these targeted therapies are antibodies that can inhibit a receptor, and some are small molecules – termed biologic therapy. Given their specific targeted nature, side effects might be less than general systemic chemotherapies.

Bevacizumab

Bevacizumab is a humanized monoclonal antibody directed against VEGF, a soluble protein involved in angiogenesis process (Ferrara et al. 2003). Bevacizumab alone as

monotherapy is ineffective; it has been shown to increase survival in patients with metastatic colorectal cancer when used with the IFL regimen (Hurwitz et al. 2004).

Cetuximab

Cetuximab is a monoclonal chimeric human/mouse IgG1 antibody directed against the extracellular domain of the EGFR, part of the ErbB family of receptors (EGFR is also known as ErbB1). EGFR is normally present on epithelium, but is overexpressed in some colorectal cancers and associated with a poorer prognosis. EGFR normally enhances pathways involved in cell growth and proliferation; thus inhibition of overexpressed EGFR should slow proliferation of cancer cells.

Activating mutations in KRAS, downstream of EGFR, has been associated with resistance to cetuximab therapy, and portends a poorer survival (Lièvre et al. 2008).

Panitumumab

Panitumumab, like cetuximab, is a monoclonal antibody directed against the extracellular domain of EGFR. However, panitumumab is a fully humanized IgG2 antibody, which may confer a smaller risk for infusion reactions over cetuximab. Colorectal cancer patients refractory to therapy had decreased tumor progression with panitumumab, and there was improved progression free survival when panitumumab was combined with FOLFIRI (Berlin et al. 2007; Van cutsem et al. 2007). Similar to cetuximab, efficacy is best with wild type KRAS patients, as those with mutant KRAS are resistant to therapy and have a poorer survival (Amado et al. 2008).

Gefitinib

Gefitinib is a small molecule that targets and inhibits intracellular EGFR tyrosine kinase activity. Unlike the extracellular portion of EGFR where monoclonal antibodies are used to block EGFR activation, gefitinib inhibits the intracellular tyrosine kinase catalytic domain of EGFR that activates downstream signaling events and effectors pathways. There is evidence that the tyrosine kinase domain may still be a useful target in cetuximab-resistant cells, suggesting that tyrosine kinase inhibitors may further modulate signaling that is not fully blocked with EGFR

antibodies (Huang et al. 2004). Gefitinib combined with FOLFOX achieved a median overall survival of twelve months in those patients who previously failed chemotherapy (Kuo et al. 2005).

Erlotinib

Erlotinib is a small molecule that competes with ATP for EGFR's tyrosine kinase domain. This inhibits receptor autophosphorylation and blocks downstream signaling from the intracellular portion of EGFR. Erlotinib has some activity as a single agent, and trials are underway as phase III studies and in combination with traditional chemotherapy regimens (Townsend et al. 2006).

Lapatinib

Lapatinib is an ATP-competitive small molecule that is a reversible inhibitor of both the ErbB1 and ErbB2 (Her2/Neu) tyrosine kinases (Ponz-Sarvisse et al. 2007). Lapatinib as a single agent showed some benefit in preventing progression of colorectal cancer metastasis in patients who progressed with prior therapy.

Sorafenib

Sorafenib is an oral multi-kinase inhibitor that has activity against Raf kinase (a serine-threonine kinase) and tyrosine kinases, including VEGF receptor, platelet derived growth factor receptor (PDGFR), c-kit, and c-Ret (Wilhelm et al. 2006). It inhibits tumor cell proliferation by targeting the Ras/Raf/extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway at the level of Raf kinase, a pathway that is often activated in colorectal cancer, and exerts its anti-angiogenic effect by targeting the receptor tyrosine kinases VEGF receptor 2, VEGF receptor 3, and PDGFR, and their associated signaling cascades (Grady and Carethers, 2008; Wilhelm et al. 2006). It is also found to induce apoptosis in some human cancer cell lines by down-regulating the levels of antiapoptotic protein myeloid cell leukemia sequence 1 (Yu et al. 2005). Single agent therapy showed some clinical benefit in patients with advanced colorectal cancer (Strumberg et al. 2005).

Aim of the work

Numerous reports describe the mir-143 and mir-145 expression as diminished in cells of tumoral origin with respect to normal counterparts in colon epithelia and in cell lines, and it has been demonstrated that these microRNA can function as oncosuppressors, by targeting a number of transcripts that may promote cell proliferation, such as the KRAS mRNA, targeted by mir-143 (Chen et al., 2009).

The genes for mir-143 and mir-145 are located in a 3kb segment on chromosome 5 and frequently display a coregulated expression profile, suggesting that they are transcribed in the same polycystron. Despite accumulating evidence on concomitant mir-143 and mir-145 downregulation in colon cancer, their function as players in a coordinated scheme of target gene regulation has seldom been studied. It has been shown that mir-143 and mir-145 cooperate in promoting smooth muscle cell differentiation while mir-145 antagonizes embryonic stem cell self-renewal (Cordes et al., 2009; Xu et al, 2009).

The antiproliferative effect of mir-143 and mir-145 though documented, has not been thoroughly investigated in an integrated framework of common targets and unique targets in common or complementary pathways. For example in colorectal cancer (CRC), the effect of mir-143 has been implicated in KRAS downmodulation, c-myc and IRS-1 might mediate the antiproliferative effect of mir-145 (Sachdeva et al., 2009; Shi et al., 2007) while IGF-1R, another target of mir-145, does not (La Rocca et al., 2009). These evidences have been proved in single overexpression experiments, and fit convincingly in the mutational landscape of CRC, with KRAS being frequently mutated and c-myc being overexpressed as a result of APC loss of function. Other mir-143 targets, such as DNMT3A (Ng et al., 2009) and ERK5 (Akao et al., 2009), have been proposed to be mediators of an antiproliferative effect, but, as for the target genes mentioned above, the relative contribution of any given target gene has not been assessed. The levels of mir-143 and mir-145 are decreased in multiple tumoral tissues such as lung, breast and lymphoma (Akao et al., 2007),

which suggests that a frequent oncogenic lesion, common to these tissues, might be implicated. One such lesion is the TP53 gene loss of function, and it has recently been demonstrated that p53 pathway activation increases the mir-143 and mir-145 levels through a posttranscriptional mechanism (Suzuki et al, 2009; Sachdeva et al., 2009)

While most reports on mir-143 and mir-145 have considered them as independent genes, our study examines the effect of their concomitant re-expression, as we speculate that they are co-transcribed and that they have partly overlapping functions/target genes.

First we have investigated the anti-proliferative effect of mir-143 and mir-145 in colon cancer cell lines after their overexpression. To this aim we have performed functional assays to investigate the differences in proliferation, migration, anchorage independent growth and tumorigenic potential, triggered by miRNA overexpression. We have then selected, among the predicted target genes, those transcripts which are particularly relevant for their putative role in CRC progression. We found that CD44, a membrane glycoprotein that is downregulated at the transcriptional level upon p53 restoration in breast cancer is a direct target of mir-143 and mir-145 in CRC cells, highlighting the focal impact of p53 in the regulation of CD44 by multiple mechanisms (Godar et al., 2008).

We have identified, as a novel mir-143 and mir-145 target in CRC cells, the KLF5 gene, which notably is required for KRAS-mediated transformation of normal colonic epithelium (Nandan et al., 2008). Furthermore, we identified as a novel mir-143 target, the BRAF gene, which is downstream of the already known target KRAS. Our results favor the hypothesis that all the target genes we investigated are mediators of the mir-143 and mir-145 tumor suppressive capabilities that we observed both *in vitro* and in *in vivo* xenograft experiments.

Materials and methods

1. Cell Culture

Colorectal cancer cell lines HCT116, SW489 and HT29 from American Type Culture Collection (ATCC) were cultivated in the recommended media. In particular, HCT116 were grown in McCoy's medium, SW480 were grown in DMEM, HT29 were grown in RPMI-1640.

The Human Embryonic Kidney 293T cell line was cultivated in IMDM. They contain the SV40 Large T-antigen, that allows for episomal replication of transfected plasmids containing the SV40 origin of replication.

All media were supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml of penicillina and 100 µg/ml of streptomycin (all the products are from Gibco).

2. Lentiviral Vectors

For TwG-miR-143-145 generation (TWG-143-5), miR-143-145 precursor DNA was amplified from human genomic DNA. We subcloned the amplified fragment spanning 2347 base pair (chr:5, 148808179-148810515) into the lentiviral vector TWEEN under the control of the cytomegalovirus promoter (CMV). TWEEN vector carries a EGFP gene under the control of the phosphoglycerate kinase (PGK) promoter used to collect transduced positive cells by FACS-sorting. We used the following primers for PCR: forward primer 5'- *tgccattgttgcaactt* -3' and reverse primer 5'- *caagagtacggcagtgctga* -3'.

For the rescue experiments we set out a lentiviral vector-based system for target gene expression. We cloned target gene (CDS), lacking of 3'UTR miR-143 and miR-145 binding site, in TWEEN-GFP or TWEEN-CFP lentiviral vectors between CMV promoter and PGK promoter/GFP or CFP cassette. In particular BRAF, CD44 and

KLF5 CDS were cloned in TWEEN-GFP vector, whereas KRAS CDS was cloned in TWEEN-CFP vector. All CDS were PCR amplified from human genomic DNA. (GeneBank reference are in Reporter assay section).

