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Identifying cancer-causing noncoding RNAs

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Citation

Le Sage, C. K. (2008, January 10). *Identifying cancer-causing noncoding RNAs*. Retrieved from <https://hdl.handle.net/1887/12553>

Version: Corrected Publisher's Version

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Identifying cancer-causing noncoding RNAs

Carlos Karel le Sage

On the cover: 'hunting the deadliest catch'

Identifying cancer-causing noncoding RNAs

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 10 januari 2008
klokke 13.45 uur

door

Carlos Karel le Sage

geboren te Sluiskil in 1979

Promotiecommissie

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Printed by Ponsen & Looijen, Wageningen, The Netherlands

The work described in this thesis was performed at the Division of Tumor Biology of the Netherlands Cancer Institute, Amsterdam, The Netherlands. This work was supported by grants from the Dutch Cancer Society (KWF). Publication of this thesis was financially supported by the Dutch Cancer Society (KWF).

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ISBN-13: 978-90-9022458-9

Voor mijn ouders

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Scope of the thesis

The first miRNA, the small-temporal lin-4 RNA, was discovered in 1993, during forward genetic experiments aimed at finding new genes involved in developmental timing of *C. elegans* larvae. Lin-4 is temporarily expressed from the first to the third larval stage, and inhibits the production of lin-14 and lin-28 proteins. In turn, the temporal decrease in lin-14 and lin-28 proteins is crucial for the correct timing of events during the larval stages. Strikingly however was the fact that lin-4 did not constitute a protein, but a noncoding RNA (ncRNA) instead. Even more remarkable was the finding that the lin-14 mRNA had multiple partial complementary sequence elements within its 3'UnTranslated Region (3'UTR), suggesting lin-4:lin-14 RNA:RNA interactions to be responsible for the clearing of lin-14 protein through a mechanism called translational repression.

Although initially regarded as a unique means of gene regulation specific to worms, the lab of Gary Ruvkun discovered a second small-temporal RNA, let-7, in the year 2000. However, let-7 was found to be expressed in both worms and fruitflies, which initiated the search for more small noncoding RNAs among different species. One year later, various studies reported the cloning and sequencing of a class of noncoding RNA genes conserved throughout species. These ncRNAs were dubbed microRNAs (miRNAs).

miRNAs are produced as long primary pol II-dependent transcripts (pri-miRNAs), that form stem-loop structures, and are cleaved in the nucleus by the RNaseIII-like enzyme Drosha to yield precursor miRNAs (pre-miRNAs). The shortened stem-loop shaped pre-miRNA is exported to the cytoplasm for further trimming by the RNaseIII-like enzyme Dicer, producing small 21-23 nt RNA duplexes. Because of thermodynamic differences between the two ends of the duplex, one strand is peeled off and incorporated into the RNA-induced silencing complex (RISC). RISC can induce translational repression or mRNA decay, depending on its composition. Nonetheless, it is the miRNA sequence, being (partially) complementary to mRNAs that guides RISC to the appropriate target(s).

In **chapter 1** the different species and functions of small noncoding RNAs in general are introduced. In **chapter 2** I present a detailed overview of the discovery and functions of microRNAs, in both normal and malignant conditions.

Whereas the number of annotated miRNA genes in humans is reaching 500, their functional cellular and organismal assignment(s) are only starting to be unraveled. Finding a relevant miRNA target proves to be very difficult, mainly because animal miRNAs are known for their partial complementarity towards targets. Based on the discovery of a handful of miRNA targets, and studies

designed to detect miRNA effectiveness, computer algorithms have been made that predict possible targets for any given miRNA. The more miRNA targets are characterized, the better the rules that constitute the algorithms will become, thereby narrowing the search for targets, and increasing the probability of finding the relevant targets. However, it is more important to know how many miRNA-mRNA interactions are involved in regulating a given cellular phenotype. Still with the current miRNA-target prediction programs, it is difficult to deduce which cellular pathways are affected by which particular miRNA. We therefore devised an experimental genome wide method to search for miRNAs that function in defined cellular pathways.

In **chapter 3** I describe the construction of a miRNA library and miRNA microarray. Using these tools we show the potential oncogenic function of the miR-372&373 family in testicular germ cell tumors. **Chapter 4** deals with a different screen where our tools were utilized to explore miRNAs that act on the 3'UTR of the tumor suppressor p27. This growth-independent way of screening led to the discovery of the miR-221&222 family as regulators of p27. Subsequent characterization of this miRNA-target pair suggests their involvement in glioblastoma tumors.

In **chapter 5** we identify a novel function for a different noncoding RNA, the human telomerase RNA. hTR is shown to be important for cells to recover from UV-mediated DNA damage.

Finally, the implications of the miRNA findings are summarized and discussed in **chapter 6**.

Chapter 1

Introduction (1)

Introduction to noncoding RNAs

Introduction to noncoding RNAs

Housekeeping ncRNAs

According to the classical view, genetic information that is stored in the DNA of organisms, is transferred to proteins. Proteins are complex molecules required for enzymatic reactions and structural functions within cells. However, since eukaryotic DNA is nuclear and protein synthesis is performed in the cytoplasm, an intermediate step is necessary, and that is where RNA comes into the play. RNA was thought to fulfill only a handful of functions, collectively important for making proteins. Fundamental to this process are copies of pieces of the genome, called messenger RNAs (mRNAs), that function as genetic templates onto which ribosomes assemble. Other forms of RNA are required for the ribosome to generate a polypeptide from one strand of mRNA. These noncoding RNAs (ncRNAs) are termed ribosomal RNA (rRNA) and transfer RNA (tRNA). The former, as the name suggests, is part of the ribosomal structure and plays a key role in the process of peptide-bond formation, while the latter literally translates triplets of mRNA nucleotides into single peptides. Together, these three RNA molecules ensure the formation of a polypeptide, which subsequently is processed and folded into its destined shape by other means. Over the past decades, breakthrough research has unmasked regulatory noncoding RNAs. These tiny and powerful ncRNAs have functional impact in all known pathways, something which enforced re-evaluation of the functional capabilities of RNA.

Genomic 'junk' makes sense after all

How can the same principal material produce so many different bodyplans? Differences in the architecture of species seem to arise from variations in the ratio between genomic output and protein production. Prokaryotes carry a gene-dense genome, and proteins dominate their genomic output (Mattick, 2004). Eukaryotes on the other hand have a lot of DNA that is not destined to be coded into proteins. These large stretches of intra- and intergenic sequences of DNA were termed 'junk', since they are either spliced out (introns) or do not seem to be transcribed at all, therefore useless to the purpose of synthesizing proteins. Then why would so many different eukaryotes with ever so sophisticated bodyplans populate the earth carrying a load of genetic

rubish in each of their nuclei? Of greater significance is to know why natural selection has favored its preservation.

Comparative analyses between genomes of micro-organisms and multicellular organisms revealed interesting relationships between complexity of the organism involved, protein output from its genome, and amount of genomic 'junk'. More complex organisms (or higher organisms) have far more nucleotides than lower species. Larger genomes harbour more protein coding genes, however the incline in protein coding genes is far less compared to the increase in genome size (Mattick, 2001). Therefore it seems that the relatively small increase in protein (including splicing variants) cannot fully account for the big increase in developmental and physiological complexity of higher eukaryotes. Strikingly, the amount of 'junk' DNA does augment in proportion to the increase in complexity (Frith et al., 2005). What is kept hidden, and (how) does this contribute to the more sophisticated architectural bodyplans of higher organisms?

In humans, while more than 40% of the genetic code is thought to be transcribed, only less than 2% is reserved for producing protein (Cheng et al., 2005). Most of the transcripts are thus classified as noncoding RNA (ncRNA). Advances have been made in different areas with respect to these ncRNAs, including classification, genomic localisation, transcription, processing, destiny and function. Following below, examples of the best known ncRNA species will be highlighted.

Noncoding RNA species

With few exceptions, RNA molecules themselves do not display enzymatic activity, and the same restriction holds true for ncRNAs. In order for ncRNAs to carry out their specific functions, proteins are assembled onto the RNA, giving rise to a so-called noncoding ribonucleoprotein (ncRNP). The RNA part specifies which targets are to be regulated, the protein members determine the fate of the targets. Different ncRNPs show different modes of behaviour towards their targets, depending on the functional qualities the protein part has to offer. On one side, the purpose of ncRNAs is to execute small tasks that include editing of RNAs, suppression of translation of mRNAs, and even destruction of target mRNAs, thereby introducing another layer of

complexity that governs protein output. On the other hand, certain ncRNA species have been assigned quite different tasks, that involve taking part in telomere synthesis, or regulation of transcriptional output through methylation of DNA and/or histones. Moreover, ncRNAs are required to finetune X chromosome gene output by initiating dosage compensation.

Taken together, ncRNA activities are crucial for gene expression and genomic stability, which is underscored by the fact that deregulated expression of ncRNAs is linked to diseases like cancer.

Small nuclear RNAs (snRNAs)

In general, small nuclear RNAs are part of the spliceosome, a large ncRNP structure involved in trimming intronic sequences from mRNAs (Matera et al., 2007). Like their nucleolar counterparts, snRNAs are 100-300 nt long non-polyadenylated ncRNAs whose functions are confined to the nucleus. After transcription they are exported to the cytoplasm where they are assembled into stable protein-containing snRNPs. The snRNP is then imported into the nucleus and accumulates in so-called Cajal bodies before being delivered at its final destination, the site of transcriptionally active chromatin, where immediate pre-mRNA processing takes place.

Small nucleolar RNAs (snoRNAs)

Unlike the scarce availability of a mere dozen of snRNAs, snoRNAs comprise a large family of over 200 ncRNAs (Matera et al., 2007). Their main task is to edit other ncRNAs, such as snRNAs and rRNA to control both spliceosome and ribosome functional output. After transcription follows immediate association with proteins to form an inactive pre-RNP. Further processing to mature active RNPs is conducted in Cajal bodies, where some snoRNPs remain to modify for example snRNAs, while others move to nucleoli to edit rRNAs.

Interestingly, one snoRNA has quite a different function. hTR (human telomerase RNA), the RNA component of the telomerase RNP, that further contains the hTERT (telomerase reverse transcriptase) protein, is a snoRNA. hTR is known to be involved in the biogenesis and localisation of telomerase, a complex that synthesizes telomere sequence repeats at chromosome ends which are required to protect telomere ends from erosion (Smogorzewska and de Lange, 2004). Chapter 5 deals with hTR, and implicates hTR in regulating the DNA damage response upon UV irradiation.

ncRNAs and dosage compensation

A different application of ncRNA by cells occurs in dosage compensation in fruitflies and mammals, a process that ensures equal transcriptional output from the X chromosomes that are unevenly distributed among males and females. Different mechanisms are deployed to achieve identical X gene transcription between sexes in both fruitfly and mammal. In male flies, the lack of a second X chromosome is compensated with a twofold transcriptional activity from the only X chromosome present. This is thought to be accomplished by a ribonucleoprotein complex called the dosage compensation complex (DCC), which contains various proteins capable of modifying chromatin structure (Gilfillan et al., 2004) and two ncRNAs, roX1 and roX2, expressed solely in male flies. The two X transcribed ncRNAs guide the RNP to X chromatin entry sites, that serve as nucleation sites from where the DCC complex spreads (Kelley and Kuroda, 2000). Hyperacetylation of histone residues appears to be the key event induced by DCC to 'super-activate' X chromosomal genes (Gu et al., 1998; Meller and Rattner, 2002).

Contrasting the fly X chromosomal super-activation is the random repression of the second X chromosome in female mammals to reduce X chromosomal gene expression to the level of that maintained in males (Heard, 2004). Although the mechanism is of opposing nature, X inactivation in placental mammals also depends on ncRNA. In this case, the X inactivation center, localized on both X chromosomes, produces two different transcripts, Xist and its antisense counterpart Tsix. Tsix seems to be involved in controlling Xist transcripts that arise from both X chromosomes. It is thought that at one X chromosome, Tsix expression is silenced, enabling Xist to bind to the X inactivation center from where it initiates and spreads chromatin remodeling. The chromatin is mainly hypoacetylated and methylated at certain lysine residues of histone molecules, and ends up as a densely packed, transcriptionally inactive chromosome. The second X chromosome is left unharmed, with Tsix in control of Xist (Heard, 2004).

Very small noncoding RNAs: RNA interference

RNA interference (RNAi) is a way of gene regulation shared by eukaryotes, and involves suppression of protein production and gene expression. The former is known to occur through extensive base-pairing with target mRNAs leading to either degradation or translational repression of the

mRNA. Silencing of gene expression on the other hand involves an intimate cooperation between both RNAi and chromatin regulatory pathways, as discussed later.

In 1990 it was discovered that introduction of a flower pigmentation transgene in *Petunia* plants did not result in the expected dark purple color, but instead led to white flowers (Napoli et al., 1990; van der Krol et al., 1990). The phenomenon was dubbed co-suppression, as the expression of both transgene and endogenous pigmentation genes bearing homologous sequences were silenced. Two pathways were found to be responsible for the observed silenced phenotype: PTGS (posttranscriptional gene silencing) (Bartel, 2004; Meister and Tuschl, 2004) and TGS (transcriptional gene silencing). Both pathways are linked in the sense that they require similar sets of molecules: small RNAs for the recognition of complementary sequences, and specialized proteins involved in the subsequent induction of silencing. PTGS was shown to be a cytoplasmic event, as the targeted transcripts were found to be degraded in the cytoplasm (de Carvalho Niebel et al., 1995). Shortly after the discovery of PTGS mediated gene regulation in plants, a similar mechanism, known as quelling, was found in the fungus *Neurospora crassa* (Cogoni and Macino, 1997).

RNA interference, the animal equivalent of PTGS/quelling, was first discovered in the nematode *Caenorhabditis elegans* (Fire et al., 1998) as a response to dsRNA (double stranded RNA). The introduction of dsRNA induced specific degradation of mRNAs carrying sequences homologous to the dsRNA. At the time, RNAi seemed constricted to worms, but soon turned out to be functional in fungi, plants (both reviewed in (Tijssterman et al., 2002)), protozoa (Ngo et al., 1998), fruitflies (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999) and vertebrates (Wianny and Zernicka-Goetz, 2000) as well.

RNA (and DNA) silencing is carried out by small noncoding RNAs, whose size ranges from 21 to 31 nucleotides. These small RNAs can be categorized into 3 classes, based on their origin (Figure 1). There are short interfering RNAs (siRNAs), repeat-associated siRNAs (rasiRNAs) or Piwi interacting RNAs (piRNAs), that were only recently discovered, and microRNAs (miRNAs). siRNAs are produced from long dsRNA precursors, while piRNAs arise from a long ssRNA sequence. miRNAs on the other hand, are made from long ssRNA precursors that fold into a

stemloop structure to become double stranded. Each class, except for the miRNAs (chapter 2) and subclasses (when existing), will be discussed below in more detail.

Small RNAs carry out their silencing abilities through base-pairing with RNA/DNA target sequences. The enzymatic reactions that drive silencing are mediated by a specialized set of proteins. These proteins interact with the target-recognizing small RNAs to form effector complexes. Among the proteins that produce mature siRNAs and miRNAs are Dicer and Drosha, with the latter reserved for miRNAs only. These RNase-III type enzymes liberate 21-24 nucleotide long RNA duplexes from long dsRNA precursors. Interestingly, different kingdoms have evolved Dicer proteins for separate tasks. For example, fruitflies are equipped with two Dicers, DCR-1 and DCR-2, for the production of miRNAs and siRNAs respectively. Worms, yeast and humans express only one Dicer, while plants carry four Dicers (DCL1-4) (Meister and Tuschl, 2004). The arising small RNA species is then bound by Argonautes, core proteins of the silencing complex. Argonautes have the ability to bind RNA sequences, cleave target mRNAs, suppress translation of mRNAs, and are involved in recruiting other proteins effective in mediating gene silencing at the posttranscriptional and transcriptional level. Different Argonaute members team up with different small RNA species, giving rise to RISC (RNA-induced silencing complex), miRNP (microRNA-ribonucleo-protein) and RITS (RNA-induced initiation of transcriptional silencing complex), complexes built around siRNAs, miRNAs, and rasiRNAs respectively (Figure 1 upper part).

Short interfering RNAs (siRNAs)

Ever since its discovery, researchers have made use of the RNA interference machinery as a tool to uncover functions of genes by knockdown technology. Still, the precise mechanism of RNAi at that time was largely unknown. A few years after RNAi discovery, endogenously expressed small interfering RNAs were found in a large variety of organisms. So far, siRNAs have been detected in protozoa, fungi, worms, fruitflies and plants, but not in mammals. siRNAs can be further divided into trans-acting siRNAs (tasiRNAs), natural antisense RNAs (nat-siRNAs), siRNAs controlling transposon expression (or rasiRNA), and small scanRNAs (all reviewed in (Kim, 2005)).