For Tet-On inducible system based experiments we used three different lentiviral vectors: pLV-rtTA, that consists of rtTA gene under the control of CMV promoter and carries a puromycin resistance gene; pLV-tTR consists of a tTR gene under the control of thymidine kinase (TK) promoter and carries a neomycin resistance gene; pLTR (Lentiviral-Tet-inducible-RFP) or pLTR-143-5 (Lentiviral-Tet-inducible-RFP-miRs) act as responders to rtTA by inserting, in a lentiviral backbone, a tetracycline response element (TRE) upstream of a minimal promoter driving the transcription of RFP or RFP/mir-143-5 respectively. TRE consists of seven repeats of tet operator (tetO). pLTR-143-5 contains the same genomic region used in TWG-143-5, cloned in the 3' untranslated (UTR) region of RFP.

3. Lentiviral Infections

Lentiviral particles were produced by calcium phosphate transfection protocol in 293T packaging cell line. The day before transfection 293T cells were plated at density of 2×10^6 cells in a T75 cell culture flask. Cells were transfected with 20 μg of lentiviral vector, 13 μg of packaging vector (pPAX) and 7 μg of vesicular stomatitis virus protein G vector (pMD2G). CaCl_2 (125 mM) and HBS (2XHBS: NaCl 280 mM, KCl 10 mM, Na_2HPO_4 1.5 mM, D(+)glucosio 12 mM, HEPES 50 mM, pH 7.1) were included in transfection mix to promote calcium phosphate/DNA precipitates formation. 24 hours post-transfection culture medium, full of calcium phosphate precipitates, was replaced with fresh growth medium. 48 hours post-transfection, viral supernatant were filtered with 0.45 μm filter and used for the following cell lines infection.

For lentivirus infection colon cancer cell lines were plated in a 6-well culture plate at 80% of confluence. The day of infection the cells growth medium was replaced with 2 ml of filtered viral supernatant. Polybrene (4 $\mu\text{g}/\text{ml}$) was used to improve infection efficiency. Cell plates were first centrifuged for 45 min at 1800 RPM at 37 $^{\circ}\text{C}$ and then were incubated for 75' at 37 $^{\circ}\text{C}$. At the end of the infection, the cells were

washed extensively and maintained in usual growth medium. The transduction efficiency was evaluated by cytofluorimetric analysis.

Colon cancer cell lines transduced with TwG or TwG-mir-143-145 lentiviral expression vectors were flow sorted after infection for GFP expression and then used for proliferation and in vivo assay.

In Tet-on inducible based system, SW480 colon cancer cell lines were infected with pLV-rtTA and pLV-tTR lentiviral expression vectors and positive cells were selected by antibiotic resistance to puromycin and neomycin, respectively. Successively cells were transduced with pLTR or pLTR-mir-143-145, were induced with the tetracycline analog doxycycline and, after two days, RFP-positive cells were flow-sorted and used for functional assay and target analysis.

Cells were sorted by BD FACSAria cell sorter according to instructions from facility instrument technicians.

4. Flow cytometry

CRC transduced with lentiviral expression vectors (TW or TW-143-5) and lentiviral inducible vectors (pLTR or pLTR-143-5) were stained with specific antibody FITC-conjugated anti-CD44 (RD System) and PE-conjugated anti-ErbB3 (RD System), used 1:20 in according to manufacture protocol.

Xenograft TWG or TWG-143-5 tumor cells were stained with specific antibody PE-conjugated anti-HLA, used 1:20 (DakoCytomation).

In rescue assay, SW480 cell lines transduced with pLTR or pLTR-143-5, and TWG-CD44 were analyzed by staining with APC-conjugated anti-CD44 used 1:20 (RD System).

FITC, PE or APC conjugated anti-human IgG antibody were used 1:20 as control (BD Bioscience).

Cells were washed with PBS and incubated with control or specific antibodies. After 1 hour of incubation, labeled cells were washed twice with PBS and fluorescence intensity was evaluated by FACScanto (Becton Dickinson, San Jose, CA).

5. RNA isolation and Real- Time PCR analysis

Total RNA was extracted with TriZol method at different time point (for a period of two weeks post-sorting). Fifty nanograms of RNA were reverse transcribed with M-MLV reverse transcriptase (Invitrogen) or TaqMan reverse transcriptase (Applied Biosystems) and cDNA was diluted 1:10 in the PCR reactions. Housekeeping gene and target gene reverse transcription was performed using oligo-dT primers (Invitrogen), while miRNA specific looped-primers were used for miR-143 and miR-145 reactions.

TaqMan microRNA assays (Applied Biosystems) for miR-143 and miR-145 were used for PCR amplification. Normalization was performed using snRNA U6 as reference (RNU6B TaqMan microRNA assay, Applied Biosystems).

Target genes of retrotranscribed RNA were amplified using 0.5 pmol of each specific primer by SYBR Green real time PCR (SYBR Green PCR Master Mix, Applied Biosystems). Normalization was performed using Peptidil Prolil Isomerase (PPIA) as reference.

Calibration was performed using cDNA samples from colon cancer cell line infected with control vector (pLTR or TWG). Values are expressed in terms of $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{calibrator}}$, ΔCT was the difference in threshold cycles between the miR or target gene and snRNAU6 or PPIA amplicons, and CT was a parameter given by ABI PRISM 7900 Sequence Detector software according manufacturer's instructions (Applied Biosystems).

Each experiment was performed in duplicate for three times.

RT-PCR primer sequences for gene target are:

Primer name	Primer forward	Primer reverse
K-Ras	cccaggtgcgggagagaggc	ctacgccaccagctccaact
B-Raf	tcccatcagtttgaacagttgtct	ctgtatggatttttatcttgcattctg
KLF5	tggagaaacgacgcatcca	gtcgcagccttcccaggta
CD44	gctctgagcatcggatttgag	tttgctgcacagatggagtt
ErB3	gaggaagaggatgtcaacggta	gaactgagaccactgaagaaagg
PPIA	ggccgcgtctccttga	agtgctcagagcacgaaaattt

6. MicroRNA target prediction

We used TargetScan (<http://genes.mit.edu/targetscan>), miRanda (<http://www.microrna.org>), and PicTar (<http://pictar.bio.nyu.edu>) for mir-143 and mir-145 target prediction. The targets were confirmed by BLAST alignment with the corresponding NCBI DNA data base for homologies between miRs and their target. Moreover, putative target were selected for their involvement in colon cancer pathogenesis.

7. Western Blots

Cells pellets were collected at different time points (for a period of two weeks post-sorting). Protein extracts were prepared by resuspending cell pellets in RIPA buffer (20 mM Tris/HCl pH 7.2, 200 mM NaCl, 1% NP40) with Protease and Phosphatase Inhibitor Cocktails I and II (Sigma-Aldrich). Samples were resolved in SDS-PAGE gels (NuPage 4-12% bis-tris Gel invitrogen), and proteins were loaded in equal amounts after measurement with Bradford assay (Biorad). Proteins were transferred to nitrocellulose and samples were analyzed by standard immunoblot procedure. After blocking, the membrane was incubated overnight at 4°C with the following primary antibody diluted in TBS/0.2% Tween with 1% BSA: anti-KRas (1:300), anti-BRaf (1:300), anti-BTEB2 (KLF5) (1:1000). All primary antibodies are from Santa Cruz Biotechnology. Anti- β -actin monoclonal antibody was used as loading control (Oncogene Research Products, San Diego, CA).

Then, the membrane was incubated with a horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse immunoglobulin antibody (1:4000) in TBS/0.2%Tween with 5% milk for 1 hour at RT.

The specific protein-antibody reaction was detected by the Super signal West Pico Chemiluminescent Substrate (Pierce).

The quantitation of protein expression was determined after normalization to β -actin, by measuring the optical density of respective band blots using the Quantity One software (Biorad).

8. Reporter assay

The potential binding sites of miR-143 and miR-145 on 3'UTR of gene target (except b-Raf where target region is in the coding sequence) were cloned immediately downstream of the stop codon of the Renilla coding sequence into multiclonal site (MSC) of a dual-luciferase vector psiCHEK-2 (Promega).

For RAS, two miR-143 3'UTR target regions of about 200 bp were amplified from human genomic DNA, corresponding to residues 2222-2436 (143Ta) and 4365-4582 (143Tb) of Genbank Acc N° NM_004985.3.

For Raf, one miR-143 coding sequence (CDS) target region of about 100 bp fragment was amplified (residues 370-552 of N° NM_004333.4).

For KLF5, one miR-143-145 3'UTR target region, of 761 bp fragment was amplified (residues 1429-2189 of N° NM_001730.3).

For CD44, one miR-143 and one miR-145 3'UTR target region, of about 200 bp was amplified (residues 2258-2438 and 3283-3515 of N° NM_001001391.1).

First, PCR fragments were cloned in pCRII-topo vector (TOPO TA Cloning Kit Dual Promoter, Invitrogen), then were transferred in psiCHEK-2 between XhoI and SpeI restriction site.

Primer sequence used are:

Primer name	Primer forward	Primer reverse
KLF5T143/145-wt	tggagaaacgacgcatcca	cccacacatttgttcaatgg
RasT143a-wt	gactcgagatggcatggtga	cccgatatcgtgctgagcttgaca
RasT143b-wt	tcggagctcttgtgggcatgtt	gctttaaagtaactgctgggttc
CD44T143-wt	gccctcgagcactgtctgggtcctac	cgcactagtcaggatggaaaaccttggga
CD44T145-wt	ccttacgtaccacttcacccacaatctt	gccactcgagtggcttgttgettttc
RafT143-wt	gggctcgagactgattttct	ctgtcgactgtttgtgggca

For mutant derivative constructs double strand oligos were generated by annealing sense and antisense mutated strands, and further ligated into psiCHEK2 digested with XhoI and SpeI. Mutation was performed in the 'seed' sequence where nucleotide 2-8 were substituted with restriction enzyme site.

Oligos sequence are:

Name	XhoI – 3'UTR oligo (s) / SpeI – 3'UTR oligo (as)
KLF5 T143-mut	<u>tcgataatgtatatggctttactcaagcagatc</u> gctagc catgacaggcagccacgtctcaacatgggt
	<u>ctagacccatggtgagacgtggctgcctgcatg</u> gctagc gatctgcttgagtaaagccatatacatta
KLF5 T145-mut	<u>tcgaaacaaacaaaagcaagaaaaccacaactaat</u> gtac aaaatgtatattttgtatattgagaaaac
	<u>ctaggtttctcaaatacaaaaatacattt</u> gtac attagttgtggtttcttgctttgtttgtt
Ras T143a-mut	<u>tcgattactatccatttctcatgttaaaagaag</u> gctagc caaactcttagtttttttttacaact
	<u>ctagagttgtaaaaaaaaaaactaagagttt</u> gctagc cttctttaacatgaagaaatggatagtaa
Ras T143b-mut	<u>tcgatgtaagttacagttgcacaagt</u> gata tcatttgattccattgatttttt
	<u>ctagaaaaaatcaatggaatacaaat</u> gata tcacttgcaaaactgtaacttaaca
CD44 T143-mut	<u>tcgaaatgtaaggagactcttctaagtct</u> atgc atcagagaccctgagttcccactcagacc
	<u>ctagggctctgagtgggaactcagggtctct</u> atgc atagacttagaagagtctcctaccatt
CD44 T145-mut	<u>tcgattctttttttctttcaactgaaagat</u> ctaga acattaggccactatgtgtgtactgcc
	<u>ctagggcagtaacaacacatagggcctaagt</u> gtctaga tctttcaagttgaaaagaaaataaaaagaa
Raf T143-mut	<u>tcgactagcctttcagtgtctacct</u> gata tcctttcagttttcaaaatcc
	<u>ctagggattttgaaaaactgaaag</u> gata tcaggtagcactgaaaggctag

The underlined nucleotides are Xho- 5'end and SpeI- 3'end protruding of the double strand oligo. Red nucleotides are 'seed' mutated site.

For transfection experiments, CRC cell lines were seeded in 24-well plate and then transfected with Lipofectamine 2000 according to manufacturer's instructions (Invitrogen).

CRC cell lines were transfected with 0.2 µg of renilla-luciferase vectors (empty psiCHEK2 and psiCHEK2-target 3'UTR/CDS wt or mutant) and 1.8 µg of miR-143-145 expression vector (empty Tw or Tw143-145). Luciferase activities was detected 48h after transfection by using Dual-Luciferase reporter assay (Promega).

Transfection efficiencies were normalized by calculating the ratio renilla/firefly. For each 3'UTR target region the relative renilla activities was calculated by dividing the values obtained in wt vector by those of mutated. Each experiment was performed in duplicate and replicated three times.

9. Proliferation assay

Transduced colon cancer cell lines (SW480, HCT116, HT29) were seeded at a density of 2000 cells per well in 96-well plates. Rates of proliferation were determined using Cell Titer Blue assay (Promega) after 0-, 3-, 4-, 5-, 6- days. Proliferation was measured by evaluating of 490nm absorbance, which is directly proportional to the number of living cells. Each experiment was done in quintuplicate and replicated three time.

10. Soft agar colony formation assay

Assays of colony formation in soft agar were done using standard protocols. Briefly, transduced CRC cell lines were seeded at same density (5×10^2 cells per well). Cells were suspended in 0.35% Agar Noble and were plated onto a layer of 0.7% Agar Noble in cell medium in 24-well tissue culture plates (Corning). The agar containing cells was allowed to solidify overnight at 37°C in 5% CO₂ humidified atmosphere. Colony formation was determined after two weeks by staining with 0,5ml of 0,005% Crystal Violet for 1 hour. Visible colonies were counted with the aid of a microscope.

11. In *vitro* cell migration assay

The motility of transduced CRC was evaluated in 24-well transwell chambers (Costar), as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well are separated by polycarbonate membranes (8 µm pore size). The lower chambers of the 24-well plate were filled with 500 µL of cell medium; 2×10^3 cells in 200 µL of the same medium was placed into the upper

compartment of wells. The transwell chambers were incubated at 37°C in 5% CO₂ humidified atmosphere for 48 h. The cells that had not invaded were removed from the upper face of the filters using cotton swabs. The cells that had invaded to the lower surface of the filters were fixed and stained with coomassie blue, and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at ×100 magnification) per filter. The experiment was repeated twice.

12. In vivo assay

Six-eight week old male NOD-SCID mice (Charles River Laboratory, Wilmington, MA) were used for examining the tumorigenicity of colon cancer cell lines transduced with TWG or TWG-143-5 lentiviral vectors. A total of 200 µl of cell suspension in Matrigel (1:1, vol/vol) was subcutaneously injected into the dorsal surface of mice (cells transduced with TWG vector on the right and cell transduced with TWG-143-5 on the left of the same mouse). The number of cells injected was 2×10^5 and three mice were inoculated for each cell line. Tumor size was monitored for 7 weeks by measuring the length (L) and width (W) with caliper. Volumes were calculated by using the following formula: $(L \times W^2) \times 0,52$. At the end of the experiment mice were sacrificed and mir-143 and mir-145 expression levels were verified by RT-PCR and FACS analysis.

13. Cytotoxicity assay

SW480 colon cancer cell lines transduced with LTR and LTR-143-5 vectors were flow sorted and plated at density of 2000 cell per well in a 96-well plate. The day after seeding cells were treated with three different concentration of 5-Fluorouracil (25, 50 and 100 uM), Oxaliplatin (5, 10 and 20 uM) and Irinotecan (5, 10 and 20 uM). Chemotherapeutic agent are Sigma (5-Fluorouracil, Oxaliplatin) and Alexis (Irinotecan). After 72 hours of treatment cell viability was assessed using Cell Titer Glo assay (Promega).

Each experiment was done in quintuplicate and replicated three times.

Results

We have started our work by investigating the anti-proliferative effect of mir-143 and mir-145 overexpression in colon cancer cell lines. We first set out a lentiviral vector-based system for mir-143 and mir-145 expression. A genomic region harboring the two miRNAs genes was cloned in a lentiviral vector TWG between a CMV promoter and a PGK promoter/GFP cassette, yielding TWG-143-5 (**Fig. 8a**). We checked the functionality of TWG-143-5 by comparing the mir-143 and mir-145 expression levels in colon cancer GFP-positive cell lines (Hct116, HT29, Sw480) infected with TWG and with TWG-143-5 respectively. Relative quantification by Real time PCR revealed that the mir-143 and mir-145 levels were increased by one and two orders of magnitude, respectively (**Fig. 8b**). This is coherent with mir-143 being the most abundant transcript in every cell type analyzed on a quantitative basis so that, increasing both microRNAs by the same amount, the relative increase is higher for the less abundant transcript.

Fig. 8b shows that high levels of mir-143 and mir-145 expression are achieved at late time points. The range of upregulation was comparable to the difference observed in mir-143 and mir-145 between normal and tumoral tissue from the intestine (Bandres et al., 2006). Hence, we proceeded to the analysis of tumoral behavior in CRC cells where these microRNAs have been restored to normal levels.

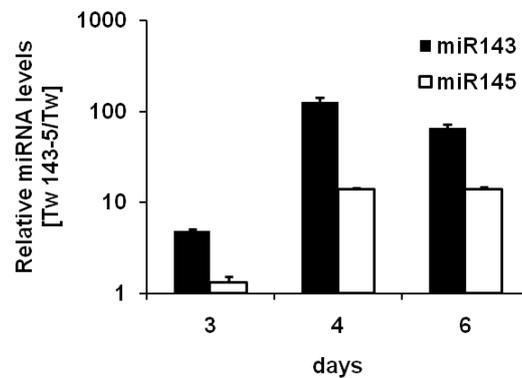
We analyzed the growth kinetics in HCT116, SW480 and HT29 cells transduced either with TWG or with TWG-143-5. Cells were flow sorted for GFP expression and then plated (2000 cells/well) in quadruplicate in a 96 well plate. Growth kinetics were analyzed over a period of one week, and in all cell lines the population transduced with TWG-143-5 displayed a limited degree of retarded growth (**Fig. 8c**). We also observed that, upon prolonged culturing (more than two week), the two populations became indistinguishable in terms of growth properties. When we analyzed the levels of mir-143 and mir-145 after the occurrence of this phenomenon, we could not detect significant differences in proliferation assays, indicating that the counter-selection operated by these microRNAs favored the emergence of cells that,

by an unknown mechanism, have restored the levels of mir-143 and mir-145 to those typical of a tumor condition.

a)



b)



c)

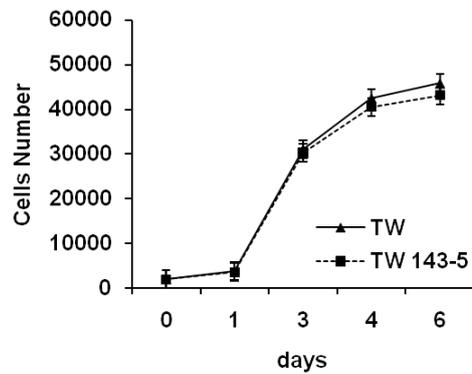


Figure 8. a) Schematic description of TWG and TWG-143-5 vectors. b) mir-143 and mir-145 relative expression levels in Hct116 cells transduced with TWG and TWG-143-5 vectors, data are normalized to control vector (empty TWG). c) Growth kinetics in Hct116 cells transduced with TWG and TWG-143-5 vectors. We show only data for Hct116 but results are similar in the other cell lines mentioned.