TasiRNAs are synthesized from long noncoding dsRNA molecules, that are the products of bidirectional transcription. TasiRNAs, expressed in

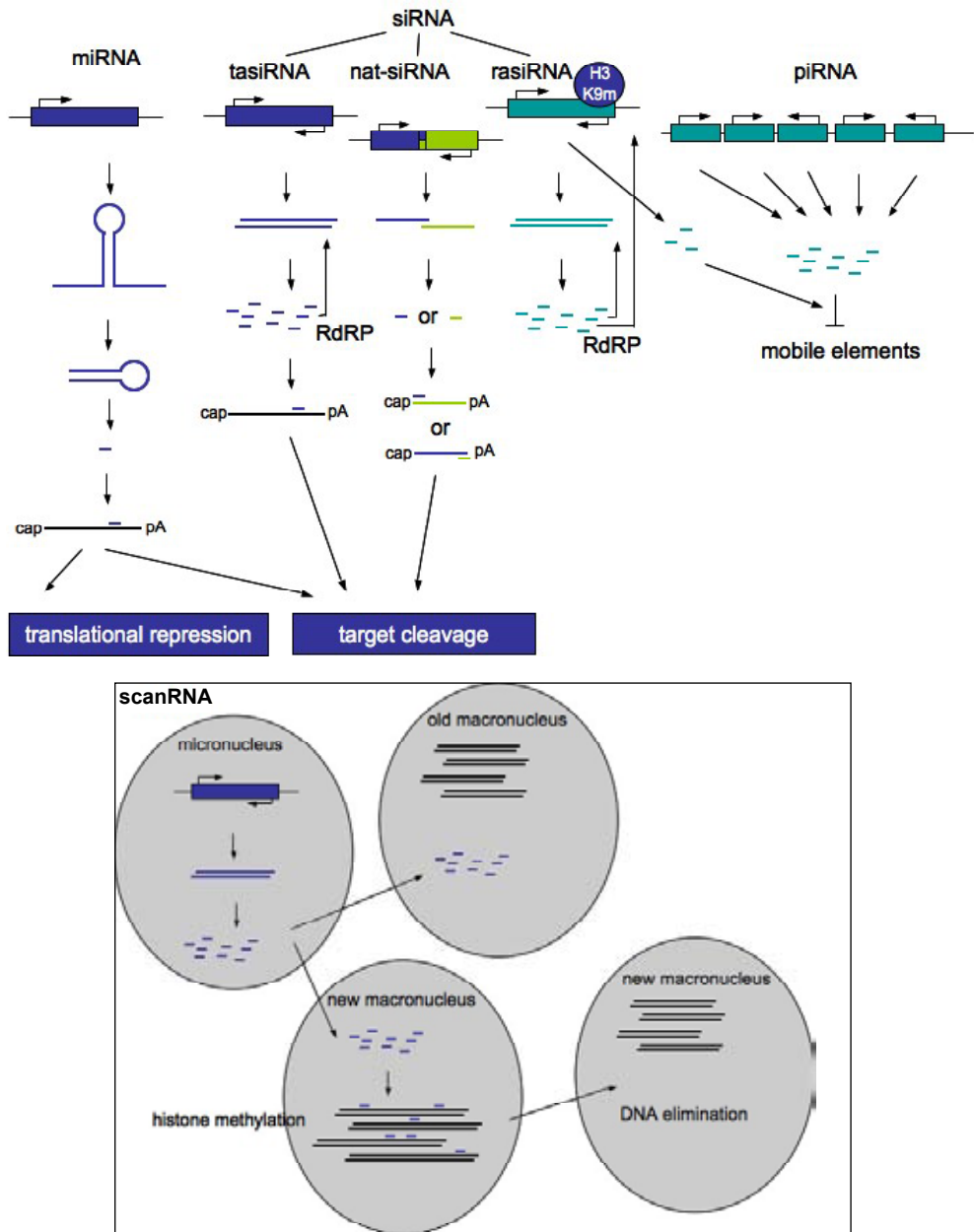


Figure 1. Schematic drawing showing the production and function of different types of small noncoding RNAs. miRNA=microRNA, tasiRNA=trans-acting siRNA, nat-siRNA=natural antisense RNA, rasiRNA=repeat-associated siRNA, piRNA=Piwi-interacting RNA, RdRP=RNA-dependent RNA polymerase

plants and nematodes only, heavily depend on RNA-dependent RNA polymerase (RdRP) function. Dicer recognizes the dsRNAs and chops them up to about 21 nucleotide long duplexes from which one strand is loaded into RISC, which directs cleavage of mRNA targets in trans.

Nat-siRNAs on the other hand, derive from dsRNAs in plants that are produced by the transcription of antisense overlapping genes. The plant dicer DCL1 is responsible for this type of siRNA, which upon expression acts in cis to guide cleavage of one of the two mRNAs to which it is complementary to (Borsani et al., 2005).

Finally, repeat-associated siRNAs (rasiRNAs) are expressed in plants, fruitflies and fission yeast (Kim, 2005). As the name suggests, these small RNAs arise from repeat sequences. RasiRNAs are transcribed as ssRNAs, that become dsRNAs through binding of a transcribed antisense sequence (similar to tasiRNAs), or is turned into a dsRNA molecule through the action of an RdRP. Then, dicer is used to slice the dsRNAs into small rasiRNAs. Instead of being loaded into RISC, required to perform posttranscriptional regulation, rasiRNAs remain in the nucleus and guide the RNAi machinery, in the shape of a RITS complex, to induce transcriptional silencing (Noma et al., 2004; Verdell et al., 2004). As discussed below, this occurs through epigenetic alterations such as DNA and histone methylations to establish heterochromatin in repetitive elements, like centromeric transposons or mobile elements (Lippman and Martienssen, 2004). Interestingly, a different class of small RNAs, the piRNAs seem to fulfill the same task, but only in the germline.

Exogenous siRNAs

Plants mainly use the RNAi machinery to combat viruses. Viruses, integrated into the genomes of plant cells have to produce RNA as part of their life cycle. However, the same RNA is used by plant cells for the production of antiviral siRNAs. In this manner, virus replication is controlled.

small RNAs and transcriptional gene silencing (TGS)

RNA interference is involved in the elimination of transcripts to prevent accumulation of mRNA in the cytoplasm and subsequent translation into protein. Moreover, it has become apparent that the RNAi machinery also negatively affects gene function at the level of transcription. This process, known as transcriptional gene silencing, was first revealed in plants in 1994, where the infection of a viroid in tobacco plants lead to *de novo* methylation of cytosines only pres-

ent in the region of RNA-bound DNA sequences (Wassenegger et al., 1994). The phenomenon was termed RNA-dependent DNA methylation (RdDM), since it depended on the production of small RNAs and the subsequent methylation of the DNA producing these small RNAs. In plants, several mechanisms can give rise to the production of dsRNAs, such as replicating RNA viruses, inverted repeat sequences or transposons. The latter requires an RdRP, which is confined to worms (Smardon et al., 2000), plants (Dalmay et al., 2000; Mourrain et al., 2000) and fungi (Cogoni and Macino, 1999), in order to produce dsRNA species. dsRNA is then processed by Dicer to produce small RNAs that are loaded into an enzyme complex termed RITS. RITS then silences the genomic source that gives rise to the small RNAs through the recruitment of chromatin regulatory factors, such as DNA- and histone-methyltransferases (Almeida and Allshire, 2005). Mutations in components of the RNAi pathway derepress virus production and transposition of transposons. Therefore, the combination of RNAi and chromatin silencing pathways ensures a refined way to prevent genome-harmful DNA sequences, like viruses and transposons, from being expressed.

For example, the centromeric regions of chromosomes in fission yeast contain transposon elements, that need to be silenced in order for kinetochores and cohesins to attach. Silencing of these elements is under the control of the RNAi machinery (Volpe et al., 2002). Indeed, knock-outs of Argonaute, Dicer and RdRP have shown to result in the derepression of expression of centromeric repeat sequences, kinetochore and cohesin mislocalisation and subsequent failure to conduct proper mitosis. Centromeric repeats give rise to small RNA species, that, analogous to plants, are used by an RdRP to produce dsRNAs. The dsRNAs are cleaved by Dicer into small RNA duplexes and one RNA strand is used by RITS to induce silencing of centromeric DNA through histone methylation (Volpe et al., 2002). However, in contrast to plants, the RNA induced silencing serves as a nucleation site from where further methylation and silencing spreads to adjacent genomic regions.

A similar mechanism, called RNAi mediated heterochromatin formation seems to exist in metazoans. RNAi has been implicated in heterochromatin formation in fruitflies. Translocation of genes placing them close to a heterochromatic region may cause inactivation of the genes by

a mechanism known as PEV (positional effect variegation). However, mutation in the Argonaute family members Piwi and Aubergine causes derepression of PEV as a consequence of reduced levels of histone methylation (Pal-Bhadra et al., 2004).

Moreover, vertebrates were found to use RNAi, for the purpose of mitosis. With the inactivation of Dicer in a chicken B-cell line carrying a human copy of chromosome 21, it was shown that human centromeric repeat transcripts accumulated, indicating a derepression of transcription of the sequences from those regions (Fukagawa et al., 2004). Together with mislocalised cohesin the total outcome was premature sister chromatid separation resulting in cell death.

Furthermore, experiments conducted with siRNAs designed to look at silencing by methylation of target genes gave differing results. When siRNAs are targeting coding regions of a gene of interest, the gene is silenced by mRNA degradation rather than TGS. However, siRNAs designed to target the promoter sequence of genes were capable of inducing methylation at those promoters, leading to TGS (Morris et al., 2004). This phenotype could be reversed by the DNA methyltransferase inhibitor 5-aza-2'-cytidine. It is still unclear which type of endogenous RNA molecules could trigger this silencing method.

In conclusion, RNA interference is capable of regulating gene expression both at the translational level, to ensure suppression of unwanted transcripts coming from viruses and transposons, and transcriptional level, where heterochromatin is maintained especially during important parts of the cell-cycle.

scanRNAs

The most extreme form of gene silencing occurs in the ciliated protozoan *Tetrahymena thermophila* (Figure 1 lower part) (Mochizuki and Gorovsky, 2004). Ciliated protozoa are single-celled organisms that possess two nuclei, a micronucleus (or germline nucleus), and a macronucleus (comparable to metazoan somatic nucleus). During conjugation, the micronucleus divides to form a new micronucleus and macronucleus. Then, in a process called DNA elimination, bidirectional transcription of the micronuclear genome (Chalker and Yao, 2001) results in the production of dsRNAs, that, depending on a Argonaute family member, are cut into scanRNAs (Mochizuki et al., 2002). These 28 nucleotide small RNAs move to the macronucleus, and recognize so-called internal eliminated segment (IES) sequences, thought to be transposons or parts of them.

Detection of such sequences by the scanRNAs, induces histone H3K9 methylation, which is required for excision of the marked sequence by specialized enzymes (Liu et al., 2004; Taverna et al., 2002). Therefore, in *Tetrahymena thermophila* ncRNA induced transcriptional gene silencing induced by epigenetic alterations, results in genetic modifications.

Piwi interacting RNAs (piRNAs)

In 2006, 5 research groups discovered another class of noncoding RNAs (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006). These Piwi-interacting RNAs (piRNAs) are different from any other small ncRNA. First, they interact with members of the Piwi subfamily of Argonaute proteins (Grivna et al., 2006) rather than with members of the Ago subfamily that are commonly associated with siRNAs and miRNAs. Second, they are about 24-31 nucleotides in length, which makes them slightly bigger than siRNAs and miRNAs that measure up from 21 to 23 nucleotides. Third, piRNAs are clustered into very distinct genomic loci, which are likely to be transcribed as long primary transcripts, that are processed to produce up to several thousands of piRNAs (Kim, 2006). Importantly, unlike siRNAs and miRNAs, piRNAs are made in a Dicer independent fashion. Indeed, fruitfly, zebrafish and mouse data have collectively provided evidence that Dicer knock-out or mutations do not influence the accumulation of piRNAs (Vagin et al., 2006). However, fruitfly Piwi mutants fail to accumulate piRNAs, which indicates that this subclass of Argonaute proteins is important for piRNA maturation.

What do piRNAs do? Clues to their role come from the tissue distribution in conjunction with the known functionalities of the interacting Argonautes. Whereas Ago members are expressed ubiquitously and interact with miRNAs and siRNAs, Piwi expression is largely constricted to germline cells, together with piRNAs. Piwi has been shown to be involved in epigenetic regulation. It inhibits retrotransposon mobility/activity in fruitfly and zebrafish germ cells that undergo gametogenesis. Interestingly, in mammals, piRNAs are expressed solely in male testis and are involved in spermatogenesis. Piwi mutants have problems in maintaining germ cell viability (fruitfly and zebrafish), oocytogenesis (fruitfly and zebrafish) and spermatogenesis (fruitfly, zebrafish and mammals). Additionally, mutant germ cells and gametocytes show derepression of transposons. Still, piRNAs may have additional functions in mammals where, compared to lower vertebrates

and invertebrates that deploy piRNAs mainly to suppress transposons, only 17-20% map to transposons (O'Donnell and Boeke, 2007). piRNAs seem to add another level of gene regulation and are required for protecting the germ line against selfish element invasion. Nonetheless, a lot of questions remain. How are piRNAs transcribed, why are there so many, and what other functions do mammalian piRNAs have other than transposon silencing? Important for making progress in understanding piRNA biology is to determine their targets.

References

- Almeida, R. and Allshire, R.C. (2005) RNA silencing and genome regulation. *Trends Cell Biol*, 15, 251-258.
- Aravin, A., Gaidatzis, D., Pfeffer, S., Lagos-Quintana, M., Landgraf, P., Iovino, N., Morris, P., Brownstein, M.J., Kuramochi-Miyagawa, S., Nakano, T., Chien, M., Russo, J.J., Ju, J., Sheridan, R., Sander, C., Zavolan, M. and Tuschl, T. (2006) A novel class of small RNAs bind to MILI protein in mouse testes. *Nature*, 442, 203-207.
- Bartel, D.P. (2004) microRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116, 281-297.
- Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R. and Zhu, J.K. (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell*, 123, 1279-1291.
- Chalker, D.L. and Yao, M.C. (2001) Nongenic, bidirectional transcription precedes and may promote developmental DNA deletion in *Tetrahymena thermophila*. *Genes Dev*, 15, 1287-1298.
- Cheng, J., Kapranov, P., Drenkow, J., Dike, S., Brubaker, S., Patel, S., Long, J., Stern, D., Tammana, H., Helt, G., Sementchenko, V., Piccolboni, A., Bekiranov, S., Bailey, D.K., Ganesh, M., Ghosh, S., Bell, I., Gerhard, D.S. and Gingeras, T.R. (2005) Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. *Science*, 308, 1149-1154.
- Cogoni, C. and Macino, G. (1997) Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. *Proc Natl Acad Sci U S A*, 94, 10233-10238.
- Cogoni, C. and Macino, G. (1999) Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature*, 399, 166-169.
- Dalmay, T., Hamilton, A., Rudd, S., Angell, S. and Baulcombe, D.C. (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell*, 101, 543-553.
- de Carvalho Niebel, F., Frendo, P., Van Montagu, M. and Cornelissen, M. (1995) Posttranscriptional cosuppression of beta-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. *Plant Cell*, 7, 347-358.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, 806-811.
- Frith, M.C., Pheasant, M. and Mattick, J.S. (2005) The amazing complexity of the human transcriptome. *Eur J Hum Genet*, 13, 894-897.
- Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T. and Os-himura, M. (2004) Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat Cell Biol*, 6, 784-791.
- Gilfillan, G.D., Dahlsveen, I.K. and Becker, P.B. (2004) Lifting a chromosome: dosage compensation in *Drosophila melanogaster*. *FEBS Lett*, 567, 8-14.
- Girard, A., Sachidanandam, R., Hannon, G.J. and Carmell, M.A. (2006) A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature*, 442, 199-202.
- Grivna, S.T., Beyret, E., Wang, Z. and Lin, H. (2006) A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev*, 20, 1709-1714.
- Gu, W., Szauter, P. and Lucchesi, J.C. (1998) Targeting of MOF, a putative histone acetyl transferase, to the X chromosome of *Drosophila melanogaster*. *Dev Genet*, 22, 56-64.
- Heard, E. (2004) Recent advances in X-chromosome inactivation. *Curr Opin Cell Biol*, 16, 247-255.
- Kelley, R.L. and Kuroda, M.I. (2000) The role of

- chromosomal RNAs in marking the X for dosage compensation. *Curr Opin Genet Dev*, 10, 555-561.
- Kennerdell, J.R. and Carthew, R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell*, 95, 1017-1026.
- Kim, V.N. (2005) Small RNAs: classification, biogenesis, and function. *Mol Cells*, 19, 1-15.
- Kim, V.N. (2006) Small RNAs just got bigger: Piwi-interacting RNAs (piRNAs) in mammalian testes. *Genes Dev*, 20, 1993-1997.
- Lau, N.C., Seto, A.G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D.P. and Kingston, R.E. (2006) Characterization of the piRNA complex from rat testes. *Science*, 313, 363-367.
- Lippman, Z. and Martienssen, R. (2004) The role of RNA interference in heterochromatic silencing. *Nature*, 431, 364-370.
- Liu, Y., Mochizuki, K. and Gorovsky, M.A. (2004) Histone H3 lysine 9 methylation is required for DNA elimination in developing macronuclei in *Tetrahymena*. *Proc Natl Acad Sci U S A*, 101, 1679-1684.
- Matera, A.G., Terns, R.M. and Terns, M.P. (2007) Noncoding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat Rev Mol Cell Biol*, 8, 209-220.
- Mattick, J.S. (2001) Noncoding RNAs: the architects of eukaryotic complexity. *EMBO Rep*, 2, 986-991.
- Mattick, J.S. (2004) RNA regulation: a new genetics? *Nat Rev Genet*, 5, 316-323.
- Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, 431, 343-349.
- Meller, V.H. and Rattner, B.P. (2002) The roX genes encode redundant male-specific lethal transcripts required for targeting of the MSL complex. *Embo J*, 21, 1084-1091.
- Misquitta, L. and Paterson, B.M. (1999) Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc Natl Acad Sci U S A*, 96, 1451-1456.
- Mochizuki, K., Fine, N.A., Fujisawa, T. and Gorovsky, M.A. (2002) Analysis of a Piwi-related gene implicates small RNAs in genome rearrangement in *Tetrahymena*. *Cell*, 110, 689-699.
- Mochizuki, K. and Gorovsky, M.A. (2004) Small RNAs in genome rearrangement in *Tetrahymena*. *Curr Opin Genet Dev*, 14, 181-187.
- Morris, K.V., Chan, S.W., Jacobsen, S.E. and Looney, D.J. (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science*, 305, 1289-1292.
- Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T.A. and Vaucheret, H. (2000) *Arabidopsis* SGS2 and SGS3 genes are required for post-transcriptional gene silencing and natural virus resistance. *Cell*, 101, 533-542.
- Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a Chimeric Chalcone Synthase Gene into *Petunia* Results in Reversible Cosuppression of Homologous Genes in trans. *Plant Cell*, 2, 279-289.
- Ngo, H., Tschudi, C., Gull, K. and Ullu, E. (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc Natl Acad Sci U S A*, 95, 14687-14692.
- Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D. and Grewal, S.I. (2004) RITS acts in cis to promote RNA interference-mediated transcriptional and posttranscriptional silencing. *Nat Genet*, 36, 1174-1180.
- O'Donnell, K.A. and Boeke, J.D. (2007) Mighty Piwis defend the germline against genome intruders. *Cell*, 129, 37-44.
- Pal-Bhadra, M., Leibovitch, B.A., Gandhi, S.G., Rao, M., Bhadra, U., Birchler, J.A. and Elgin, S.C. (2004) Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science*, 303, 669-672.
- Smardon, A., Spoerke, J.M., Stacey, S.C., Klein, M.E., Mackin, N. and Maine, E.M. (2000) EGO-1 is related to RNA-directed RNA polymerase and functions in germline development and RNA interference in *C. elegans*. *Curr Biol*, 10, 169-178.
- Smogorzewska, A. and de Lange, T. (2004) Regulation of telomerase by telomeric proteins. *Annu Rev Biochem*, 73, 177-208.