In order to observe the effect of mir-143 and mir-145 shortly after their enforced expression, we decided to set up an inducible system for microRNAs expression.

We used the Tet-On gene expression system technology (Gossen et al, 1995) by engineering colon cancer cell lines with three different lentivirus expression vectors: pLV-rtTA, pLV-tTR and pLTR-143-5 (or pLTR related control).

rtTA (reverse tetracycline-controlled transactivator) is a fusion protein consisting of rTetR (the reverse mutant of TetR) and VP16 activation domain (from herpes simplex virus). rtTA binds specifically to tetracycline responsive element (TRE) and activates transcription in the presence of doxycyclin. tTR (tetracycline-controlled transcriptional repressor) is a fusion protein consisting of the TetR and KRAB-AB domain of Kid-1. It binds specifically to the TRE and suppresses transcription in absence of Dox. pLV-tTR is designed to prevent unregulated mir-143 and mir-145 expression in the Tet-On system. pLTR-143-5 (or pLTR) is the response plasmid of rtTA which expresses RFP/miR143-miR145 (or only RFP) under the control of TRE. In pLTR-143-5 the genomic region used in TWG-143-5 was cloned in the 3' untranslated (UTR) region of RFP (**Fig. 9a**). In absence of doxycyclin tTR binds TRE and miRNAs transcription is repressed. In presence of doxycyclin rtTA binds TRE and miRNAs expression is activated.

First, SW480 colon cancer cell lines were infected with pLV-rtTA and pLV-tTR vectors. Positive cells were selected by antibiotics resistance to puromycin and neomycin respectively, and then were infected either with pLTR or its derivative pLTR-143-5. After infection, cells were induced with the tetracycline analog doxycycline and, after two days, RFP-positive cells were flow-sorted and used in functional assays.

For proliferation assay, cells were plated (2000 cells/well) in quadruplicate in a 96 well plate. Inducible expression of mir-143 and miR-145 had an inhibitory effect on the growth properties of Sw480 cell line (**Fig. 9b**). Overexpression of mir-143 and mir-143 were confirmed by Real Time-PCR (**Fig. 9c**).

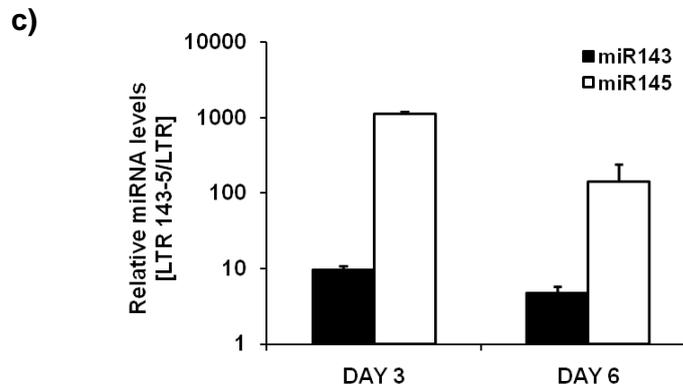
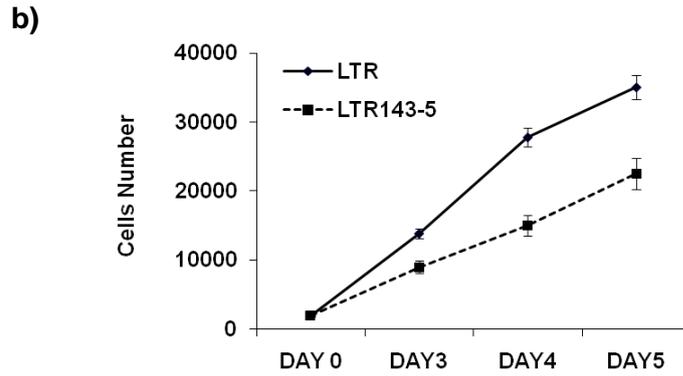
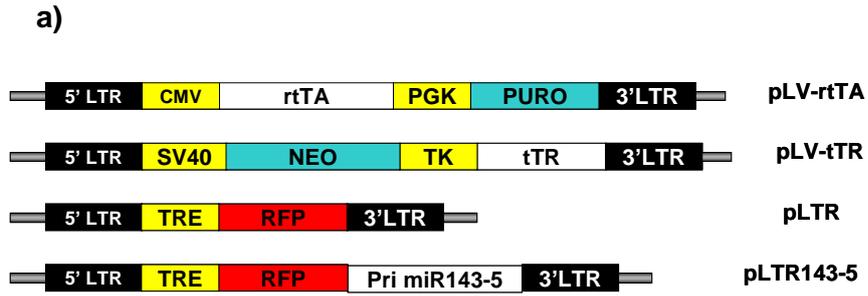


Figure 9. a) Schematic description of pLox-rtTA, pLox-tTR, pLTR and pLTR 143-5 vectors. b) Growth kinetic of Sw480 cell line infected with constructs in a) after doxycycline induction. The day of the sorting being day 0. c) Relative miR-143 and miR-145 expression level in Sw480 infected with construct in a) after doxycycline induction, data are normalized to control vector (empty pLTR).

We then examined the migration ability of SW480 cell lines transduced with pLTR or pLTR-143-5 expression vector. 2×10^3 cells, obtained as previously described, were plated in a 24-transwell microporous chamber, and incubated for 48h. We observed a dramatic reduction in the migration capabilities of the cell population overexpressing mir-143 and mir-145, at a high extent which could not be attributed merely to a decrease in the proliferation, given the short duration of the assay (**Fig. 10**).

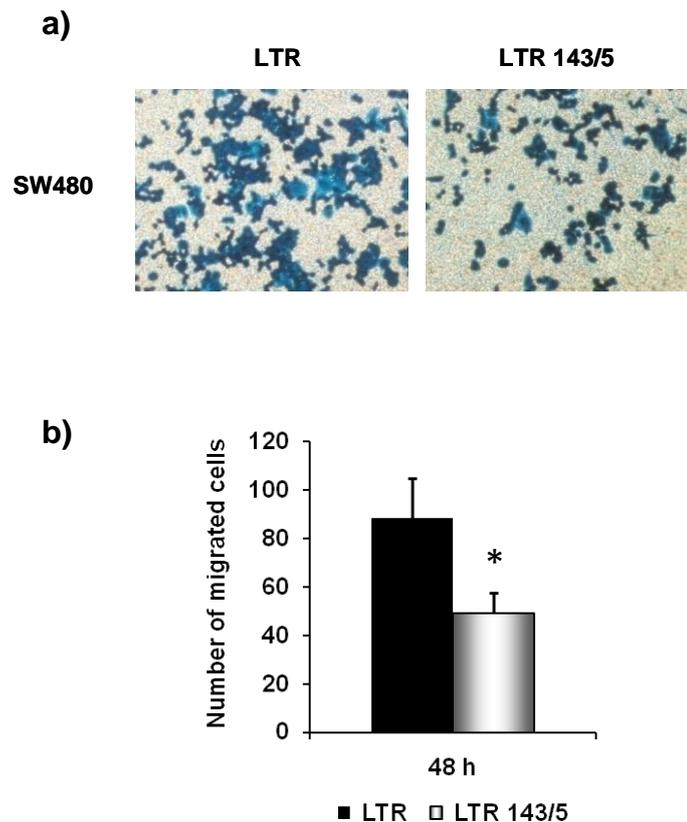


Figure 10. a) Sw480 pLTR and pLTR-143-5 cell lines fixed and stained with coomassie blue in transwell chambers. b) Graph shows the number of migrated cells in Sw480 cell line, 48h post-sorting. (* $p < 0,05$).

The same cells used for the migration assay were used to assess whether reinforced expression of mir-143 and mir-145 might influence the ability to grow in an anchorage-independent manner. To this aim, 5×10^2 cells were plated in soft agar, then, after 16 days, plates were stained with Crystal violet and colonies were counted

(**Fig. 11a**). The histogram shows a decrease in anchorage independent growth ability in SW480 pLTR-143-5 compared to the control vector transduced cells (**Fig. 11b**).

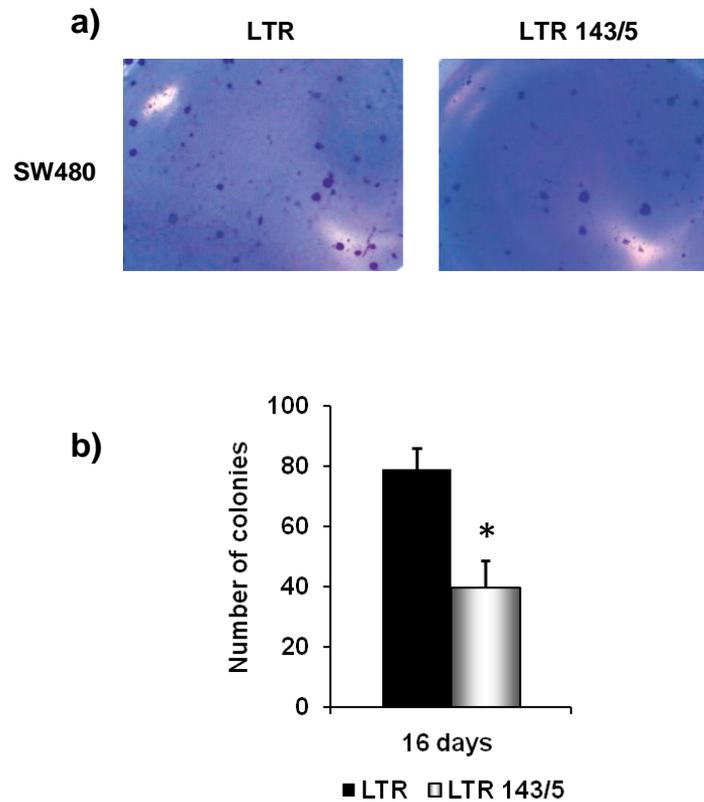


Figure 11. a) Sw480 pLTR and pLTR-143-5 cell lines in soft agar, after 16 days. b) Graph shows the number of colonies formed in semisolid medium in Sw480 cell lines, 16 days post-sorting. (* $p < 0,05$).

At this point we asked if the restoration of mir-143 and mir-145 expression level might influence the sensibility to the most commonly used chemotherapeutic drugs for the treatment of colon neoplasia. To this aim, we performed a toxicity assay with SW480 LTR and LTR-143-5 cell lines. Sorted cells were plated and treated with three different concentrations of 5-fluorouracil, irinotecan and oxaliplatin. The results are shown in **Fig. 12**, where a dramatic increase in the effect of the drug after 72h of treatment is observed as cell death, indicating that mir-143 and mir-145 do not

only affect proliferation of CRC cells, but also their survival, opening the possibility that they influence a set of genes with pleiotropic properties.

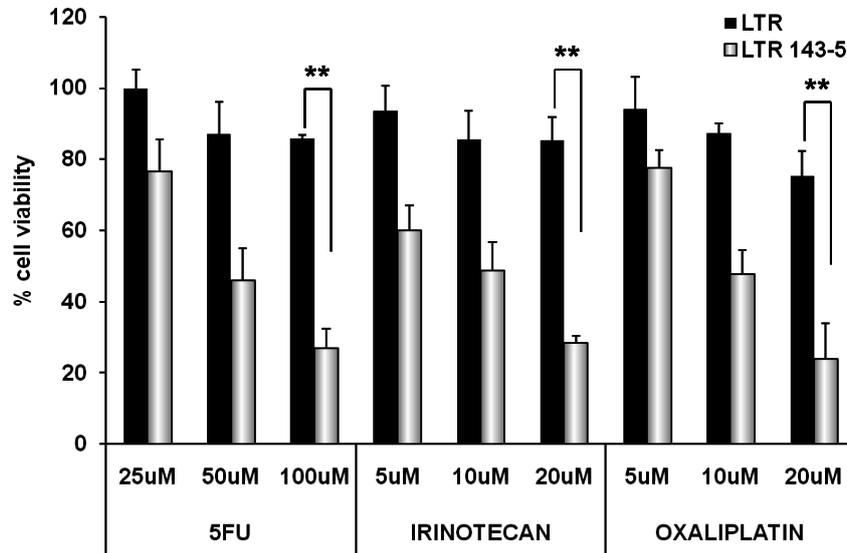


Figure 12. Chemosensitivity of Sw480 cell lines overexpressing mir-143 and mir-145 compared to control cells after 72 hours of treatment with the drugs and the doses indicated. (** p < 0,01)

Having confirmed that the coordinated expression of the mir-143 and mir-145 transcripts provokes a decrease in tumorigenic properties of SW480 colon cancer cell lines in the standard culture conditions, we endeavored to translate our findings in an *in vivo* model of tumor growth. To this aim, colon cancer cells lines (Hct116, HT29 and SW480), transduced with either TWG or TWG-143-5, were injected subcutaneously in SCID mice, and tumor formation was monitored over seven weeks. As shown in **Fig. 13a**, both cell populations are able to form tumor *in vivo*, but the tumors overexpressing mir-143 and mir-145 display a significant delay in their growth, which is possibly due to the time required for the outgrowth of a clone that escapes the growth inhibitory effect, as already detected in prolonged *in vitro* culturing. This is further supported by the fact that, once animals are sacrificed and

their tumors are evaluated for mir-143 and mir-145 levels (**Fig. 13b**), the difference in levels between those originated from TWG-transduced cells and those originated from TWG-143-5 are blunted, despite both populations being GFP-positive (**Fig. 13c**). Indeed, oncosuppressor functions are supposed to impart to tumoral cells a disadvantage that, depending on its strength, might manifest in a different range of phenotypes, from no growth to slightly reduced, but the possibility exists that, especially in gene transfer experiments, escape from a critical condition is achieved through transgene silencing.

The above experiments favour the hypothesis that mir-143 and mi-145 loss of expression might represent an oncogenic event that, as recently demonstrated, could be attributed at least partly to p53 loss of function (Suzuki et al, 2009; Sachdeva et al., 2009).

To understand more deeply the mechanism by which these microRNAs can behave as oncosuppressors, we tried to establish whether any of their putative targets might represent a crucial function in colon cancer oncogenesis. We identified, among the target genes predicted by TargetScan (Lewis et al., 2003), some transcripts which were particularly suggestive for the role that they play in colon cancer progression. Among these, k-Ras (targeted by mir-143) is activated by mutations in 50% of colorectal cancer patients (Arber et al., 2000; Bos et al, 1987), b-Raf (targeted by mir-143) is the immediate downstream effector of k-Ras in the mitogen-associated protein kinase (MAPK) pathway, Klf5 (targeted by mir-143 and mir-145) is a tissue-specific transcription factor which mediates the transformation of normal colonic epithelium by oncogenic k-Ras (Nandan et al., 2008), ErbB3 (targeted by mir-143 and mir-145) encodes EGFR3, a receptor of EGF which stimulates the MAPK pathway through Ras and is highly expressed in colon cancer cell lines (Wu et al., 2009). Finally, CD44 (targeted by mir-143 and mir-145), is a plasma-membrane protein with an important role in cell survival and migration, and is repressed by p53. While the effect of mir-143 on k-Ras in colorectal cancer tissues has been proposed elsewhere, the remaining targets are novel, with the exception of Klf5 which was demonstrated to be a target of mir-145 in vascular smooth muscle cells (Cheng et al., 2009). Notably, the b-Raf transcript has a target site for mir-143 in its coding region, which is neglected by most target prediction engines (**Fig. 14**).

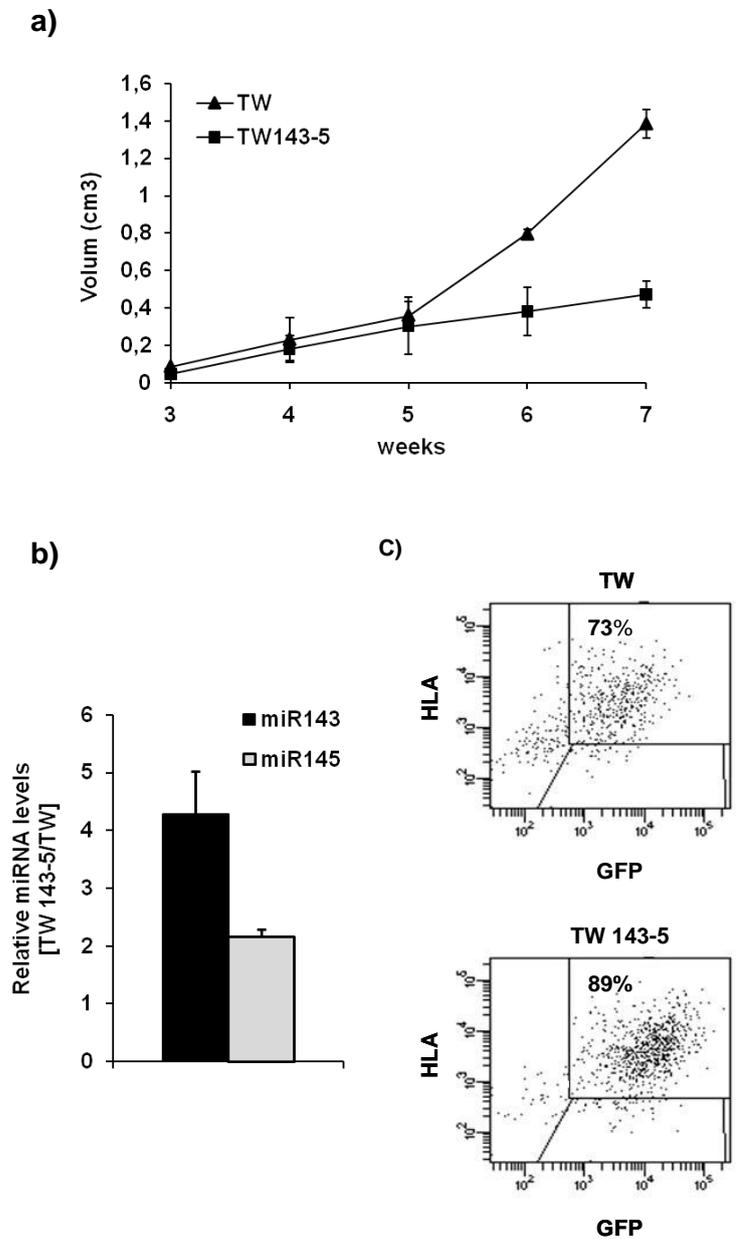


Figure 13. **a)** Tumor volume in SCID mice inoculated with TW or TW-143-5 HT29 cell lines. **b)** Relative mir-143 and mir-145 expression level in tumor xenograft cells after seven weeks. Data are normalized to control vectors (empty TW). **c)** FACS analysis of GFP expression in tumor xenograft cells after mice sacrifice.

assayed the levels of native, processed membrane proteins as these are more informative for the functional consequences. Cells were therefore incubated with the appropriate FITC and PE conjugated antibodies, followed by facs analysis. Although ErbB3 appeared not affected by mir-143 and mir-145 induction (**Fig. 15b**), CD44 was 20% diminished in pLTR-143/5 cells induced with doxycyclin compared to pLTR control (**Fig. 15c**).