Taverna, S.D., Coyne, R.S. and Allis, C.D. (2002) Methylation of histone h3 at lysine 9 targets programmed DNA elimination in *Tetrahymena*. *Cell*, 110, 701-711.

Tijsterman, M., Ketting, R.F. and Plasterk, R.H. (2002) The genetics of RNA silencing. *Annu Rev Genet*, 36, 489-519.

Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. and Zamore, P.D. (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. *Science*, 313, 320-324.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N. and Stuitje, A.R. (1990) Flavonoid genes in *Petunia*: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell*, 2, 291-299.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I. and Moazed, D. (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. *Science*, 303, 672-676.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I. and Martienssen, R.A. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science*, 297, 1833-1837.

Wassenegger, M., Heimes, S., Riedel, L. and Sanger, H.L. (1994) RNA-directed *de novo* methylation of genomic sequences in plants. *Cell*, 76, 567-576.

Watanabe, T., Takeda, A., Tsukiyama, T., Mise, K., Okuno, T., Sasaki, H., Minami, N. and Imai, H. (2006) Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. *Genes Dev*, 20, 1732-1743.

Wianny, F. and Zernicka-Goetz, M. (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol*, 2, 70-75.

Chapter 2

Introduction (2)

Immense promises for tiny molecules: uncovering miRNA functions

Cell Cycle (2006)

Immense promises for tiny molecules: uncovering miRNA functions

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With the human genome fully sequenced, the need to obtain functional knowledge of many genes became apparent. Amongst them is a large set of recently discovered genes with powerful promise: the microRNA family. Accumulating data assigned functions in a wide array of biological processes to some miRNAs. Here we review the main approaches used to identify and validate miRNAs and describe ways to discover their functions. As potential effectors of many cellular pathways, mis-expression of miRNAs has been implicated in human cancer.

In the beginning

The era of the microRNome, or genomic regions harboring microRNA (miRNA) genes, began with the discovery of two nematode genes identified by a screen for worms with defects in the larval stage transition (Chalfie et al., 1981; Horvitz and Sulston, 1980). Required for traversing the first larval stage (L1), it was found that one of these genes, *lin-4*, negatively regulates the other gene, *lin-14* (Lee et al., 1993; Wightman et al., 1991; Wightman et al., 1993). Surprisingly, *lin-4* did not constitute a protein but rather a small non-coding RNA, the sequence of which bares high complementarity with several sites present in the 3'UTR (UnTranslated Region) of *lin-14*. As a consequence of *lin-4* expression, *lin-14* protein levels diminished, with no apparent difference in mRNA levels. Therefore, this novel posttranscriptional form of gene regulation was explained as translational repression.

Seven years later, further investigation of regulatory genes that define worm developmental timing events led to the discovery of the second noncoding small RNA, *let-7*. Through inhibition of *lin-41* protein expression, *let-7* is required for late larval development (Reinhart et al., 2000; Slack et al., 2000). Unlike *lin-4*, *let-7* was clearly conserved across the animal kingdom, pointing to a broad existence of small noncoding RNAs (Pasquinelli et al., 2000). Because of their function as regulators of specific developmental stages in worms, *lin-4* and *let-7* were first dubbed small temporal RNAs. With the subsequent identification of many more small noncoding RNAs expressed in a broad spectrum of metazoans and

predicted to be involved in processes covering almost all cellular contexts, these 2 founding members now belong to a super-family of genes called microRNAs.

miRNA biogenesis

Most, if not all, miRNAs are produced by a polymerase II-dependent transcription (Cai et al., 2004; Lee et al., 2004). The primary transcript (called pri-miRNA) folds into a characteristic hairpin, that is cleaved by the nuclear RNaseIII-like enzyme Droscha in complex with DGCR8 giving rise to a ~70 nucleotides (nt) precursor miRNA intermediate (pre-miRNA). The pre-miRNA is exported to the cytosol for further cleavage by Dicer, another RNaseIII-like enzyme, to produce the final and functional ~22 nt long, mature miRNA (Denli et al., 2004; Hutvagner et al., 2001; Lee et al., 2003). This short single-strand RNA is used by miRISC (miRNA-associated multiprotein RNA Induced Silencing Complex) to bind target mRNAs at their 3' UTRs (Hutvagner and Zamore, 2002; Mourelatos et al., 2002). Depending on the degree of complementarity and number of binding sites, the mRNA can be silenced by translational inhibition or RNA cleavage mechanisms (Hutvagner and Zamore, 2002). In either case, the miRNA-mRNA duplexes are relocated from the cytosol to so-called P-bodies, where RNA silencing takes place (Liu et al., 2005; Sen and Blau, 2005).

miRNA identification

(i) Forward genetics

Several approaches have been undertaken in the

process of miRNA gene-function discovery. Loss-of-function mutations giving rise to an abnormal phenotype have led to the identification of *lin-4* and *let-7* in worms. An additional forward genetic screen in worms identified the *lisy-6* miRNA and its involvement in neuronal cell fate (Johnston and Hobert, 2003). Similar studies in *Drosophila melanogaster* mutants defective in regulating apoptosis and proliferation identified the bantam miRNA locus (Brennecke et al., 2003). Moreover, miR-14 was found in mutant flies affected for both cell death and fat storage (Xu et al., 2003). Although forward genetic screening provides an unbiased approach for finding genes whose mutation is causative for the observed phenotype, thereby directly coupling gene to function, it is not the most optimal approach for miRNA gene discovery. This is mainly because forward genetics depends on gene disrupting mutations that are not likely to occur in all miRNA genes, and because of the tendency of miRNAs to exist in families that share similar sequences and functions.

(ii) miRNA gene cloning

Direct cloning of miRNAs is an approach that resulted in the discovery of most of the known miRNA genes in various organisms (e.g. worm, fly, mammal and fish, <http://microrna.sanger.ac.uk/sequences/index.shtml>). This method is not dependent on phenotypes or functions and can therefore be applied to any organism. However, cloning is not an unbiased method and is restricted to miRNAs that are expressed in the cells and tissues examined. In addition, highly expressed miRNAs are easier to clone than poorly expressed miRNAs.

(iii) Computer-based predictions

Complementary to the gene cloning approach are the algorithms developed for the identification of miRNAs. Different laboratories have designed several computer programs to detect novel miRNAs. Programs such as miRseeker and miRScan use algorithms to search for RNA sequences that fold into hairpin structures as indication of a potential miRNA (Lai et al., 2003; Lim et al., 2003b). Utilization of miRseeker and miRScan led to the identification of miRNAs in *Drosophila* and *C. elegans* respectively (Lai et al., 2003; Lim et al., 2003a; Lim et al., 2003b). Another successful approach was phylogenetic shadowing that resulted in the discovery and prediction of hundreds of miRNA genes (Berezikov et al., 2005). Phylogenetic shadowing is a technique that determines the level of conservation

of each nucleotide within a given sequence. This approach is based on the better conservation of the 70 nucleotides miRNA precursor sequences compared to sequences flanking the precursor or in the hairpin loops.

Due to comparative analysis between species, many of the mentioned algorithms overlook miRNA genes unique to a certain species. Using a conservation-independent approach, the computer algorithm deployed on the human genome by the group of Bentwich (Bentwich et al., 2005) yielded additional miRNA candidates, many of which specific to primates.

Validation of predicted miRNAs

With the computerized identification of many novel candidate miRNAs, the emphasis lies on validating the expression of these sequences predicted to form hairpins. Different methods are available for doing so. Among these are different forms of gene cloning and sequencing techniques and validation methods involving RNA detection through hybridization assays, such as northern blot, RNase protection assay and a method based on signal-amplifying ribozymes (Lee et al., 2002; Hartig et al., 2004). High-throughput methods, such as microarray and bead-based profiling (Lu et al., 2005), rely on sensitivity and specificity and can therefore be used for identification, validation and expression level of predicted miRNAs. Various microarray studies used specifically designed oligonucleotides spotted on glass slides to which miRNAs complementary in sequence from size-fractionated RNA can hybridize. In the bead-based profiling method miRMASA (<http://gene.genaco.com/miRNA.htm>), oligonucleotides are coupled to beads, each possessing a unique color, which can be selectively monitored and quantified by flow cytometry.

In an attempt to bypass conservation-dependent computer predictions and subsequent validation for each independent prediction, Cummins and colleagues combined SAGE (serial analysis of gene expression) with direct miRNA cloning to discover novel miRNA genes in human colorectal cells (Cummins et al., 2006). The approach involves isolation of small RNA species, followed by ligation of specialized linkers to enable RT-PCR with biotinylated primers. The linkers are enzymatically cleaved and removed by binding to streptavidin-coated magnetic beads. Released tags are concatenated, cloned, and sequenced. Despite depending on miRNA expression, this method yielded over 100 new and validated

miRNAs and promises to be an efficient tool for the identification of novel miRNAs expressed in human tissues.

Still, some miRNA genes might be low expressed, in only a few cell types or in a short time frame.

A way to examine the potential of miRNA expression by a gene locus is through vector-based ectopic expression. In many cases, a genomic DNA fragment including the miRNA with the addition of a minimum of 60 basepairs from each side is sufficient to demonstrate miRNA expression (Chen et al., 2004; Voorhoeve et al., 2006).

miRNA function

(i) Translational repression, mRNA degradation, or both?

As mentioned above, deletion or reintroduction of the *lin-4* miRNA gene in *C. elegans* did not affect *lin-14* mRNA levels but suppressed gene expression, proposing translational inhibition as the sole mechanism (Wightman et al., 1993). Similarly, *let-7* was predicted to suppress the *lin-41* gene by means of translational repression (Reinhart et al., 2000; Slack et al., 2000). More recent, closer investigation into the mechanism of mRNA-target inhibition by miRNAs led to some controversy. Two groups have used reporter constructs that drive the production of mRNA with 3'UTR sequences to which the studied miRNA/siRNA can interact. The target sequence was designed in such a way that target-miRNA or target-siRNA interactions were predicted to contain a central bulge (non-complementary region), important for miRNA/siRNA mediated translational repression. In an initial study, it was determined that miRNA-mediated repression of a reporter target through imperfect binding, depends on the 5' terminal m7G-cap of the mRNA (Pillai et al., 2005). When the translation of the reporter target was driven by an IRES (internal ribosome entry site) sequence, the transcribed mRNA was completely insensitive to miRNA-induced repression. Therefore, it was concluded that miRNAs might prevent the initiation step of translation. Opposing to this was a study based on a reporter construct to which a partially complementary siRNA was designed (Petersen et al., 2006). First it was demonstrated that IRES dependent translation of a target mRNA could be inhibited by miRNA repression. Second, translational repression depended on polyribosome drop off, while translation is occurring. Therefore, it was proposed that miRNA-mediated repression is induced during translation initiation.

However, both studies presented their findings based on a reporter construct that has artificial

miRNA recognition sites, which could be different from natural target mRNAs. Clearly, more experiments are required to identify the precise mechanism through which miRNAs elicit translation inhibition.

In spite of the early reports, evidence is now accumulating arguing that animal miRNAs can also induce mRNA destruction. Close inspection of the regulation of endogenous targets of *let-7* and *lin-4* miRNAs in *C. elegans* demonstrated the capacity of these miRNAs to decrease the mRNA levels of their respective target genes *in vivo* (Bagga et al., 2005). Additionally, microarray analysis on the effects of miRNAs on mRNA levels revealed that delivery of a single miRNA to human cells could reduce the levels of many target transcripts (Lim et al., 2005). miR-124 is expressed in brain cells, whereas miR-1 is preferentially expressed in muscle cells. Delivery of miR-124 to HeLa cells shifted the mRNA expression profile towards that of brain cells, while miR-1 expression created a muscle-like mRNA profile in HeLa cells. Interestingly, two recent papers studying miR-125b and *let-7* in human cells, and miR-430 in zebrafish, shed more light on the actual mechanism by which miRNAs direct target degradation. Both studies report the ability of miRNAs to accelerate deadenylation of target mRNAs, both *in vitro* (Wu et al., 2006) and *in vivo* (Giraldez et al., 2006). It has been described that mRNA destruction involves loss of poly-A tail prior to removal of the 5' terminal cap, resulting in degradation by an exonuclease (Teixeira et al., 2005). Therefore, miRNA-mediated mRNA degradation might follow a similar pathway, although further investigation is required to identify the components involved.

Taken together, accumulating data point out that certain miRNAs can induce degradation of at least some of their target mRNAs. However, it is still unclear whether this degradation is restricted to particular miRNAs or will end up to be a global mechanism for gene suppression by miRNAs in animals. Second, although recent advances, the exact mechanisms by which miRNAs induce translation inhibition and target mRNA destruction are not completely clear. Last, the rules that determine degradation over translational repression are still unknown, simply because only a handful of functional miRNA-target pairs have been identified that serve as an example of how miRNAs deal with mRNA targets. An interesting thought would be that a particular miRNA might induce translational inhibition and mRNA degradation of one target gene while inducing the

translational repression of another, depending on the nature of the interaction between miRNA and target. This idea was already pointed out in a study by the group of Steve Cohen (Brennecke et al., 2005). They proposed that three categories of *in vivo* functional miRNA-target pairs exist. miRNAs may bind their targets by base-pairing with strong 5' and 3' ends, strong 5' end only or weak 5' end and compensatory strong 3' end of the miRNA. Thus, while a lot of progress was made recently, a better understanding of the dynamics and rules governing miRNA-target interaction will facilitate the process of predicting and finding functional miRNA targets in animals.

(ii) Linking miRNAs to biologically relevant targets

The founding members of the miRNA family *lin-4* and *let-7* were discovered through loss-of-function mutational studies in *C. elegans*, linking miRNA directly to a biological function. Since then, several other miRNAs were identified in a similar forward genetic approach, such as *Isy-6* (Johnston and Hobert, 2003), *bantam* (Brennecke et al., 2003) and *miR-14* (Xu et al., 2003), which were discussed above. However, with the existence of hundreds of miRNAs, it seems very unlikely that forward genetic screens can assign functionality to each and every miRNA gene for at least two reasons. First, miRNAs are small genes and are therefore not likely to be hit by mutagens. Second, due to the tendency of miRNAs to appear in families that may exhibit a high degree of redundancy, mutational disruption of a given miRNA might not result in a phenotype.

To facilitate the understanding of miRNA function, researchers turned to target prediction computer algorithms. The basal principles that underlie these target prediction programs are as follows. (1) The most relevant miRNA sequence for target prediction is thought to be nucleotides 2-8 (the miRNA seed) in the miRNA 5' end, with a compensatory role for the 3' end. (2) The local structure of the target mRNA, with preferable accessibility to the miRNA. (3) Minimal amount of large bulges and G:U wobbles. (4) Thermodynamic stability of the miRNA-mRNA interaction. (5) Interspecies conservation. (6) Multiple miRNA recognition sites per target mRNA. In general, the current prediction algorithms take into account at least 3 of the described parameters. The differences in parameter choice and settings for building an algorithm seem to be the reason for different target outputs when the various prediction programs are challenged with the same input miRNA. Nonetheless, all the avail-

able programs have one thing in common: they predict over 100 possible targets per miRNA. However, the biological relevance of each predicted target remains questionable. The current validation methods include the use of reporter constructs with the target 3'UTR, overexpression or knockdown experiments of the target gene, mutational studies and mRNA-expression arrays (Bentwich, 2005) and references therein). As mRNA-expression array analysis is the only high-throughput method available to cope with the functional validation of predicted targets for a given miRNA, and miRNAs prominently function to suppress protein translation, the development of a high-throughput protein expression method is clearly required to validate and understand the function of a given miRNA.

Another strategy to identify target mRNAs for miRNAs was devised by the lab of Robert Weil (Vatolin et al., 2006) circumventing the need for computational predictions. In short, they used miRNAs in complex with target mRNA templates as primers for synthesizing cDNA from human cells. Sequence analysis showed that the recovered cDNA molecules were consisting of defined mRNAs bound by the miRNA, suggesting functional miRNA-mRNA interaction. However, as this analysis relies on interaction rather than on its outcome, the remaining questions are whether all the detected interactions function to suppress gene expression and to what extent functional interactions were missed.