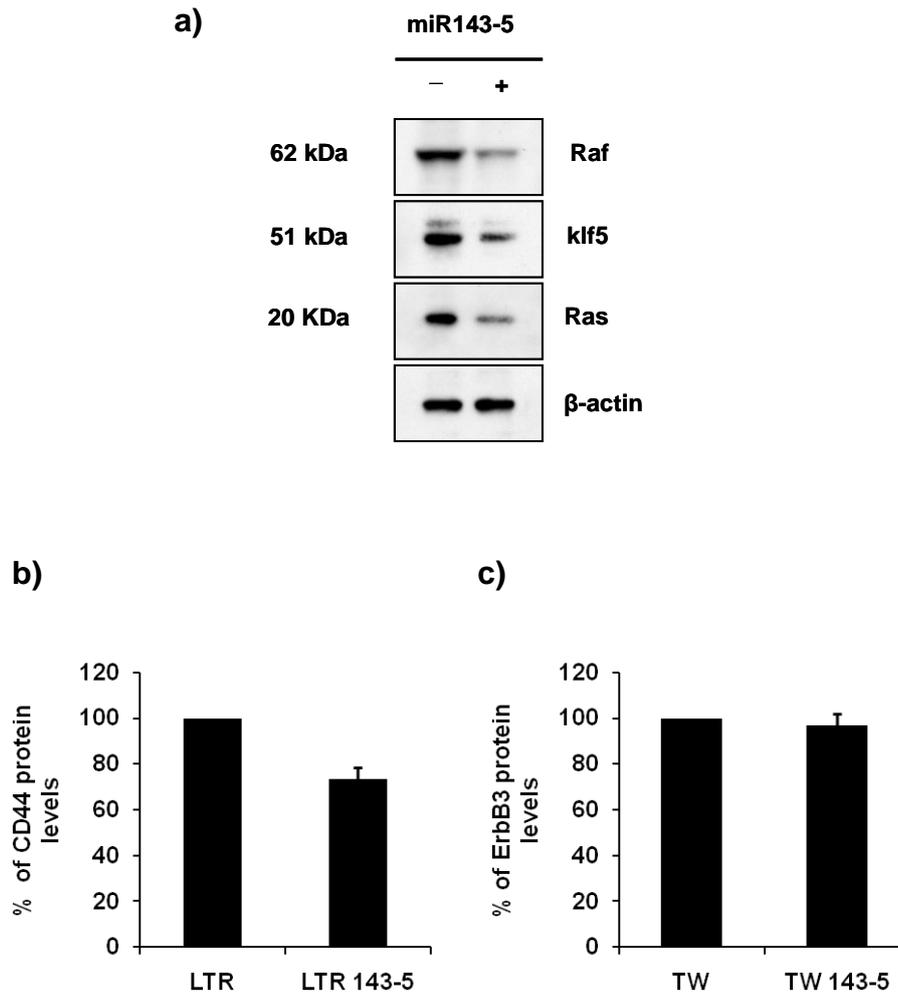


Figure 15. a) k-Ras, b-Raf and Klf5 protein levels revealed by immunoblotting in SW480 cell line transduced with pLTR or pLTR-143-5, 10 days post-sorting, β -actin is used as loading control. b) CD44 protein levels assayed by immunostaining in SW480 cell lines transduced with pLTR or pLTR-143-5, 10 days post-sorting. c) ErbB3 protein levels assayed by immunostaining in SW480 cell lines transduced with TW or TW 143-5, 10 days post-sorting.

We then investigated whether the decrease in the target proteins could be attributed to the direct action of the overexpressed microRNA on the target transcripts. To do this, we first performed the relative quantification of target transcripts in the cell lines used for the protein analysis experiments, secondly we created luciferase-based reporter constructs bearing part of the target transcript 3'UTR/CDS after the renilla reporter protein stop codon (**Fig. 17a**).

Our results show that the mRNA for the target proteins remain unaffected by microRNA overexpression, so that the mechanism leading to protein downregulation is essentially posttranscriptional in nature (**Fig. 16**).

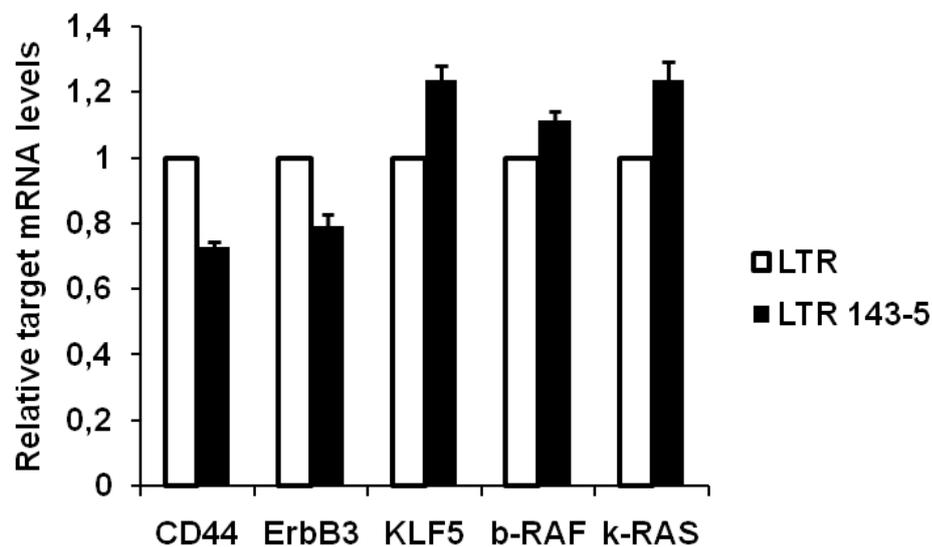


Figure 16. Relative expression levels of gene target in SW480 LTR-143-5 compared to SW480 pLTR, 10 days post-sorting.

When we performed the reporter gene experiments, we compared the activity of the target 3'UTR/CDS construct with that of a cognate construct bearing a mutation of the “seed” sequence, that is, the sequence that base pairs with residues 2-8 of the microRNA. We thus obtained the relative strength of inhibition for the microRNA/target site pair, showing that all the target sites we found were genuine (**Fig. 17b**). However, for Raf we didn't observe any inhibition of luminescent signal in

miRNA/wt target site pair compared with mutant, probably because the putative mir-143 binding site is in the CDS of the gene target. Moreover we analyzed two target binding site in 3'UTR of Ras, but only one (143a) seem to be active.

This data provided the molecular basis for the assumption that mir-143 and mir-145, being coregulated and sharing targets and affected pathways, can be considered as part of a system of gene repression that integrates in the oncosuppression mechanism elicited by p53.

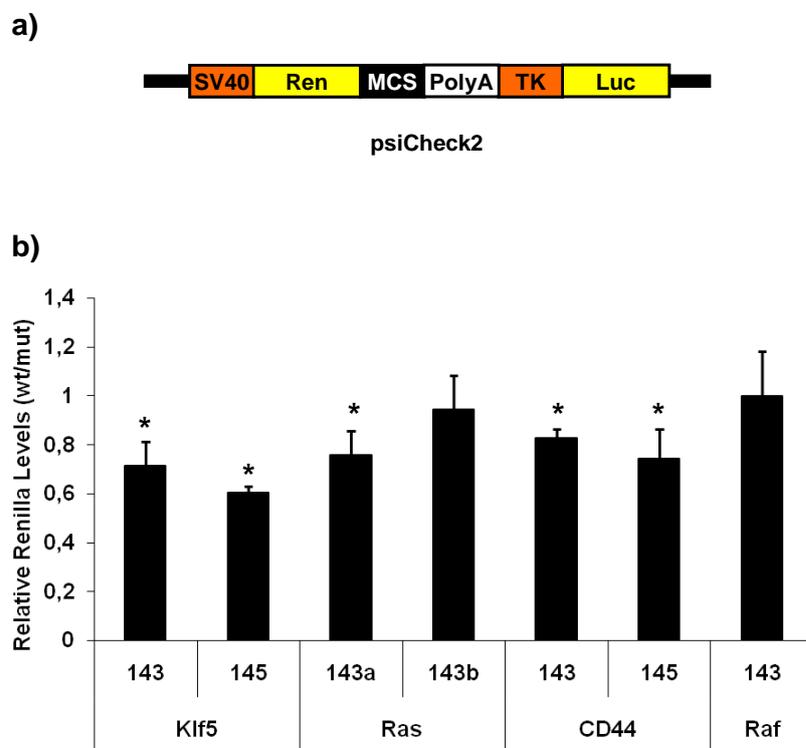


Figure 17. a) Schematic description of the reporter construct used for the validation of mir-143 and mir-145 base pair on the target transcripts. The putative 3'UTR/CDS miR-143 and -145 binding site wt and mutated were cloned in MCS (multicloning site) b) Relative renilla activities was calculated by dividing the values obtained in 3'UTR/CDS binding site wt vector by those of mutated. (* $p < 0,05$).

The subset of target genes we identified is centered in the MAPK activation pathway, with the exception of CD44 being at least partly connected with the suppression of growth by p53. To ascertain whether decrease in any of these proteins mediates the oncosuppressive properties of the mir-143-145 system, we performed proliferation and migration assay with the concomitant transduction of lentiviral constructs that allow the restoration of single target genes, so as to observe partial or total phenotype recovery.