Recently, our lab devised a reverse genetic approach to search for miRNAs that function to alter a certain cellular phenotype (Figure 1). This was done through the development of a miRNA-expression library (miR-Lib) as a tool to perform genetic screens. Together with miR-Array, microarray slides printed with miRNA sequences corresponding to all cloned miRNAs, miR-Lib provides a genome wide approach to search for miRNAs functioning in specific cellular pathways (Voorhoeve et al., 2006).

(iii) miRNAs in cancer

Since it has been predicted that miRNAs might regulate up to 30% of all protein coding genes, it is very likely that they are involved in controlling or fine-tuning many cellular pathways. Therefore, it is not surprising that altered expression of miRNAs can be harmful to cells, tissues and organisms. Recent findings have linked both miRNA-machinery and miRNA-function to cancer. Pertaining to the first are Dicer and Argonaute that were found to be deleted in a certain subset of tumors. Dicer protein levels were found

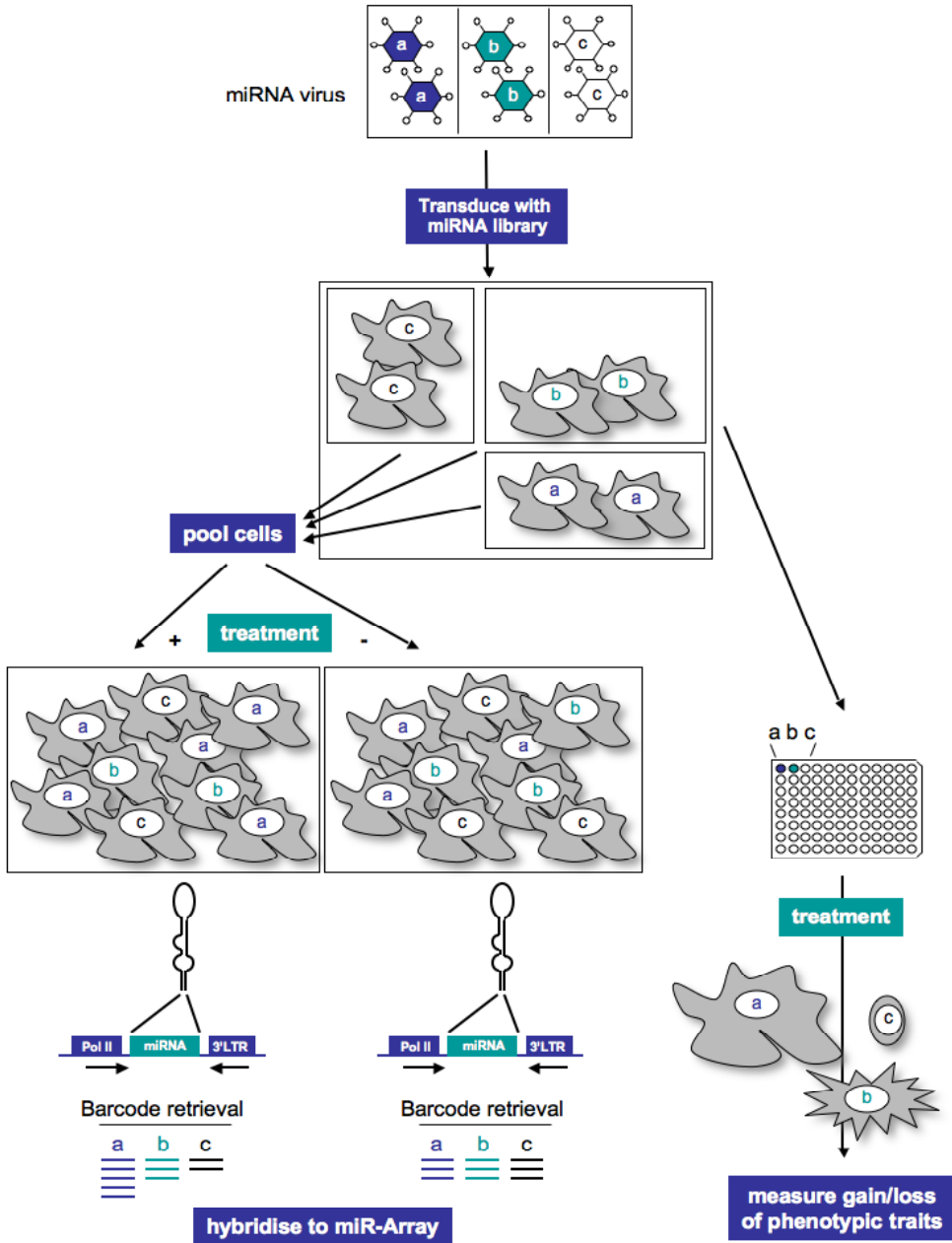


Figure 1. Schematic drawing of miRNA-genetic screen methods. Cells are transduced with individual miRNA vectors (miR-Vecs), drug selected and then either pooled or left unpooled. Pooled cell populations are then subjected to a growth-affecting treatment and its influence on the abundance of each miR-Vec is examined by a microarray experiment with miR-Array (Voorhoeve et al., 2006). Alternatively, the individual miR-Vec transduced cells are separately treated and phenotypically scored. This allows the evaluation of miRNA effects on growth-independent treatments.

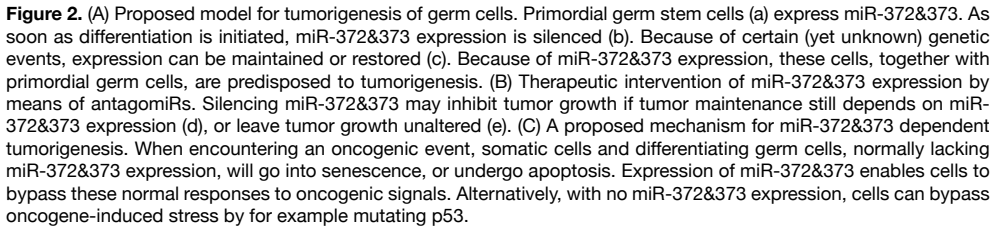
to be reduced, most notably in poor differentiated lung tumors (Karube et al., 2005). Another study showed that elimination of wildtype Dicer expression in chicken DT40 cells resulted in premature sister chromatid separation, indicating a possible initial step in tumorigenesis (Fukagawa et al., 2004). Yet, Kanellopoulou et al. demonstrated the effect of the loss of Dicer expression in mouse embryonic stem cells. Although no apparent cell death was observed, embryonic stem cells were unable to differentiate into the three germ layer types as compared to normal embryonic stem cells, implicating Dicer involvement in development. Last, the expression of Hiwi, the human homologue of the (*Drosophila*) Argonaute family member PIWI, has been correlated with tumorigenesis. The expression of HIWI was found to be enhanced in a subset of testicular germ cell tumors, the seminomas (Qiao et al., 2002).

Different studies have linked specific miRNAs to cancer. Interestingly, over half of the miRNA genes are located at sites in the genome known to be frequently amplified, deleted or translocated (Calin et al., 2004). Some notable miRNAs identified to harbor an oncogenic function are miR-155 and miR-21. The expression of miR-155 (product of the BIC (bicaudal) gene) was reported to be upregulated in patients suffering from Burkitt's lymphoma and Hodgkin's lymphoma (Metzler et al., 2004), where it was correlated with overexpression of the Myc oncogene (Tam et al., 2002). *In vitro* work demonstrated that miR-155 expression cooperates with Myc in lymphomagenesis, most notably by shortening the latency by which lymphomas occurred. Lately, mice transgenic for miR-155, whose expression is targeted to B cells, were shown to develop B cell lymphomas (Costinean et al., 2006), emphasizing the potential of miR-155 in human malignancies. miR-21 was found to be upregulated in both glioblastoma (Ciafre et al., 2005) and breast tumors (Iorio et al., 2005). Glioblastoma cells are resilient to apoptosis, and this was shown to be dependent on miR-21 expression (Chan et al., 2005). Indeed, with the reduction of miR-21 by means of 2'-O-methylated oligos, apoptosis was induced in these cells. Furthermore, a recent study proposed c-Myc as transcriptional activator of the miR-17-92 polycistron, a cluster of six miRNAs (O'Donnell et al., 2005). Two miRNAs from this cluster, miR-17-5p and miR-20a were shown to negatively regulate the transcription factor E2F1, a gene known to be transcriptionally activated by c-Myc. The involved miRNAs induced by c-Myc seemingly counteracted c-Myc function to suppress tumor growth. The same cluster of miRNAs

was shown to have an opposing function in a different study. Haematopoietic stem cells from Eμ-myc transgenic mice were transduced with a retrovirus harboring the miR-17-19b-1 cluster, and reconstituted in mice to allow tumor development. These mice developed tumors at a much earlier onset compared to control mice, implicating an oncogenic rather than a tumor suppressive role for these miRNAs (He et al., 2005).

Recently, our lab has contributed to the list of miRNAs with oncogenic potential (Voorhoeve et al., 2006). Human BJ primary fibroblasts can be transformed by the depletion of p53 and p16 combined with expression of telomerase, small t and RAS^{V12} (Voorhoeve and Agami, 2003). In response to oncogenic stress (expression of RAS^{V12}), BJ cells undergo a growth arrest termed senescence. A screen, designed to identify miRNAs that could bypass oncogenic stress in this system led to the discovery of two oncogenic miRNAs, miR-372 and miR-373. Part of this function was mediated through the inhibition of LATS2, the large tumor suppressor gene 2. The mechanism of LATS2 protein reduction involved the combination of mRNA decay and translation inhibition.

Interestingly, the seed sequence of miR-372 and miR-373 is identical, and is evolutionary conserved to zebrafish, where the miRNAs bearing these sequences are part of the miR-430 family (Giraldez et al., 2005). A recent focus on the function of the miR-430 family demonstrated that the expression of this miRNA family is an important determinant in marking the maternal-to-zygotic transition during zebrafish development (Giraldez et al., 2006). miR-430 was shown to be responsible for the clearance of many maternal mRNAs just after the onset of zygotic transcription. After having served this purpose, the expression of most of the miR-430 family members diminish beyond the detection level. In humans, however, the expression of miR-372 and miR-373 is found in embryonic stem cells and in TGCTs, testicular germ cell tumors (Suh et al., 2004; Voorhoeve et al., 2006). We hypothesise that abnormal expression of miR-372 and miR-373 contribute to the tumorigenic phenotype of TGCTs (Figure 2), at least in part by inhibiting LATS2 expression. It is possible that the normal function of the miR-372 family is to regulate many genes and contributes in maintaining undifferentiated cellular states (e.g. stem cell), its deregulation may induce changes in cellular phenotypes through a limited number of targets. Clearly, more experiments are required to elucidate the function of miR-372 family in stem cells and in tumorigenesis.



With the broad function of miRNAs and the increase in miRNA research tools, it is to be expected that the number of miRNAs that are causally involved in different aspects of the cancerous process will increase in the near future.

Another way to unravel miRNA functions is by specific inhibition of their expression. Interference with the expression of a specific miRNA

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et al. deployed a library of miRNA inhibitors to screen for miRNA dependent roles in growth and apoptotic pathways in a human ovarian cancer cell line (Cheng et al., 2005). While miR-223 knockdown inhibited the retinoic acid dependent differentiation of human granulocytes (Fazi et al., 2005), miR-375 is specifically expressed in pancreatic islet cells and was shown to be an inhibitor of glucose-stimulated insulin secretion (Poy et al., 2004). With the knockdown of miR-375, insulin secretion increased dramatically. An important *in vivo* step was made when LNA-AMOs targeting the large RNA polymerase II subunit were injected locally to tumor-bearing mice. With continuous administration of these oligonucleotides, it was shown that tumor growth could be inhibited *in vivo* (Fluiter et al., 2003).

Whereas the previously described AMOs require constant delivery to ensure miRNA silencing, the biological activity of so-called antagomiRs can last for more than 3 weeks following one administration (Krutzfeldt et al., 2005). AntagomiRs are AMOs conjugated to cholesterol, and when injected into mice were shown to specifically inhibit the expression of miRNAs throughout the organism (except brain) (Krutzfeldt et al., 2005). Altogether, compared with the initial forward genetic screens in *C. elegans*, these new and high-throughput loss-of-function approaches, using tools that interfere specifically with miRNA expression, impose novel resources of studying miRNA functionality within the living organism. Inhibitors of miRNAs turn out to be useful tools to detect miRNA function. Similar to the classical approaches to identify gene function, such as mutation analysis, gene knockout or overexpression, change of phenotype is the direct readout. An important question is whether knockdown of miRNAs in *in vitro* and *in vivo* systems will lead to a phenotype. The future will point out to what extent miRNA knockdown in model organisms such as worm, fly and even mouse will help in demonstrating the biological relevant functions of miRNAs.

An interesting report compared the appearance of miRNA targets and anti-targets within cells (Stark et al., 2005). The results that emerged showed that whenever miRNAs are expressed in a given cell type or tissue, potential targets are not expressed. Anti-targets, or mRNAs seemingly unaffected by miRNAs, maintain avoidance by having very short 3'UTRs that harbor less miRNA recognition sites per kb 3'UTR length compared to target mRNAs (Stark et al., 2005). Therefore, it was speculated that whenever cells face the decision of having to differentiate, miRNAs might

aid in the process by clearing unwanted transcripts. Later on, the same miRNAs are required for clearing residual transcripts. In other words, miRNAs might function to create and maintain the differentiated identity of cells. In this view, miRNA inhibition might usually not result in a dramatic change of phenotype. The phenotypic change from one cell state into the other is more likely to have occurred at an earlier stage, possibly as early as embryogenesis. This is underscored by the initial finding of miRNAs through worm mutator phenotypes. These miRNAs were needed for the larvae to carry out differentiation to transit into the next phase of development. Therefore, to identify miRNA function by inhibition, interference should be carried out at exactly the right moment.

Concluding remarks

With the discovery of a new class of genes, predicted to regulate a large set of protein coding genes involved in different cellular processes, the miRNA family possesses three challenges. The identification and validation of all members of this family. The assignment of a function for each of the miRNA genes. To use this knowledge for developing novel therapeutic approaches. While the first challenge seems to be close to completion, the second and third are still far from reach. Nonetheless, the development of high throughput miRNA-expression, knockout and inhibitory tools are big steps in the right direction. Moreover, while the use of miRNA data is very helpful in diagnosing diseases such as cancer, their usefulness to treatment decisions still needs to be demonstrated. Last, the development of miRNA inhibitors with abilities to penetrate the cell's membrane and affect gene expression may open up new avenues in therapy. It remains to be seen whether hopes raised by the discovery of miRNAs will meet their expectations.

References

- Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R. and Pasquinelli, A.E. (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*, 122, 553-563.
- Bentwich, I. (2005) Prediction and validation of microRNAs and their targets. *FEBS Lett*, 579, 5904-5910.
- Bentwich, I., Avniel, A., Karov, Y., Aharonov, R., Gilad, S., Barad, O., Barzilai, A., Einat, P., Einav, U., Meiri, E., Sharon, E., Spector, Y. and Bentwich, Z. (2005) Identification of hundreds of conserved and nonconserved human microRNAs.

Nat Genet, 37, 766-770.

Berezikov, E., Guryev, V., van de Belt, J., Wienholds, E., Plasterk, R.H. and Cuppen, E. (2005) Phylogenetic shadowing and computational identification of human microRNA genes. *Cell*, 120, 21-24.

Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B. and Cohen, S.M. (2003) bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell*, 113, 25-36.

Brennecke, J., Stark, A., Russell, R.B. and Cohen, S.M. (2005) Principles of microRNA-target recognition. *PLoS Biol*, 3, e85.

Cai, X., Hagedorn, C.H. and Cullen, B.R. (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *Rna*, 10, 1957-1966.

Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M. and Croce, C.M. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*, 101, 2999-3004.

Chalfie, M., Horvitz, H.R. and Sulston, J.E. (1981) Mutations that lead to reiterations in the cell lineages of *C. elegans*. *Cell*, 24, 59-69.

Chan, J.A., Krichevsky, A.M. and Kosik, K.S. (2005) microRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res*, 65, 6029-6033.

Chen, C.Z., Li, L., Lodish, H.F. and Bartel, D.P. (2004) microRNAs modulate hematopoietic lineage differentiation. *Science*, 303, 83-86.

Cheng, A.M., Byrom, M.W., Shelton, J. and Ford, L.P. (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res*, 33, 1290-1297.

Ciafre, S.A., Galardi, S., Mangiola, A., Ferracin, M., Liu, C.G., Sabatino, G., Negrini, M., Maira, G., Croce, C.M. and Farace, M.G. (2005) Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem Biophys Res Commun*, 334, 1351-1358.

Costinean, S., Zanesi, N., Pekarsky, Y., Tili, E.,

Volinia, S., Heerema, N. and Croce, C.M. (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in Eμ-miR155 transgenic mice. *Proc Natl Acad Sci U S A*.

Cummins, J.M., He, Y., Leary, R.J., Pagliarini, R., Diaz, L.A., Jr., Sjoblom, T., Barad, O., Bentwich, Z., Szafranska, A.E., Labourier, E., Raymond, C.K., Roberts, B.S., Juhl, H., Kinzler, K.W., Vogelstein, B. and Velculescu, V.E. (2006) The colorectal microRNAome. *Proc Natl Acad Sci U S A*, 103, 3687-3692.

Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F. and Hannon, G.J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature*, 432, 231-235.

Esau, C., Kang, X., Peralta, E., Hanson, E., Marcussan, E.G., Ravichandran, L.V., Sun, Y., Koo, S., Perera, R.J., Jain, R., Dean, N.M., Freier, S.M., Bennett, C.F., Lollo, B. and Griffey, R. (2004) microRNA-143 regulates adipocyte differentiation. *J Biol Chem*, 279, 52361-52365.

Fazi, F., Rosa, A., Fatica, A., Gelmetti, V., De Marchis, M.L., Nervi, C. and Bozzoni, I. (2005) A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPα regulates human granulopoiesis. *Cell*, 123, 819-831.

Fluiter, K., ten Asbroek, A.L., de Wissel, M.B., Jakobs, M.E., Wissenbach, M., Olsson, H., Olsen, O., Oerum, H. and Baas, F. (2003) *In vivo* tumor growth inhibition and biodistribution studies of locked nucleic acid (LNA) antisense oligonucleotides. *Nucleic Acids Res*, 31, 953-962.

Fukagawa, T., Nogami, M., Yoshikawa, M., Ikano, M., Okazaki, T., Takami, Y., Nakayama, T. and Osimura, M. (2004) Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat Cell Biol*, 6, 784-791.

Giraldez, A.J., Cinali, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., Hammond, S.M., Bartel, D.P. and Schier, A.F. (2005) microRNAs regulate brain morphogenesis in zebrafish. *Science*, 308, 833-838.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J. and Schier, A.F. (2006) Zebrafish miR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*, 312, 75-79.

- Hartig, J.S., Grune, I., Najafi-Shoushtari, S.H. and Famulok, M. (2004) Sequence-specific detection of microRNAs by signal-amplifying ribozymes. *J Am Chem Soc*, 126, 722-723.
- He, L., Thomson, J.M., Hemann, M.T., Hernandez-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J. and Hammond, S.M. (2005) A microRNA polycistron as a potential human oncogene. *Nature*, 435, 828-833.
- Henry, S.P., Geary, R.S., Yu, R. and Levin, A.A. (2001) Drug properties of second generation antisense oligonucleotides: how do they measure up to their predecessors? *Curr Opin Investig Drugs*, 2, 1444-1449.
- Horvitz, H.R. and Sulston, J.E. (1980) Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. *Genetics*, 96, 435-454.
- Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T. and Zamore, P.D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science*, 293, 834-838.
- Hutvagner, G. and Zamore, P.D. (2002) A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, 297, 2056-2060.
- Iorio, M.V., Ferracin, M., Liu, C.G., Veronese, A., Spizzo, R., Sabbioni, S., Magri, E., Pedriali, M., Fabbri, M., Campiglio, M., Menard, S., Palazzo, J.P., Rosenberg, A., Musiani, P., Volinia, S., Nenci, I., Calin, G.A., Querzoli, P., Negrini, M. and Croce, C.M. (2005) microRNA gene expression deregulation in human breast cancer. *Cancer Res*, 65, 7065-7070.
- Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D. and Slack, F.J. (2005) RAS is regulated by the let-7 microRNA family. *Cell*, 120, 635-647.
- Johnston, R.J. and Hobert, O. (2003) A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. *Nature*, 426, 845-849.
- Karube, Y., Tanaka, H., Osada, H., Tomida, S., Tatematsu, Y., Yanagisawa, K., Yatabe, Y., Takamizawa, J., Miyoshi, S., Mitsudomi, T. and Takahashi, T. (2005) Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Sci*, 96, 111-115.
- Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K.G., Tuschl, T., Manoharan, M. and Stoffel, M. (2005) Silencing of microRNAs *in vivo* with 'antagomirs'. *Nature*, 438, 685-689.
- Lai, E.C., Tomancak, P., Williams, R.W. and Rubin, G.M. (2003) Computational identification of *Drosophila* microRNA genes. *Genome Biol*, 4, R42.
- Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75, 843-854.
- Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. and Kim, V.N. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature*, 425, 415-419.
- Lee, Y., Jeon, K., Lee, J.T., Kim, S. and Kim, V.N. (2002) microRNA maturation: stepwise processing and subcellular localization. *Embo J*, 21, 4663-4670.
- Lee, Y.S., Kim, H.K., Chung, S., Kim, K.S. and Dutta, A. (2005) Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. *J Biol Chem*, 280, 16635-16641.
- Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J. and Carthew, R.W. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, 117, 69-81.
- Lim, L.P., Glasner, M.E., Yekta, S., Burge, C.B. and Bartel, D.P. (2003a) Vertebrate microRNA genes. *Science*, 299, 1540.
- Lim, L.P., Lau, N.C., Garrett-Engle, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S. and Johnson, J.M. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, 433, 769-773.
- Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B. and Bartel, D.P. (2003b) The microRNAs of *Caenorhabditis elegans*. *Genes Dev*, 17, 991-1008.

- Liu, J., Valencia-Sanchez, M.A., Hannon, G.J. and Parker, R. (2005) microRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol*, 7, 719-723.
- Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R. and Golub, T.R. (2005) microRNA expression profiles classify human cancers. *Nature*, 435, 834-838.
- Metzler, M., Wilda, M., Busch, K., Viehmann, S. and Borkhardt, A. (2004) High expression of precursor microRNA-155/BIC RNA in children with Burkitt's lymphoma. *Genes Chromosomes Cancer*, 39, 167-169.
- Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M. and Dreyfuss, G. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev*, 16, 720-728.
- O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V. and Mendell, J.T. (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*, 435, 839-843.
- Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degan, B., Muller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E. and Ruvkun, G. (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature*, 408, 86-89.
- Petersen, C.P., Bordeleau, M.E., Pelletier, J. and Sharp, P.A. (2006) Short RNAs repress translation after initiation in mammalian cells. *Mol Cell*, 21, 533-542.
- Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E. and Filipowicz, W. (2005) Inhibition of translational initiation by let-7 microRNA in human cells. *Science*, 309, 1573-1576.
- Poy, M.N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., Macdonald, P.E., Pfeffer, S., Tuschl, T., Rajewsky, N., Rorsman, P. and Stoffel, M. (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature*, 432, 226-230.
- Qiao, D., Zeeman, A.M., Deng, W., Looijenga, L.H. and Lin, H. (2002) Molecular characterization of Hiwi, a human member of the Piwi gene family whose overexpression is correlated to seminomas. *Oncogene*, 21, 3988-3999.
- Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R. and Ruvkun, G. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, 403, 901-906.
- Sen, G.L. and Blau, H.M. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*, 7, 633-636.
- Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R. and Ruvkun, G. (2000) The lin-41 RBCC gene acts in the *C. elegans* heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol Cell*, 5, 659-669.
- Stark, A., Brennecke, J., Bushati, N., Russell, R.B. and Cohen, S.M. (2005) Animal microRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. *Cell*, 123, 1133-1146.
- Suh, M.R., Lee, Y., Kim, J.Y., Kim, S.K., Moon, S.H., Lee, J.Y., Cha, K.Y., Chung, H.M., Yoon, H.S., Moon, S.Y., Kim, V.N. and Kim, K.S. (2004) Human embryonic stem cells express a unique set of microRNAs. *Dev Biol*, 270, 488-498.
- Tam, W., Hughes, S.H., Hayward, W.S. and Besmer, P. (2002) Avian bic, a gene isolated from a common retroviral site in avian leukosis virus-induced lymphomas that encodes a noncoding RNA, cooperates with c-Myc in lymphomagenesis and erythroleukemogenesis. *J Virol*, 76, 4275-4286.
- Teixeira, D., Sheth, U., Valencia-Sanchez, M.A., Brengues, M. and Parker, R. (2005) Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA*, 11, 371-382.
- Vatolin, S., Navaratne, K. and Weil, R.J. (2006) A novel method to detect functional microRNA targets. *J Mol Biol*.
- Vester, B. and Wengel, J. (2004) LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA. *Biochemistry*, 43, 13233-13241.
- Voorhoeve, P.M. and Agami, R. (2003) The tumor-suppressive functions of the human INK4A locus. *Cancer Cell*, 4, 311-319.

Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A.J., Stoop, H., Nagel, R., Liu, Y.P., van Duijse, J., Drost, J., Griekspoor, A., Zlotorynski, E., Yabuta, N., De Vita, G., Nojima, H., Looijenga, L.H. and Agami, R. (2006) A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell*, 124, 1169-1181.

Wightman, B., Burglin, T.R., Gatto, J., Arasu, P. and Ruvkun, G. (1991) Negative regulatory sequences in the *lin-14* 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev*, 5, 1813-1824.

Wightman, B., Ha, I. and Ruvkun, G. (1993) Post-transcriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell*, 75, 855-862.

Wu, L., Fan, J. and Belasco, J.G. (2006) microRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A*, 103, 4034-4039.

Xu, P., Vernooy, S.Y., Guo, M. and Hay, B.A. (2003) The *Drosophila* microRNA *mir-14* suppresses cell death and is required for normal fat metabolism. *Curr Biol*, 13, 790-795.

Chapter 3

A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors

Cell (2006)

A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors

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Endogenous small RNAs (miRNAs) regulate gene expression by mechanisms conserved across metazoans. While the number of verified human miRNAs is still expanding, only few have been functionally annotated. To perform genetic screens for novel functions of miRNAs we developed a library of vectors expressing the majority of cloned human miRNAs and created corresponding DNA barcode arrays. In a screen for miRNAs that cooperate with oncogenes in cellular transformation we identified miR-372 and miR-373, each permitting proliferation and tumorigenesis of primary human cells that harbor both oncogenic RAS and active wildtype p53. These miRNAs neutralize p53-mediated CDK inhibition, possibly through direct inhibition of the expression of the tumor-suppressor LATS2. We provide evidence that these miRNAs are potential novel oncogenes participating in the development of human testicular germ cell tumors by numbing the p53 pathway, thus allowing tumorigenic growth in the presence of wildtype p53.

Introduction

Since their discovery, the functions of only a handful of microRNAs (miRNAs) have been determined (recently reviewed in (Zamore and Haley, 2005)). Relevant to carcinogenesis, it was found that let-7 inhibits RAS expression and in lung tumors negatively correlates with RAS levels (Johnson et al., 2005). Furthermore, the oncogenic potential of the miR-17-92 cluster was demonstrated (He et al., 2005; O'Donnell et al., 2005). This cluster is amplified in lymphomas (Ota et al., 2004) and its introduction accelerates tumorigenicity by an as yet undefined process. These findings demonstrate the powerful ability of small RNAs to alter cellular pathways and programs. However, the small number of miRNAs with a known function stresses the need for a systematic screening approach to identify more miRNA functions.

The difficulties in deciphering the mechanism of action of miRNAs with an unknown function and deducing their activity from their sequence is largely due to the complex relationship with their target genes. In general, target genes containing sequences that are completely complementary

to the miRNA will be degraded by an RNA-interference mechanism, whereas targets with partial complementary sequences at their 3'UTR will be subjected to translation inhibition and to a lesser extent also to mRNA degradation (Bagga et al., 2005; Doench and Sharp, 2004; Lim et al., 2005; Pillai et al., 2005). In mammals, a near-perfect complementarity between miRNAs and protein coding genes almost never exists, making it difficult to directly pinpoint relevant downstream targets of a miRNA. Several algorithms were developed that predict miRNA targets, most notably TargetScanS, PicTar and miRanda (John et al., 2004; Lewis et al., 2005; Robins et al., 2005). These programs predict dozens to hundreds of target genes per miRNA, making it difficult to directly infer the cellular pathways affected by a given miRNA. Furthermore, the biological effect of the downregulation depends greatly on the cellular context, which exemplifies the need to deduce miRNA functions by *in vivo* genetic screens in well-defined model systems.

The cancerous process can be modeled by *in vitro* neoplastic transformation assays in primary human cells (Hahn et al., 1999). Using this sys-

tem, sets of genetic elements required for transformation were identified. For example, the joint expression of the telomerase reverse transcriptase subunit (hTERT), oncogenic H-RAS^{V12} and SV40-small t antigen, combined with the suppression of p53 and p16INK4A was sufficient to render primary human fibroblasts tumorigenic (Voorhoeve and Agami, 2003). Recently, these neoplastic transformation assays were used to uncover novel human tumor suppressor genes (Kolfschoten et al., 2005; Westbrook et al., 2005).

Moreover, oncogenes, such as H-RAS^{V12}, provoke a stress response in primary cells that results in an irreversible growth arrest, termed premature senescence (Serrano et al., 1997). The senescent phenotype was recently shown to play a role in the protection from tumor development *in vivo* (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). The elimination of this protective mechanism by for example the suppression of the p53 and p16INK4A pathways permits continued proliferation of the modified primary cells in the presence of the oncogenic event, consequently leading to tumorigenicity (Voorhoeve and Agami, 2003). Here, we use this model system to perform a functional genetic screen to identify miRNAs that act as oncogenes in tumorigenesis. We characterize two miRNAs whose expression can substitute for the loss of wildtype (wt) p53 that is needed to overcome oncogene-mediated arrest and implicate their involvement in the formation of testicular germ cell tumors.

Results

miR-Vec - A vector-based miRNA expression system

To identify novel functions of miRNAs we constructed a retroviral vector for miRNA expression (miR-Vec) following a previously described approach (Chen et al., 2004). We inserted ~500 bp fragments spanning a given miRNA-genomic region in a modified pMSCV-Blasticidin vector, such that they are placed under the control of a CMV promoter (Figure 1A). To examine miRNA expression from the miR-Vec system, a miR-24 mini-gene containing virus was transduced into human cells. Expression was determined using an RNase protection assay (RPA) with a probe designed to identify both precursor and mature miR-24 (Figure 1B). Figure 1C shows that cells transduced with miR-Vec-24 clearly express high levels of mature miR-24 whereas little expression was detected in control-transduced cells. Furthermore, we confirmed the consistency of

miRNA expression driven by miR-Vec by cloning eight miR-Vec plasmids expressing randomly chosen miRNAs. With one exception all constructs yielded high expression levels of mature miRNAs (Figure S1). Notably, very little pre-miRNA accumulation was detected in all cases, indicating the efficient processing of the ectopically expressed miRNAs in the cells.

Next, we examined the functionality of the miR-Vec system to suppress gene expression by using both GFP, tagged with a sequence complementary to miR-19, and luciferase containing either the wt 3'UTR of G6PD, a predicted miR-1 target, or control with two mutated miR-1 binding sequences (Lewis et al., 2003). Using fluorescence microscopy and luciferase assays we observed potent and specific miRNA activity expressed from each miR-Vec (Figure S2). These results demonstrate the general applicability of miR-Vec to drive functional miRNA expression.

miR-Lib and miR-Array

We subsequently created a human miRNA expression library (miR-Lib) by cloning almost all annotated human miRNAs into our vector (Rfam release 6, Supplementary data S3). Additionally, we made a corresponding microarray (miR-Array) containing all miR-Lib inserts, which allows the detection of miRNA effects on proliferation.

To test the sensitivity of screens with miR-Lib and miR-Array, we transduced modified primary BJ fibroblasts expressing ecotropic receptor and immortalized with hTERT (BJ-ET) with a mixture of 197 different miR-Vecs and mixed them in a ratio of 400:1 with BJ-ET cells containing both miR-Vec-311 and a knockdown construct for p53 (p53^{kd}, Figure 1D). Previously, we have shown that in a period of two weeks, BJ-ET-p53^{kd} cells increase 4-5 fold in number compared with BJ-ET cells (Voorhoeve and Agami, 2003). In accordance, we observed an approximately four fold increase in miR-311 signal, indicating that our procedure is sensitive enough to detect mild growth differences (Figure 1E).