Target genes lacking mir-143 and mir-145 binding site were cloned in a lentiviral vector TW-GFP or TW-CFP between a CMV promoter and a PGK promoter/GFP or CFP cassette. First, colon cancer cell lines (Hct 116, Sw480, Ht29) were infected with inducible vector based-system (pVL-rtTA, pVL-tTR) and positive cells were selected by antibiotic resistance. Next, cells were infected with the lentivirus for constitutive expression of the mir-targeted gene (TW-target) and positive cells were collected by FACS-sorting. Finally, cells were infected with pLTR or pLTR-143-5 vector, induced with doxycycline and after two days RFP-positive cells were flow-sorted and used for the next assays.

In functional rescue assays, target gene restoration induced an increase in terms of proliferation and migration ability of colon cancer cell lines compared with cells where only miR-143 and mir-145 are overexpressed. Proliferation and migration enhancement is comparable to cells infected with pLTR only (**Fig 18-19**).

We confirmed k-Ras, b-Raf, Klf5 protein overexpression by immunoblotting and CD44 protein overexpression by immunostaining. **Fig 20** shows that target protein levels increase when mir-143 and mir-145 are expressed in association with TW-target vectors compared with cells where miRNAs are overexpressed alone.

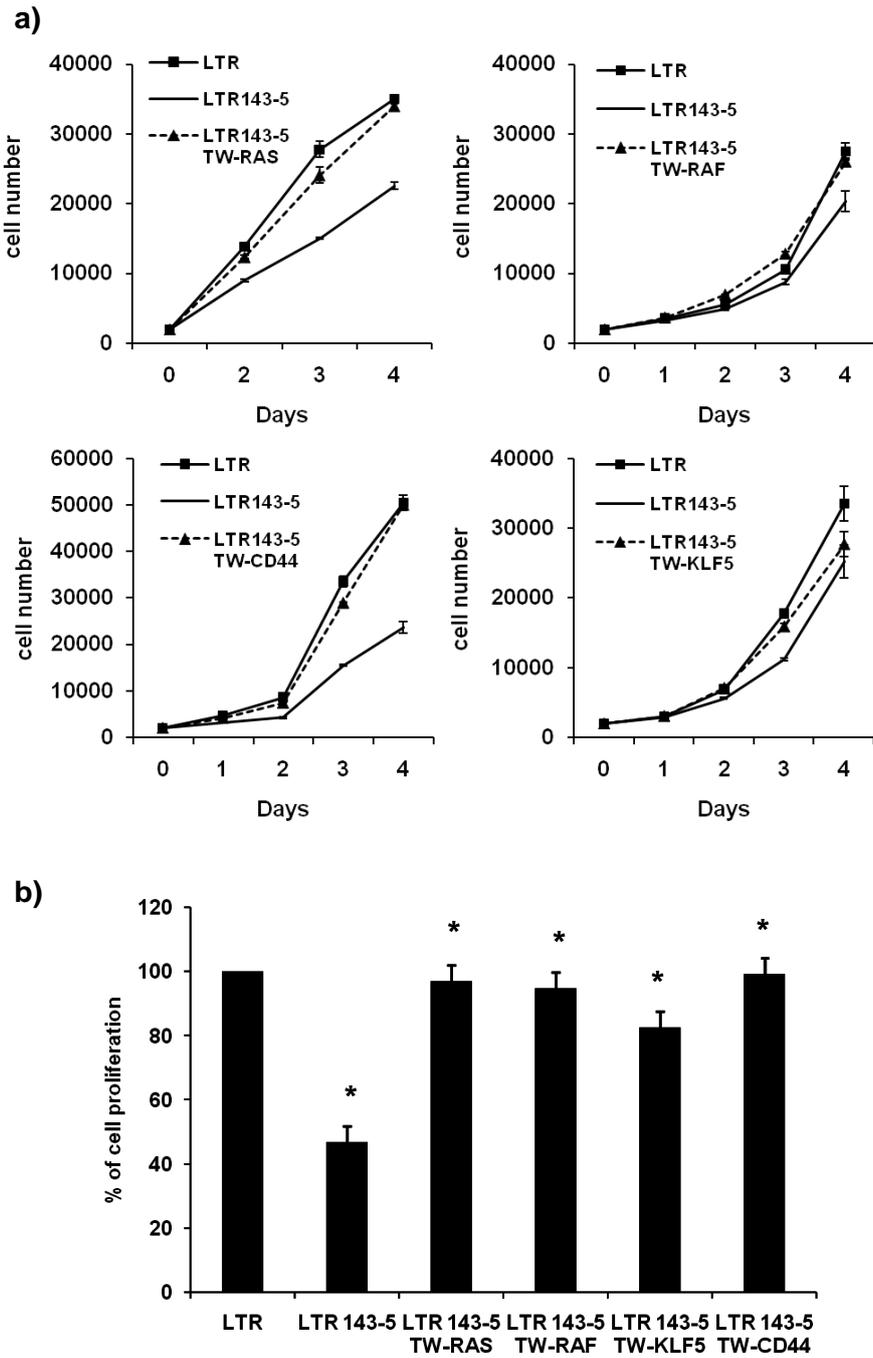


Figure 18. a) Growth kinetic of Sw480 cell line infected with pLTR, pLTR 143-5 or pLTR 143-5/TW-target lentiviral vectors indicated. b) Graph shows percentage of proliferation SW480 cell line infected with indicated target at 4 day post-sorting. (* $p < 0.05$).

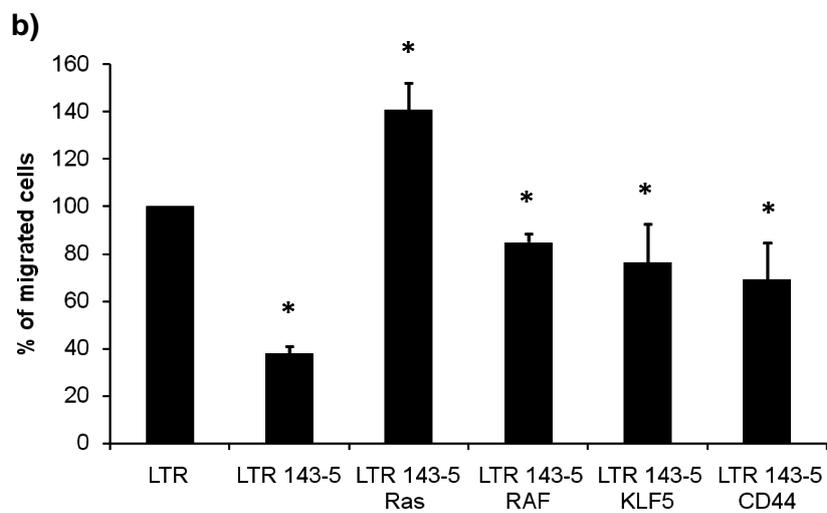
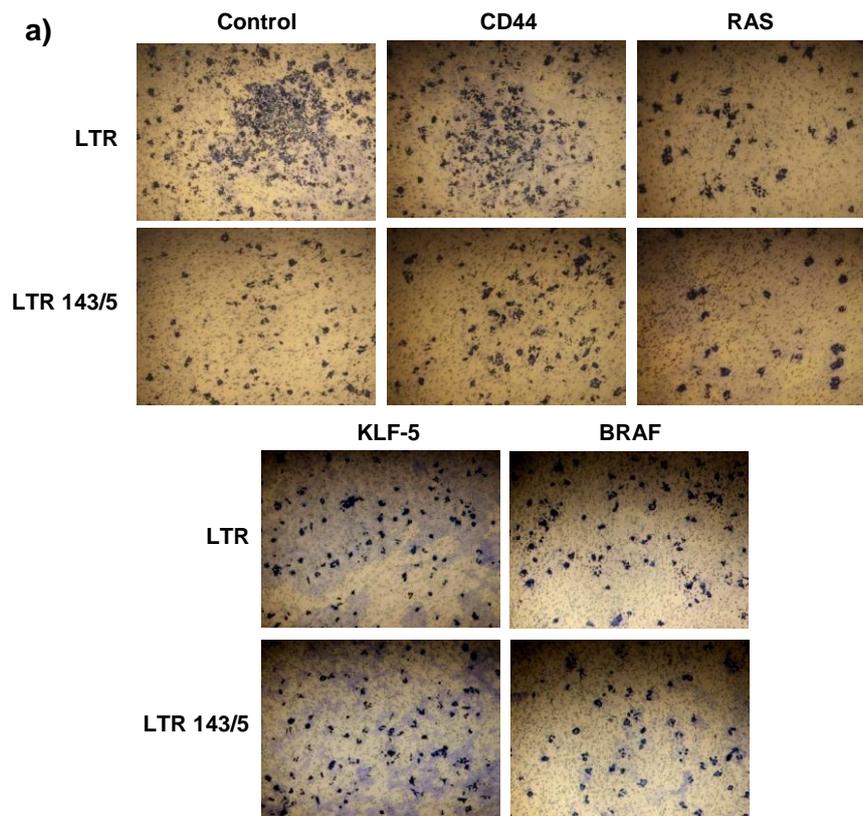


Figure 19. **a)** Migrated SW480 cell line fixed and stained with coomassie blue 48 hours post-sorting. **b)** Percentage of migrated cells infected with indicated vectors 48 hours post- sorting.

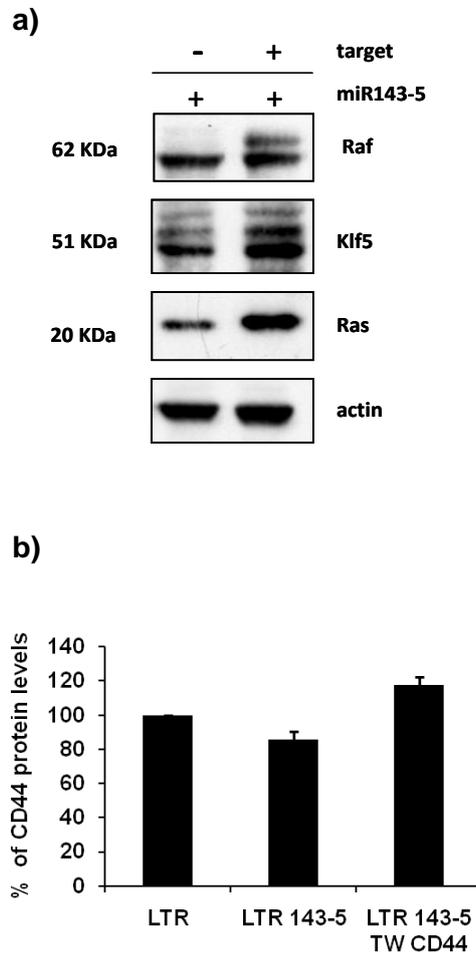
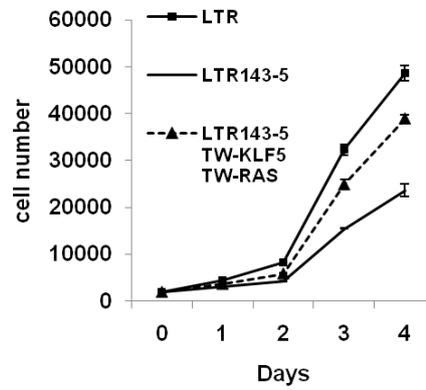


Figure 20. **a)** k-Ras, b-Raf and Klf5 protein levels revealed by immunoblotting in colon cancer cell lines with or without TW-target overexpression vectors, 10 days after the last sorting. **b)** CD44 protein levels revealed by FACS analysis in colon cancer cell lines with or without TWG-CD44 overexpression vectors, 10 days after the last sorting.

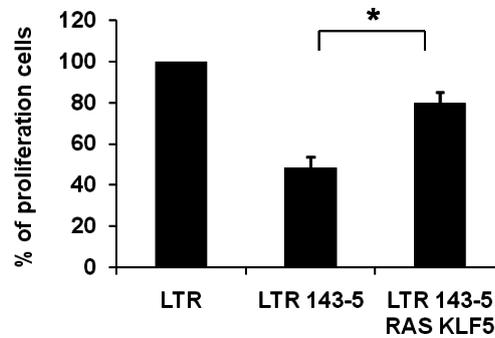
However, none of them can, by itself, allow for the full restoration of the proliferation capabilities that are affected by mir-143 and mir-145. We also performed double coexpression experiments with Klf5 and k-Ras, as a synergistic effect was expected based on previous evidences (Nandan et al., 2008). Proliferation was partially restored also in this case (**Fig. 21**). We cannot exclude that expressing the two transgenes over their physiological levels might lead to compensation of b-Raf and CD44 deficit. When we examined the effect of target restoration on the migration capabilities of CRC cells overexpressing mir-143 and mir-145, we could observe that restoring RAS expression was sufficient to completely recover the ability of CRC cells to migrate, while a weaker effect was seen upon overexpression of CD44, BRAF and KLF5. Again, it might be inferred that the weak effect is dependent on nonphysiological levels attained through the use of a strong promoter, and that a recovery in the proliferation capabilities might slightly affect the readout of the migration assay.

Rescue assay results favor the hypothesis that downmodulation in all target genes is responsible for the oncosuppressive properties of mir-143 and -145.

a)



b)



c)

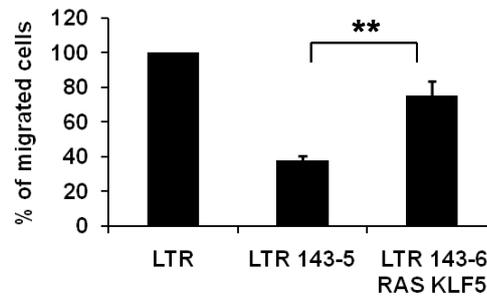


Fig 21. a) Growth kinetic of Sw480 cell lines infected with pLTR, pLTR 143-5 or pLTR 143-5, TWC-Ras and TWG-klf5 lentiviral vectors. b) Percentage of proliferation SW480 cell line in a) at 4 day post-sorting. c) Percentage of SW480 migrated cells infected with the same vectors as in a) 48 hours post- sorting.

Discussion

Deregulation of gene expression is currently one of the most studied molecular features of diseased tissue, both for diagnostic and therapeutical purposes. The identification of recurrent patterns of differential gene expression in normal and diseased tissue has helped to clarify that gene profiles are intertwined with the array of mutation that are causative to the genesis of a given pathology. Some of the most known oncogenic lesions, for example those leading to loss of function of TP53 or to c-Myc overexpression, have been found to entail the differential expression of a subset of microRNAs that might be active players in the mechanism of oncogenesis or oncosuppression, hence representing a point of therapeutic intervention. To distinguish between microRNAs whose downregulation is a mere bystander event to the oncogenic lesion, and those which are intrinsic to oncosuppression, functional assays can be performed, where their transcript are re-expressed back to normal levels.

Most reports on mir-143 and mir-145 have considered them as independent genes, while our study examines the effect of their concomitant re-expression, as we speculate that they are co-transcribed and that they have partly overlapping functions/target genes. We found that enhanced expression of mir-143 and mir-145 in CRC cells might lead to oncosuppression by altering the cells' ability to proliferate, migrate, grow in an anchorage independent manner, and undergo apoptosis upon genotoxic stimulation. Coherent with these observations, our experiment of *in vivo* xenotransplantation show that enhanced expression of mir-143 and mir-145 provokes a sensible delay in the growth of subcutaneous tumors. We have also found that re-establishment of mir-143 and mir-145 oncosuppressor functions in tumoral cell lines, can impose a proliferation block/delay that, upon prolonged culturing, might eventually result in a counter-selection effect, in our case loss of transgene expression. Hence, we speculate that for long term (more than two weeks) studies of microRNAs with oncosuppressor function, a reversible and

controllable expression system might be more appropriate than a constitutive one although, in the real manifestations of tumoral pathology, loss of oncosuppressor expression is supposed to be permanent.

The definition of the subset of microRNA target genes that mediate an oncosuppressor or oncogenic effect is one of the most intriguing aspects of this field of study, as it is probably the element that by itself can explain most of the mechanism of action which eventually need to be tackled by a therapeutical instrument. Reports abound where a single microRNA-target gene interaction is claimed to be responsible for a given effect, although search engines typically return hundreds of target transcripts to a single microRNA query. In our study of the mir-143 and -145 regulatory action, we tried to focus on a larger number of targets chosen for being cotargeted by the two transcripts and for belonging to the IGF/EGF signal transduction pathway. We found that, in addition to k-RAS, a previously known mir-143 target (Chen et al., 2009), the two microRNAs downregulate two important mediators downstream of k-RAS. Both microRNAs do indeed target the KLF5 transcription factor, which synergizes with k-RAS in colorectal transformation (Nandan et al., 2008), and mir-143 targets BRAF, the immediate downstream mediator of KRAS activation. Notably, we have found a target site for mir-143 in the coding sequence (CDS) of the BRAF mRNA. Search engines normally neglect this region, as well as the 5' UTR, and we believe this is detrimental to the comprehension of microRNA biology, considering that other articles have reported the occurrence of microRNA target sites in regions other than the 3'UTR (Tay et al., 2008). In addition to that, we found that both microRNAs target CD44, a receptor that transduces survival signals to the cell. To establish whether the decrease in any of these target proteins was dispensable for mir-143 and mir-145 action, we expressed exogenous target genes along with the microRNAs. Given the intrinsic complexity of such an approach, we have investigated the single contribution of the downregulation in target protein levels on the overall oncosuppression phenomena we observed. We have thus found that any decrease in single target proteins is involved to a different extent in the phenotype of growth suppression and, based also on published evidence, we conclude that the microRNAs mir-143 and -145 act in a concerted manner to suppress the IGF-EGF receptor pathway at multiple levels. It

has been shown that the regulatory network of a microRNA comprises a small number of genes that are heavily affected at the protein level, and a larger group of genes that are mildly downregulated (Baek et al., 2008; Selbach et al., 2008), where secondary effects are to be counted as well. Although we believe that other mir-143 and mir-145 targets could be implicated in cell growth and survival, we propose that the concerted action on the EGF-IGF signaling pathway and on the CD44 survival pathway can account for a substantial part of the oncosuppression properties of this regulatory system. Microarray profiling experiments are underway to observe whole transcriptome changes in CRC cells that are forced to re-express mir-143 and -145 and further define the affected pathways.

MicroRNAs have emerged as key players in posttranscriptional gene regulation, and as indispensable mediators of several important biological processes, including tumorigenesis and tissue homeostasis and regeneration (Esquela-Kerscher et al., 2006). In their unique chemistry lies a great advantage over protein-encoding genes: it is becoming evident that, if a gene therapy would be required, their synthesis, stability, and delivery to target cells is much more feasible and immediate than for proteins. Very recently, it has been shown that the use of an adeno-associated virus for restoration of a microRNA in a murine model of hepatocellular carcinoma has a tremendous impact on tumor progression (Kota et al., 2009). Besides viral-based therapy, other studies support the possibility that microRNAs can be delivered systemically to target organs, without the risk of immune reaction (Yun et al., 2003). This prompts for extensive investigation of the potential role of microRNAs in disease and as instruments of molecular medicine.

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