Expression of miR-372 and miR-373 protects from oncogenic stress

In response to mitogenic signals from oncogenes, such as RAS^{V12}, primary human cells undergo a growth arrest (Figure 2A, (Serrano et al., 1997)). In contrast, primary cells lacking functional p53 efficiently overcome this arrest. This escape from oncogene-induced senescence is a prerequisite for full transformation into tumor cells. To identify miRNAs that can interfere with this process and thus might contribute to the development of tu-

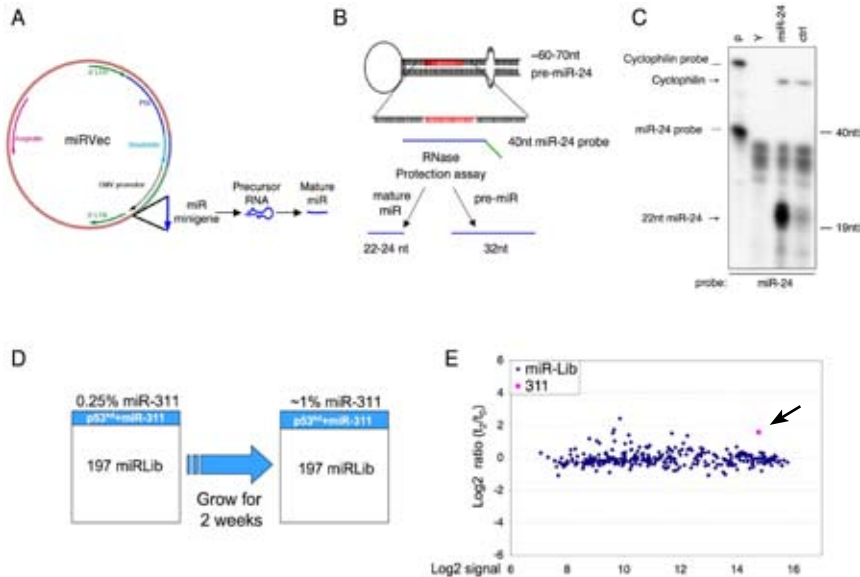


Figure 1. Tools for functional genetic screens with human miRNAs. (A) The miR-Vec miRNA expressing system. Transcription of the minigene mimics the pri-miRNA which is subsequently processed to a mature miRNA. (B) The RPA technique used to detect precursor and mature miRNAs in this study. (C) RPA was performed on RNA extracts from primary human BJ cells stably transduced with miR-Vec-24 and miR-Vec-ctrl. We used a probe against cyclophilin to control for loading. (P=10% input probe, Y=Yeast control RNA). (D and E) BJ-ET-p53^{kd} cells were transduced with miR-Vec-311 and BJ-ET cells were transduced with a mix of 197 other miR-Vecs. Both populations were drug selected, mixed in a ratio of 1:400 and left to grow for two weeks. A barcode experiment was done comparing cells right after mixing (t_0) and after 2 weeks in culture (t_2). The log2 of the ratio of the signals between t_2 and t_0 was plotted against the average signal to visualize outliers. The signal derived from the spot corresponding to miR-311 is indicated by the arrow.

mor cells, we transduced BJ-ET fibroblasts with miR-Lib and subsequently transduced them with either RAS^{V12} or a control vector (Figure 2B). After 2 or 3 weeks in culture, senescence-induced differences in abundance of all miR-Vecs were determined with the miR-Array. Figure 2C shows that in three independent experiments the relative abundance of three miR-Vecs increased reproducibly in the RAS^{V12}-expressing population. These hits corresponded to three constructs derived from one genomic region expressing miRNAs 371, 372, 373 and 373* (Figure 2D). Due to the close proximity of miR-371 and miR-372 in the genome (within 0.5 kilobase) two largely overlapping constructs encoded both miRNA-371 and 372 (miR-Vec-371&2). The third construct did not overlap with miR-Vec-371&2 and encoded miRNA-373 and 373*. Interestingly, the mature miR-373 is a homologue of miR-372, and neither share obvious homology with either miRNA-371 or miR-373* (Figure 2D). This suggests that miR-372 and miR-373 caused the observed selective growth advantage. Next, we verified miRNA function in a cell-growth

assay. First, we verified the expression of both miR-371 and 372 by miR-Vec-371&2 and miR-373 by miR-Vec-373 (Figure 2E). We then transduced BJ-ET cells with miR-Vec-371&2, miR-Vec-373, p53^{kd} or a vector control, and then with RAS^{V12}. As expected, control cells ceased proliferating in response to RAS^{V12} whereas p53^{kd} cells continued to proliferate (Figure 2F). The expression of either miR-371&2 or miR-373 allowed cells to continue proliferating in the presence of oncogenic stress, validating the effect observed with the miR-Array.

Oncogene-induced senescence is characterized by the appearance of cells with a flat morphology that express senescence associated SA- β -Galactosidase. Indeed, control RAS^{V12}-arrested cells showed relatively high abundance of flat cells expressing SA- β -Galactosidase (Figures 2G and H). Consistent with the cell growth assay, very few cells showed senescent morphology when transduced with either miR-Vec-371&2, miR-Vec-373 or control p53^{kd}. Altogether, these data show that transduction with either miR-Vec-371&2 or miR-Vec-373 prevents RAS^{V12}-induced

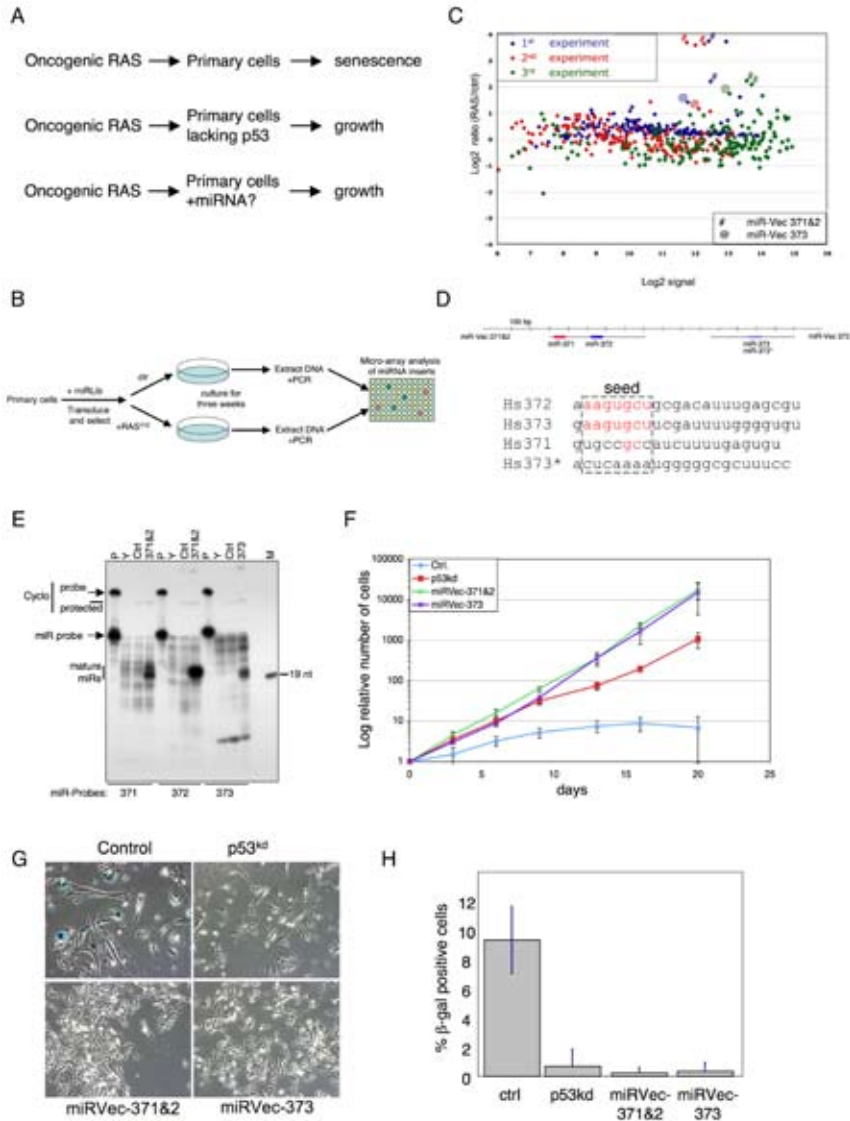


Figure 2. Identification of miR-Vecs that inhibit oncogene-induced senescence. (A) The effects of oncogenic RAS^{V12} on cellular growth. (B) A flow-chart of the screen. Cells transduced with the miR-Lib were grown for two to three weeks in the presence or absence of RAS^{V12}. Subsequently, the population of inserts in each condition was recovered and compared using miR-array. (C) Three independent miR-Array experiments were performed. The position of the reproducibly upregulated miR-Vecs is indicated for each experiment. (D) The miR-371-3 genomic organization and the sequences of the mature miRNAs expressed from this locus. For comparison, the nucleotides 2-8 (seed) of the miRNAs are boxed. (E) RPA analysis of RNA from BJ-ET cells containing the indicated miR-Vecs. F. BJ-ET cells containing the indicated vectors were transduced with RAS^{V12}, drug selected and subjected to a growth assay. Standard deviations from three independent transductions are shown. (G) The cells from F were stained 10 days after RAS^{V12} transduction to detect SA-β-Galactosidase expression. (H) The percentage of SA-β-Galactosidase positive cells was counted in three independent dishes.

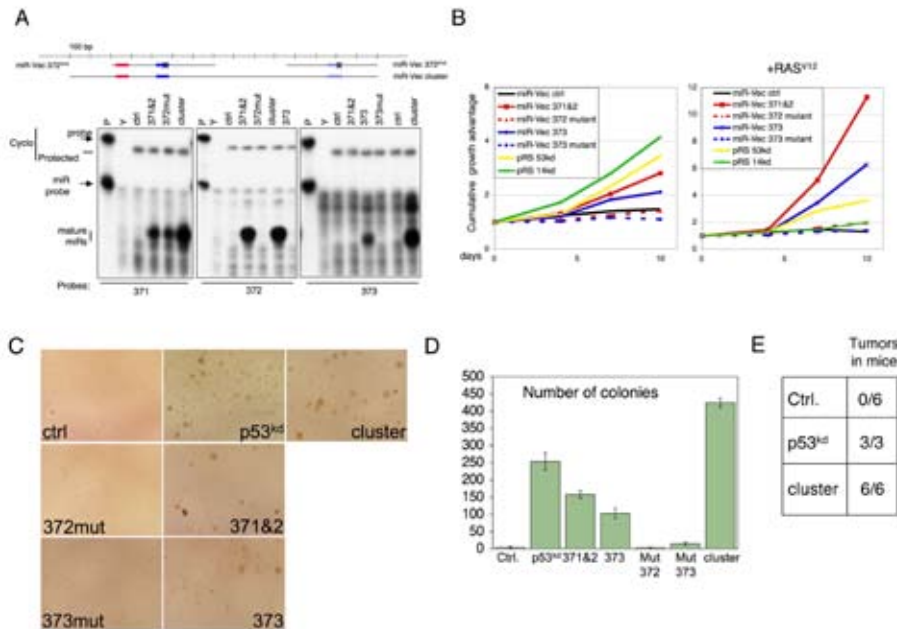


Figure 3. miR-372&373 collaborate with oncogenic RAS^{V12} to transform primary human cells. (A) The mature miR-372&373 sequences were mutated in their corresponding miR-Vecs and their expression was examined by RPA. miR-Vec cluster is a construct encompassing miRs-371-3. (B) The indicated YFP-containing vectors were transduced in BJ-ET-RAS^{V12}-ERTAM cells. The cumulative growth advantage was determined in the absence or presence of tamoxifen (+RAS^{V12}). (C+D) BJ-ET cells containing SV40 small t, p16INK4A knockdown, RAS^{V12} and the indicated constructs were plated in soft agar and colonies were photographed and counted after 3 weeks. The average and standard deviation of three independent dishes is shown. (E) The cluster, p53^{kd} and control cell populations from panel C were injected subcutaneously in athymic nude mice and tumor growth was scored 5 weeks later.

growth arrest in primary human cells. The independent identification of constructs encoding two very similar miRNAs (miR-372 and miR-373) suggests that they (but not miR-371 or miR-373*) are required to cause this phenotype. To test this, we mutated the sequences of miR-372 and miR-373. As demonstrated by RPA, miR-Vec-372mut and miR-Vec-373mut indeed failed to express miR-372 and miR-373, respectively (Figure 3A). Note that miR-Vec-372mut still expressed miR-371 to a similar extent as the original miR-Vec-371&2. We then tested these constructs in a YFP-competition assay to detect possible growth advantages conferred by the miRNAs on BJ cells in the absence or presence of RAS^{V12} (Figure 3B). For this purpose we used a miR-Vec vector that expresses YFP instead of a blasticidin resistance marker, and compared the growth rates of YFP-tagged and untagged cells within one population. Increase in time of the YFP-positive cells within the population indicates

a growth advantage conferred by the additional genetic unit encoded by the YFP vector. We also generated BJ-ET cells expressing the RAS^{V12}-ERTAM chimera gene, which is only active when tamoxifen is added (De Vita et al., 2005), and transduced them with either YFP-tagged wt or mutant miR-Vec-371&2 and miR-Vec-373 constructs, as well as p53^{kd}, p14ARF^{kd} or control vectors. Figure 3B shows that even without activating RAS^{V12} (no tamoxifen added) both miR-Vec-371&2 and miR-Vec-373 conferred a growth advantage to cells, although to a lesser extent than observed with p53^{kd} or p14ARF^{kd}. Once RAS^{V12} was activated, the growth advantage of cells with miR-Vec-371&2 and 373 increased dramatically, indicating that these constructs allowed growth of cells in the presence of oncogenes while the rest of the population ceased to proliferate. In accordance with previously published data (Voorhoeve and Agami, 2003), reducing p53, but not p14ARF expression, was sufficient to overcome the on-

cogenic stress. Consistent with our assumption that miR-372&373 are the active miRNAs, mutating their mature sequence abrogated their growth advantage. This shows that miR-372&373, but not miR-371 or miR-373*, caused stimulation of proliferation and resistance to oncogenic stress.

Expression of miR-372 and miR-373 transforms primary human cells

Suppression of cellular senescence is essential for tumorigenesis. We therefore examined whether the ectopic expression of miR-372&373 is sufficient to replace loss of p53 in transformation of cells. A hallmark of cellular transformation is the ability of tumor cells to grow anchorage independently in semi-solid medium and as tumors in model mice (Hahn et al., 1999; Hanahan and Weinberg, 2000). Indeed, in a soft agar assay, modified primary human BJ-ET cells expressing hTERT, SV40-small t, RAS^{V12} and shRNA-knockdowns for p53 and p16INK4A showed potent ability to grow in an anchorage independent manner (Figures 3C and 3D). To mimic the expression of the complete miR-371-373 gene cluster, we made a miR-Vec expressing all miRNAs from one cluster (miR-Vec-cluster, see Figure 3A for expression). Similar to the knockdown of p53, the ability to grow in soft agar was also observed for cells containing miR-Vec-cluster, miR-Vec-371&2 or miR-Vec-373, but not miR-Vec-372mut or miR-Vec-373mut (Figures 3C and 3D). Moreover, the cells containing the miR-371-373 cluster grew efficiently as tumors in athymic nude mice (Figure 3E). These results demonstrate that miR-372&373 collaborate with RAS in transformation in a manner that resembles p53 inactivation.

Importantly, our results so far indicate that the expression of miR-372&373 did not reduce the activity of RAS^{V12}, as these cells were still growing faster than normal cells and were tumorigenic, for which RAS activity is indispensable (Hahn et al., 1999; Kolfshoten et al., 2005). Therefore, the miRNA mediated circumvention of the activation of p53 can in principle be obtained at a level upstream of p53, on p53 itself or downstream. To shed more light on this aspect, we examined the effect of miR-372&373 expression on p53 activation in response to oncogenic stimulation. For this experiment we used BJ-ET cells containing p14ARF^{kd} because, following RAS^{V12} treatment, in those cells p53 is still activated but more clearly stabilized than in parental BJ-ET cells (Voorhoeve and Agami, 2003), resulting in a sensitized system for slight alterations in p53 in response to RAS^{V12}. Figure 4A shows that following RAS^{V12}

stimulation p53 was stabilized and activated and its target gene p21^{Cip1} was induced in all cases, indicating an intact p53 pathway in these cells. Therefore, it is unlikely that the miRNAs act on a factor upstream of p53 or on p53 itself to suppress the cellular response to oncogenic RAS. Increased levels of p21^{Cip1} inhibit CDK activity causing cells to arrest in G1 phase (el-Deiry et al., 1993) whereas suppression of p21^{Cip1} allows cells to grow in the presence of RAS^{V12} (Figure S4A). To test whether p21^{Cip1} was still functional in the miRNA-transduced cells we examined CDK2 activity using an IP kinase assay (Figure 4B). In both miR-372&373 expressing cells, CDK2 remained active following RAS^{V12} induction whereas it was inhibited in the control cells (Figure 4B). In contrast, miR-372&373-transduced cells were still sensitive to inhibition of CDK activity by roscovitin (Figure S4B). This indicates that the presence of miR-372&373 acts as a molecular switch to make CDK2 resistant to increased levels of the cell-cycle inhibitor p21^{Cip1}.

Both p53 and p21^{Cip1} play a major role in the DNA damage response to ionizing radiation (IR) (Weinert, 1998). Since cells expressing miR-372&373 proliferate in the presence of increased p21^{Cip1} levels, we examined their response to damaged DNA. In the presence of both miRNAs, and irrespective of RAS^{V12} expression, IR-induced a cell-cycle arrest that was indistinguishable from control cells, whereas the suppression of p53 expression allowed, as expected, continuous DNA replication (Figure 4C and data not shown). These results indicate that although miR-372&373 confer complete protection to oncogene-induced senescence in a manner similar to p53 inactivation, the cellular response to DNA damage remains intact.

Potential role of miR-372 and miR-373 in human cancer

Based on the above results, we hypothesized that miRNA-372&373 may participate in tumorigenesis of some tumors that retain wt p53 and are sensitive to DNA-damaging treatments. One such tumor type is the testicular germ cell tumor of adolescents and adults (TGCT), known for the presence of wt p53 in the majority of cases, and known to be generally sensitive to chemotherapies as well as irradiation (Kersemakers et al., 2002; Masters and Koberle, 2003; Mayer et al., 2003). In addition, these tumors harbour an embryonic stem (ES) cell signature (Almstrup et al., 2004), which correlates with the reported ES-cell expression pattern of the miR-371-3 cluster (Suh et al., 2004). We therefore examined a number of

cell lines originating from TGCTs for the expression of the miR-371-3 cluster. Four out of seven cell lines expressed this cluster (Figures 4D and S5). This result is significant as no clear expression of the miR-371-3 cluster was detected in any of the somatic cell lines we tested (originating from breast, colon, lung and brain tumors, Figure S6).

TGCTs are divided into seminomas, nonseminomas and spermatocytic-seminoma according to their origin, clinical behaviour and chromosomal constitution (Oosterhuis and Looijenga, 2005). The nonseminomas can be composed of embryonal carcinoma (EC, the stem cell component), teratocarcinoma (TC, somatic differentiation) and yolk sac tumor and choriocarcinoma (YS and CH, extra-embryonal tissues). All the cell lines we tested were derived from nonseminomatous tumors, as there are no other type of TGCT cell lines available so far. To substantiate our results and extend them to other TGCT types we examined a panel of primary seminomas, nonseminomas and spermatocytic seminomas for the expression of miR-372. Figures 4D, and S7 show that most seminomas (28/32) had a clear miR-372 expression, about two thirds (14/21) of the nonseminomas expressed miR-372 and expression was observed in neither RNA from the spermatocytic-seminoma tumors (data not shown) nor from the normal testis tissue panel. Noteworthy is the fact that endogenous expression of miR-372 reached levels that are comparable to those driven by miR-Vec-372 (Figure 4D), indicating the biological relevance of our system in primary human fibroblast cells. Within the nonseminoma samples, both pure and mixed histologies were present (Supplementary data S7). The RPA analysis showed that high expression of miR-372 correlated with a larger EC component. To further investigate this connection, we performed *in situ* miRNA hybridizations on tissue sections of 10 representative TGCTs and found in all cases miR-372&373 to be strictly localized to the EC component, as judged by morphology and immunohistochemistry with CD30 and Oct3/4 (Figure 4E and data not shown). Both seminomas and the EC component of nonseminomas share features with ES cells. To exclude that the detection of miR-371-3 merely reflects its expression pattern in ES cells, we tested by RPA miR-302a-d, another ES cells-specific miRNA cluster (Suh et al., 2004). In many of the miR-371-3 expressing seminomas and nonseminomas, miR-302a-d was undetectable (Figures S7 and S8), suggesting that miR-371-3 expression is a selective event during tumorigenesis.

Interestingly, we noted a correlation between cluster expression and p53 status in the TGCT cell lines (Figure 4F). Whereas all three cluster-expressing cell lines contained high wt-p53 levels, NTERA2 has low wildtype p53 levels and NCCIT lost one p53 allele while the second allele is mutated (Burger et al., 1998). To strengthen the p53 connection seen in the TGCT cell lines, we examined p53 mutations in exons 5 to 8 in the primary tumors, where the majority of mutations are found. In the nonseminoma panel, no mutations were detected. In contrast, two out of four miRNA 372-negative seminomas had an inactivating mutation in the p53 gene (SE20 in exon 8 and SE28 in exon 5. Figures 4F and S7), a rare phenomenon in TGCT (Kersemakers et al., 2002). In contrast, none of the thirteen miRNA 372&373-expressing seminomas that we examined contained mutations in p53. Altogether, these results strongly suggest that the expression of miR-372&373 suppresses the p53 pathway to an extent sufficient to allow oncogenic mutations to accumulate in TGCTs.

We then decided to test directly the correlation between the p53 pathway and miR-372&373 expression in TGCTs. It was technically not possible to sufficiently and persistently inhibit the expression of both miR-372 and miR-373 by methylated miRNA-oligos or knockdown vectors against the loop of the precursors (as judged by miR-372 and miR-373 luciferase reporter targets, data not shown). As an alternative approach, we used NCCIT, an embryonal carcinoma derived cell line containing only a mutated, non-functional, p53 that expresses very low amounts of the miR-371-3 cluster (Figure 4D and (Burger et al., 1998)). We activated the p53 pathway by transfecting NCCIT cells with a p21-RFP construct that inhibits Cyclin E/CDK2 activity and examined the effects of miR372/3 by cotransfecting miR-Vec-371-3 (cluster). As expected, overexpression of p21-RFP caused accumulation of cells in G1 whereas cotransfection of Cyclin E/CDK2 allowed cells to continue proliferating in the presence of p21-RFP (Figure 4G). Significantly, cells cotransfected with the miR-Vec-cluster showed a phenotype similar to that observed with Cyclin E/CDK2. This result demonstrates the ability of miR-372 and miR-373 to overcome a p21-mediated cell-cycle arrest in TGCTs and substantiates the correlation between these miRNAs, CDK and the p53 pathway.

miR-372 and miRNA-373 regulate LATS2 expression

Our results thus far indicate that the miR-371-

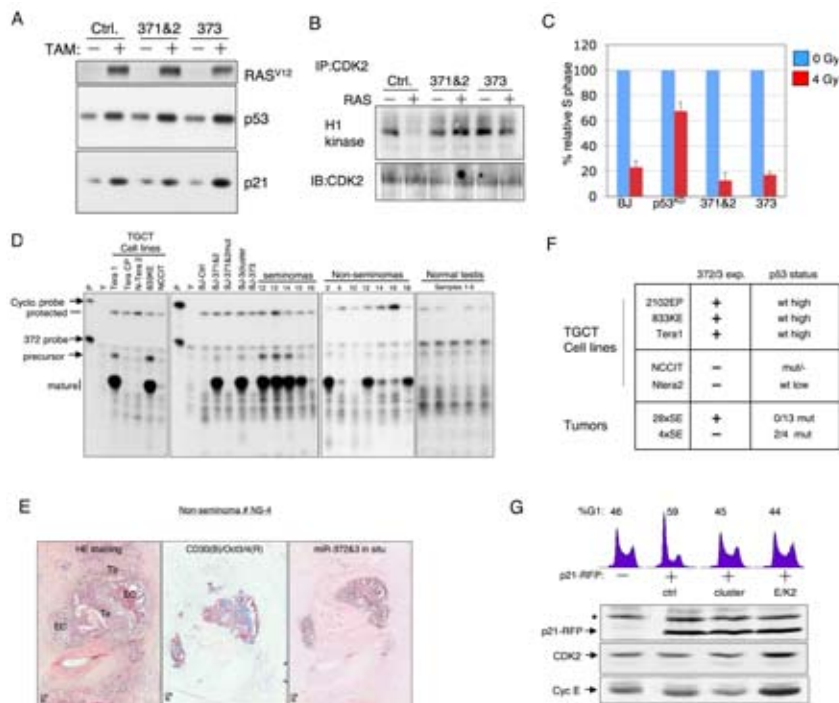


Figure 4. miR-372 and miR-373 sustain CDK2 activity in TGCT. (A) BJ-ET-p14ARF^{kd}-RAS^{V12}ERTAM cells containing the indicated miR-Vecs were cultured for a week in the presence or absence of tamoxifen (TAM), harvested and subjected to immunoblot analyses to detect p21^{Cip1}, p53 and RAS^{V12}ER. (B) The same polyclonal populations as in A were harvested and CDK2 kinase activity was measured using an IP-kinase protocol with Histone H1 as a substrate. Equal pulldown of CDK2 was checked by immunoblot of the same samples (lower panel). (C) BJ-ET cells containing either a control vector (BJ) or the indicated constructs were irradiated (4 Gy), labeled with BRDU and subjected to flow cytometric analysis. The percentage of BRDU positive cells relative to the unirradiated cells is shown. SD is from three independent experiments. (D) The expression of miR-372 was detected by RPA in RNA extracts from several TGCT cell lines as well as from primary seminoma and nonseminoma tumors, and from normal testis tissues. (E) *In situ* hybridization on a nonseminoma of mixed histology to detect miR-372&373 expression. The probe was developed with NBT/BCIP blue and the section was counterstained with FastRed. The EC component of the tumor was morphologically determined using HE-counter staining and by immunohistochemistry with anti-CD30 and Oct3/4 antibodies of the next sections. (F) Summary of the p53 status in several TGCT cell lines and primary seminomas (in the latter only exons 5 to 8 were examined). (G) NCCIT cells were cotransfected with H2B-GFP and the indicated constructs. Cell-cycle profiles of the GFP-positive population were examined after 4 days using flow cytometry. Also shown is an immunoblot analysis of cells from the same experiment with antibodies against p21^{Cip1}, CDK2 and cyclin E.

3 cluster suppresses an inhibitor of CDK activity and that this function is important for the development of TGCTs. To start to identify relevant targets of miR-372&373 we took advantage of the fact that miRNAs may cause limited destruction of their target mRNAs apart from inhibiting their translation (Lim et al., 2005). We performed an mRNA-expression array analysis comparing RAS^{V12}-expressing BJ-ET cells either containing p53^{kd} or expressing the miR-371-3 cluster (Figure 5A). We chose this setup as both cell

types proliferate in the presence of oncogenic stress, thus canceling out the profound effects of cells going into senescence. We first looked in the p53^{kd} cells and found p53 itself and many of its transcriptional targets to be down regulated compared to the cluster expressing cells (Figure 5A). This independently confirms our previous results (Figure 4A) indicating that miR-372&373 do not directly inhibit p53 activity. From the list of genes whose expression was 2 or more fold lower in the cluster expressing cells we used tar-

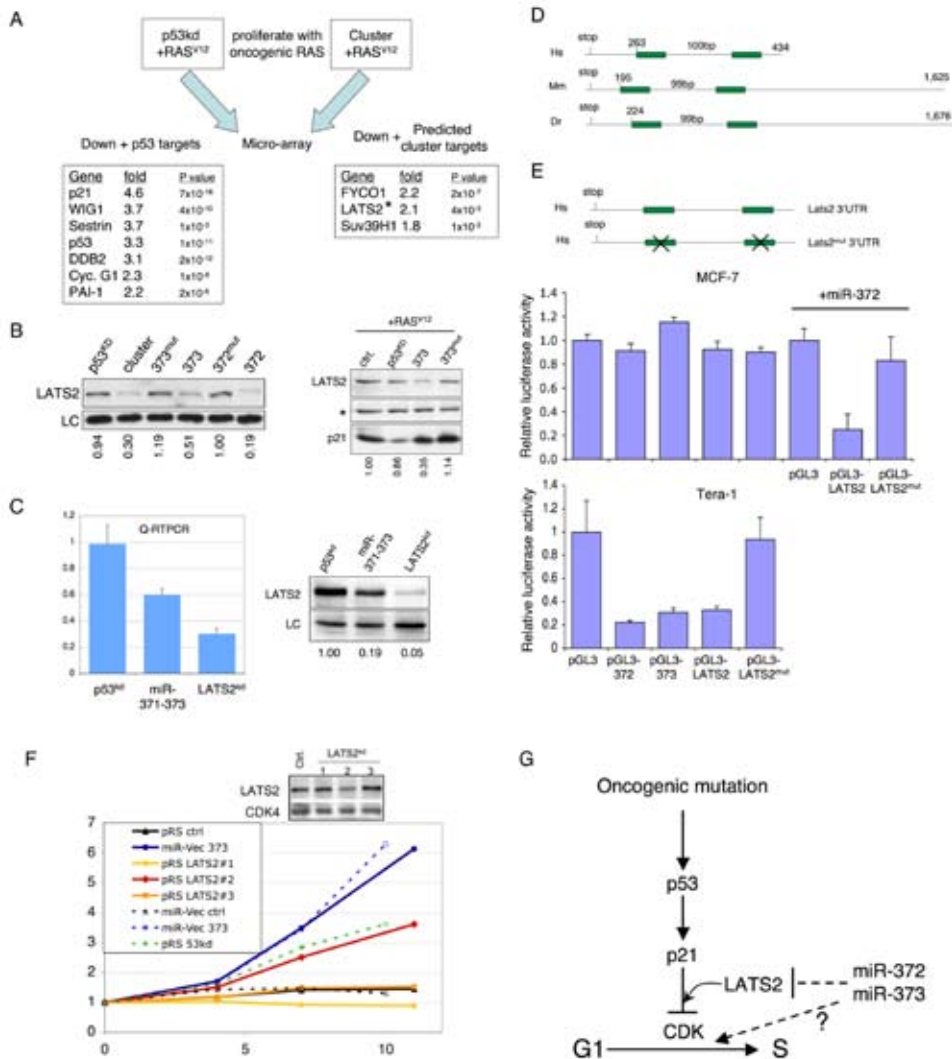


Figure 5. Inhibition of LATS2 expression by miR-372 and miR-373. (A) RNA was extracted from BJ-ET cells expressing RAS^{V12} and containing either a p53^{kd} construct or miR-Vec-371-3 (cluster), and compared using oligo-expression arrays. Listed are genes whose expression was down regulated in the p53^{kd} cells and are known transcriptional targets of p53 as well as genes whose expression was suppressed in the cluster expressing cells and are predicted TargetScanS targets of miRNA-372&373. LATS2^{miR}, the reduction was verified by Q-RT-PCR (Figure S9). (B+C) BJ-ET cells containing the indicated constructs were analyzed by immunoblot analysis or by Q-RT-PCR. Band intensity was calculated by densitometry. (D) The 3'UTRs of LATS2 in human (Hs), mouse (Mm) and zebrafish (Dr) are shown, and the predicted miR-372&373 target sequences are marked. (E) The indicated vectors were transfected in miR-372&373 positive (Tera1) and negative (MCF-7) cell lines. The relative Firefly luciferase levels (divided by Renilla control and compared to pGL3) are shown. SD are from three independent experiments. (F) Cumulative growth advantage assay was performed as described in Figure 3B in RAS^{V12}-expressing cells transfected with the indicated vectors. For comparison, data from Figure 3B is included (dashed lines). An accompanying immunoblot shows that only LATS2^{kd}#2 is functional. (G) A schematic model showing the mechanism through which miR-372&373 can suppress an oncogene-activated p53 pathway.

get prediction programs to find possible direct targets of miR-372&373. We identified three miR-372&373 predicted targets (FYCO1 (FYVE and coiled coil containing protein 1), Suv39-H1 and LATS2, Figure 5A). Interestingly, while nothing is known about the function of the FYCO1 protein, both Suv39-H1 and LATS2 have been connected in the past to RAS^{V12}-mediated transformation. It was recently shown that lymphocytes from mice nullizygous for Suv39-H1 are resistant to oncogene-induced senescence (Braig et al., 2005). However, the mechanism underlying this effect and its conservation to other tissues and to man are not known. Most promising seemed the LArge Tumor Suppressor homologue 2 (LATS2), a serine-threonine kinase whose deletion in flies accelerates cellular proliferation and tumorigenic development (Justice et al., 1995; Xu et al., 1995). In mice, a similar activity was seen in LATS2^{-/-} mouse embryonic fibroblasts (McPherson et al., 2004), whereas its overexpression was shown to inhibit cyclin E/CDK2 activity and RAS^{V12}-mediated transformation (Li et al., 2003). Additionally, loss of LATS2 stimulated reduplication, an activity comparable to that observed when cyclin E is overexpressed in the absence of p53 (Fukasawa et al., 1996; Tarapore and Fukasawa, 2002; Toji et al., 2004). Finally, in human breast cancer, hypermethylation of the LATS2 promoter was associated with an aggressive phenotype of the tumors (Takahashi et al., 2005). These observations suggest that the suppression of LATS2 explains at least in part the sustained activity of CDK in the presence of high p21^{Cip1} levels in miR-372&373-expressing cells.

To investigate the possibility that miR-372 and miR-373 suppress the expression of LATS2 we performed immunoblot analysis of cells expressing wt and mutant miR-372&373, the cluster and the controls p53^{kd} and empty vector. Both in the absence of RAS^{V12} and its presence, a significant reduction in LATS2 protein level was observed upon miR-372&373 expression (Figure 5B). Using quantitative RT-PCR and immunoblot analysis we observed a 2 fold effect on LATS2 RNA levels and 4-5 fold on protein levels by the miR-371-3 cluster (Figure 5C). As a control we used a LATS2 knockdown construct (see Figure 5F). These results show that a combined effect of RNA destruction and translation inhibition is used by miR-372&373 to silence LATS2.

miR-372&373 was predicted to bind two sites in the 3'UTR of LATS2 that are highly conserved between human, mouse and zebrafish (Figure 5D). To further substantiate LATS2 as a direct target of miR372&373 we cloned its 3'UTR

downstream of the Firefly luciferase gene (pGL3-LATS2). We transfected either pGL3-LATS2 or the controls pGL3-372 and pGL3-373 (containing a miR- complementary sequence in their 3'UTR) or pGL3 into Tera1 and MCF-7 cells (respectively positive and negative for miR-371-3, Figures 4D and S6). As predicted, the 372&373 complementary sequences mediated strong inhibition of luciferase expression in Tera1 cells. Significantly, a potent inhibition of luciferase activity was also mediated by the 3'UTR of LATS2 in either MCF-7 ectopically expressing miR-372 or in Tera1 cells but not by a construct mutated at both miR-372-predicted target sites. These results indicate that LATS2 is indeed a direct target of miR-372&373. Next, we tested whether LATS2 is a functional target of miR-372&373 using a YFP-competition assay. Indeed, inhibition of LATS2 conferred a growth advantage to cells expressing RAS^{V12} (Figure 5F). The overall effect was less than the effect of the miR-Vec 373 but comparable to loss of p53. Therefore, these results point to LATS2 as a mediator of the miR-372 and miR-373 effects on cell proliferation and tumorigenicity, although they do not exclude the participation of other direct miR-targets, such as Suv39-H1, in these processes. Further investigation should enumerate the exact role of LATS2 down regulation and the possible participation of other miR-372&373-targets in the overall observed miR-effect on cellular transformation.

Discussion

Functional genetic screens for miRNAs

We developed a miRNA-expression vector library and a corresponding barcode array to detect miRNAs whose expression modifies a defined cellular pathway. We demonstrate here the power of this technology by the identification of miRNA-372 and miRNA-373 as potential oncogenes that collaborate with oncogenic RAS in cellular transformation (Figure 5G). However, this strategy is also suitable for the identification of miRNAs that regulate other cellular pathways resulting in a proliferation or survival difference, such as the DNA damage response, differentiation, sensitivity to growth factors and resistance to anticancer drugs. Furthermore, the miR-Lib tool can be used in a single-well format to identify growth-independent phenotypes.

Collaboration of miRNA372&373 and RAS^{V12} in tumorigenesis

Sustained proliferation of cells in the presence of oncogenic signals is a major leap towards tumorigenicity (Hanahan and Weinberg, 2000). We

found miR-372&373 to collaborate with RAS^{V12} and stimulate a full-blown neoplastic transformation phenotype. However, whereas in the majority of the cases neoplastic transformation will require inactivation of p53 (for example by expression of HPV E6, HDM2 or mutant p53) miR372&373 uniquely allowed transformation to occur while p53 was active. This indicates that miRNA-372&373 do not block RAS^{V12} signals but rather allow cells to proliferate irrespective of p53 activation and induction of p21^{Cip1}.

The expression of miR-372&373 results in prevention of the CDK inhibition that is caused by the oncogenic stress response. In both primary human fibroblasts and in a TGCT-derived cell line, cells expressing miR-372&373 were insensitive to elevated levels of the cell-cycle inhibitor p21^{Cip1}. Although the exact mechanism responsible for this effect is still unclear, we suggest that suppression of LATS2 is an important factor. Indeed, the expression of LATS2 is directly controlled by miR-372&373 and its activity is important for RAS^{V12}-induced senescence. However, further investigation is required to demonstrate the exact mechanism of LATS2 action and whether there are other targets of miR-372&373, such as Suv39-H1, that are relevant to this phenotype.

Correlations to other miRNAs

Based on the seed sequence, the miR-372&373 gene family also includes miR-93 and miR-302a-d. As these may share a broad range of target genes, they may also share many functions. Indeed, preliminary results show that similar to miR-372&373, albeit with minor differences, both miR-93 and miR-302a-d can effectively target the LATS2 3'UTR and bypass oncogene-induced senescence (manuscript in preparation).

Role of miR372&373 in TGCT development

Our results suggest that during transformation the activities of miR-372&373 circumvented the need to mutate p53, leading to a DNA-damage-sensitive transformed phenotype. These characteristics of miR-372&373-transformed primary human cells therefore suggest a role in wt p53-tumor genotypes that are also sensitive to chemotherapies, including irradiation. Indeed, TGCTs conform to this profile (Masters and Koberle, 2003). This could for instance be a result of high mdm2 levels, as was previously suggested for mouse teratocarcinomas (Lutzker and Levine, 1996). However, by several criteria mouse teratocarcinomas are counterparts of human germ cell tumors of neonates and infants rather than TGCT

(Oosterhuis and Looijenga, 2005). Indeed, while the first show high mdm2 expression levels, this was not demonstrated in TGCT (Mostert et al., 2000). Therefore, it is highly significant that we found that miR-372&373 expressing TGCTs did not contain mutated p53 alleles whereas a subset of miR-371-3 negative primary TGCTs and cell lines did. Altogether, these provide a strong indication that there is no selective advantage to mutate p53 during TGCT development when the miR-371-3 cluster is expressed.

The potent role of miR-372&373 in cellular transformation and potentially in TGCT development raises the possibility that they may play a similar role in somatic tumors. To this end, we determined the expression of the miR-371-3 cluster in several distinct somatic tumor cell lines and found little evidence for their expression (Figure S6). Consistent with our results, clear miR-371-3 cluster expression was observed in only 1 out of 70 leukemia tumors examined by others (Lu et al., 2005). It therefore seems that miR-372&373 expression is a rare event in somatic tumors. Whether such a role can be seen with the other members of the miR-372&373 family remains an open possibility.

Although both miR-372&373 and miR-302a-d clusters are expressed in ES cells (Suh et al., 2004) the miRNA-302 cluster is not expressed in many of these primary seminomas and nonseminomas (Figure S7). It is therefore most likely that the expression of miR-371-3 in primary TGCT is not merely a remnant of their ES cell phenotype but rather a selective event during TGCT tumorigenesis.

Function of miRNA372&373 in embryonic stem cells

Our results suggest a link between the expression of miR-372&373 in embryonic stem cells and their function in cellular proliferation in these cells. miR-372&373 may facilitate rapid growth of stem cells by suppressing the expression of CDK inhibitors. Intriguingly, *Drosophila* germ cells and mouse embryonic stem cells require miRNAs to proliferate (Forstemann et al., 2005; Hatfield et al., 2005). The proliferation defect in *Drosophila* mutants that lack miRNAs could be alleviated by loss of dacapo, the *Drosophila* p21^{Cip1} homologue (Forstemann et al., 2005; Hatfield et al., 2005).

Our results indicate that due to enhanced tolerance to oncogenic mutations, deregulated expression of miR-372&373 predisposes cells for accumulation of carcinogenic events. Thus, the expression of these miRNAs must be carefully controlled during differentiation to prevent

progression to cancer. Which factors control miR-371-3 expression during differentiation and whether their activity is causally related to development of TGCTs remains to be explored. Nevertheless, our experiments stress the importance of a strong downregulation of factors that maintain rapid cell proliferation, as in the absence of this downregulation safeguard mechanisms against oncogene emergence are functionally impaired.

Materials and methods

Constructs

pMSCV-Blast and pMSCV-YFP were made by replacing the puromycin resistance marker of pMSCV-Puro (Clontech) with a PCR product encoding the blasticidin resistance gene from cDNA6/TR (Invitrogen) or YFP from pEYFP-N1 (Clontech), respectively. pRetrosuper (pRS)-Blast was generated by replacing the 3'LTR from pMSCV-Blast with the 3'LTR from pRS-Hyg (Voorhoeve and Agami, 2003).

miR-Vec-Ctrl was made by deleting the MCS and the PGK-promoter from pMSCV-Blast, followed by insertion of the CMV promoter from pcDNA-3.1+ and a stuffer DNA derived from the first 211 nt of hTR downstream of the resistance marker. miR-Vec-YFP was cloned similar to miR-Vec-Ctrl, only starting from pMSCV-YFP. pBabe-Puro-RAS^{V12} and pBabe-Puro, pMSCV-GFP-st, pRS-GFP, pBabe-H2B-GFP, pCMV-cyclin E, pCMV-CDK2 and pBabe-RAS^{V12}ERTAM were described before (De Vita et al., 2005; Voorhoeve and Agami, 2003). p53^{kd}, p16^{kd}, p14ARF^{kd}, p21^{Cip1kd} shRNA constructs were described before (Duursma and Agami, 2005; Voorhoeve and Agami, 2003). pMSCV-Blast RAS^{V12}-ERTAM was made by subcloning RAS^{V12}-ERTAM into pMSCV-Blast. p21-RFP was produced by cloning p21 to the N-terminus of dsRFP. The constructs encoding Luciferase-3'-G6PD wt and mut were a kind gift of David Bartel (Lewis et al., 2003).

The miRNA minigenes were PCR amplified from genomic human DNA, cloned downstream of the CMV promoter in miR-Vec and sequence verified. The primers used for the genomic PCR amplification of the individual miRNA minigenes, the miR-Vec-cluster and the miR-Vec mutants are listed in Figure S3.

LATS2 knockdown constructs were cloned to pRetrosuper (pRS)-YFP (Brummelkamp et al., 2002). Targeting sequences are shown in Figure S3.

miR-Array

Genomic DNA was isolated from BJ-ET cells with the DNeasy Tissue Kit (Qiagen). The inserts were

recovered by PCR using primers listed in Figure S3. The PCR product was purified and 500 ng was labeled using ULS-Cy3 or Cy5 (Kreatech) and hybridized to the miR-Array according to the manufacturers instructions (see <http://microarrays.nki.nl>). As the amount of spots was too small to normalize automatically, the red and green signals were normalized by hand in Excel. For each spot the log2 of the red and green ratio, as well as the log2 of the square root of the product of the two signals was calculated. Outliers were picked and listed, and compared across three independent experiments.

miRNA detection

RNase protection assays were performed using the mirVana miRNA probe construction and detection kits (Ambion) according to the manufacturers instructions. 2.5 -10 µg of RNA was used per reaction. Primers to make the RPA probes are listed in Figure S3. The antisense cyclophilin probe contained nucleotides 149–46 of Accession # BC013915.

In situ hybridizations were performed with a mix of LNA oligos against miR-372 and miR-373 (Exiqon) according to the manufacturers instructions.

Cell culture and antibodies

Primary BJ fibroblasts with an ecotropic receptor Neo and pBabe-Puro-hTert (BJ-ET) (Voorhoeve and Agami, 2003) or pBabe-H2B-GFP-hTert (BJ-ET) (Kolfsochten et al., 2005) were grown in DMEM plus 10% FCS and antibiotics. NCCIT cells were grown in RPMI plus 10% FCS and antibiotics.

Retrovirus was made by calcium-phosphate transduction of EcoPack 2 cells (Clontech) and harvesting 40 and 64 hours later. BJ cells were selected with the relevant selective medium 48 hours after transduction for at least a week. In the case of RAS^{V12}-encoding retroviruses, the selection was continued for the entire duration of the experiment.

Antibodies used were DO-1 (p53), F5 (p21^{Cip1}), F235 (RAS), M20 (cyclin E), M2 (CDK2) from Santa Cruz Biotechnology and 3D10 (LATS2 (Toji et al., 2004)). Western blots were scanned and quantified using AIDA software (Raytek, Sheffield, UK).

Genetic screen

BJ-ET cells were transduced with a mixture of 197 miR-Vec vectors, drug selected for a week and transduced independently three times with pBabe-Puro-RAS^{V12} or pBabe-Puro. Cells from

the independent transductions were propagated for two or three weeks before genomic DNA was isolated.

Growth assay

BJ-ET cells were transduced with miR-Vec or pRS-Blast constructs, drug selected for a week, transduced with pBabe-Puro-RAS^{V12} and drug selected for three days. 3×10^5 cells were plated in triplicates in 6 cm dishes and propagated twice a week. SA- β -Galactosidase activity was assessed 10 days after RAS^{V12} transduction, as described (Kolschoten et al., 2005). Three times two hundred cells were scored for SA- β -Galactosidase activity.

Soft agar assay and tumorigenic growth in mice

BJ-ET cells were transduced to more than 80% with pMSCV-GFP-st, pRS-Hyg-p16^{kd}, drug selected, transduced with the various miR-Vec retroviruses or pRS-Blast-p53^{kd}, drug selected again and transduced with pBabe-Puro-RAS^{V12}. After a week, the cells were either plated in triplicates in soft agar and macroscopically visible colonies were counted after 3 weeks or 10^6 cells were injected subcutaneously into athymic nude mice.

Cumulative growth advantage assay

BJ-ET cells were transduced with pMSCV-Blast-RAS^{V12}-ERTAM, drug selected and transduced with miR-Vec-YFP or pRS-GFP constructs. Efficiency of transduction (starting at 20-60%) was assessed by FACS in FL1, and cells were plated with and without 10^{-7} M 4-OHT-Tamoxifen. Cells were propagated and percentage positive cells was measured twice a week. The relative growth advantage was calculated as described (Voorhoeve and Agami, 2003).

IP-kinase assay and flow cytometry

IP-kinase assay and flow cytometry were performed as described (Agami and Bernards, 2000).

Expression-array analysis and target prediction

Total RNA from BJ-ET-st-p16^{kd}-RAS^{V12} cells either expressing a p53^{kd} shRNA or the miR-Vec-cluster, was extracted using Trizol (Invitrogen) and hybridized to an oligo microarray using a standard protocol (<http://microarrays.nki.nl>). The genes that decreased 2 fold or more were further screened for possible miR-372&373 target sites using a local version of the TargetScan algo-

rithm (Lewis et al., 2003) with default parameters (<http://www.mekentosj.com/targetscanner>).

Acknowledgments

We thank Martijn Kedde and Hugo Horlings for technical help, Ron Kerkhoven and Mike Heimerikx for support in microarray analysis, Steve de Jong for reagents, Wigard Kloosterman for help in miRNA *in situ* protocol, J. Wolter Oosterhuis for supportive work on histology and Alexandra Pietersen for critical reading of the manuscript. This work was supported by grants from the Dutch Cancer Society to P.M.V, C.S and R.A. and the EURYI award to R.A.

References

- Agami, R., and Bernards, R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell-cycle arrest in response to DNA damage. *Cell* 102, 55-66.
- Almstrup, K., Hoei-Hansen, C. E., Wirkner, U., Blake, J., Schwager, C., Ansorge, W., Nielsen, J. E., Skakkebaek, N. E., Rajpert-De Meyts, E., and Leffers, H. (2004). Embryonic stem cell-like features of testicular carcinoma *in situ* revealed by genome-wide gene expression profiling. *Cancer Res* 64, 4736-4743.
- Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 122, 553-563.
- Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A. H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C. A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 436, 660-665.
- Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550-553.
- Burger, H., Nooter, K., Boersma, A. W., Kortland, C. J., and Stoter, G. (1998). Expression of p53, Bcl-2 and Bax in cisplatin-induced apoptosis in testicular germ cell tumor cell lines. *Br J Cancer* 77, 1562-1567.
- Chen, C. Z., Li, L., Lodish, H. F., and Bartel, D. P. (2004). microRNAs modulate hematopoietic lineage differentiation. *Science* 303, 83-86.
- Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K.,

- Dotan, Z. A., Niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W., et al. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 436, 725-730.
- Collado, M., Gil, J., Efeyan, A., Guerra, C., Schumacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M., et al. (2005). Tumor biology: senescence in premalignant tumors. *Nature* 436, 642.
- De Vita, G., Bauer, L., da Costa, V. M., De Felice, M., Baratta, M. G., De Menna, M., and Di Lauro, R. (2005). Dose-dependent inhibition of thyroid differentiation by RAS oncogenes. *Mol Endocrinol* 19, 76-89.
- Doench, J. G., and Sharp, P. A. (2004). Specificity of microRNA target selection in translational repression. *Genes Dev* 18, 504-511.
- Duursma, A., and Agami, R. (2005). p53-Dependent regulation of Cdc6 protein stability controls cellular proliferation. *Mol Cell Biol* 25, 6937-6947.
- el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* 75, 817-825.
- Forstemann, K., Tomari, Y., Du, T., Vagin, V. V., Denli, A. M., Bratu, D. P., Klattenhoff, C., Theurkauf, W. E., and Zamore, P. D. (2005). Normal microRNA maturation and germline stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol* 3, e236.
- Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. F. (1996). Abnormal centrosome amplification in the absence of p53. *Science* 271, 1744-1747.
- Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999). Creation of human tumor cells with defined genetic elements. *Nature* 400, 464-468.
- Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100, 57-70.
- Hatfield, S. D., Shcherbata, H. R., Fischer, K. A., Nakahara, K., Carthew, R. W., and Ruohola-Baker, H. (2005). Stem cell division is regulated by the microRNA pathway. *Nature* 435, 974-978.
- He, L., Thomson, J. M., Hemann, M. T., Hernandez-Monge, E., Mu, D., Goodson, S., Powers, S., Cor-don-Cardo, C., Lowe, S. W., Hannon, G. J., and Hammond, S. M. (2005). A microRNA polycistron as a potential human oncogene. *Nature* 435, 828-833.
- John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C., and Marks, D. S. (2004). Human microRNA targets. *PLoS Biol* 2, e363.
- Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K. L., Brown, D., and Slack, F. J. (2005). RAS is regulated by the let-7 microRNA family. *Cell* 120, 635-647.
- Justice, R. W., Zilian, O., Woods, D. F., Noll, M., and Bryant, P. J. (1995). The *Drosophila* tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes Dev* 9, 534-546.
- Kersemakers, A. M., Mayer, F., Molier, M., van Weeren, P. C., Oosterhuis, J. W., Bokemeyer, C., and Looijenga, L. H. (2002). Role of P53 and MDM2 in treatment response of human germ cell tumors. *J Clin Oncol* 20, 1551-1561.
- Kolfschoten, I. G., van Leeuwen, B., Berns, K., Mullenders, J., Beijersbergen, R. L., Bernards, R., Voorhoeve, P. M., and Agami, R. (2005). A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. *Cell* 121, 849-858.
- Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15-20.
- Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell* 115, 787-798.
- Li, Y., Pei, J., Xia, H., Ke, H., Wang, H., and Tao, W. (2003). Lats2, a putative tumor suppressor, inhibits G1/S transition. *Oncogene* 22, 4398-4405.
- Lim, L. P., Lau, N. C., Garrett-Engle, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S., and Johnson, J. M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769-773.
- Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B. L., Mak, R. H., Ferrando, A. A., et al. (2005). microRNA expression profiles classify human cancers. *Nature* 435, 834-838.

- Lutzker, S. G., and Levine, A. J. (1996). A functionally inactive p53 protein in teratocarcinoma cells is activated by either DNA damage or cellular differentiation. *Nat Med* 2, 804-810.
- Masters, J. R., and Koberle, B. (2003). Curing metastatic cancer: lessons from testicular germ-cell tumors. *Nat Rev Cancer* 3, 517-525.
- Mayer, F., Stoop, H., Scheffer, G. L., Scheper, R., Oosterhuis, J. W., Looijenga, L. H., and Bokemeyer, C. (2003). Molecular determinants of treatment response in human germ cell tumors. *Clin Cancer Res* 9, 767-773.
- McPherson, J. P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak-Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J., et al. (2004). Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. *Embo J* 23, 3677-3688.
- Michaloglou, C., Vredeveld, L. C., Soengas, M. S., Denoyelle, C., Kuilman, T., van der Horst, C. M., Majoor, D. M., Shay, J. W., Mooi, W. J., and Peepers, D. S. (2005). BRAF^{E600}-associated senescence-like cell-cycle arrest of human naevi. *Nature* 436, 720-724.
- Mostert, M., Rosenberg, C., Stoop, H., Schuyer, M., Timmer, A., Oosterhuis, W., and Looijenga, L. (2000). Comparative genomic and *in situ* hybridization of germ cell tumors of the infantile testis. *Lab Invest* 80, 1055-1064.
- O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435, 839-843.
- Oosterhuis, J. W., and Looijenga, L. H. (2005). Testicular germ-cell tumors in a broader perspective. *Nat Rev Cancer* 5, 210-222.
- Ota, A., Tagawa, H., Karnan, S., Tsuzuki, S., Karpas, A., Kira, S., Yoshida, Y., and Seto, M. (2004). Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res* 64, 3087-3095.
- Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by let-7 microRNA in human cells. *Science* 309, 1573-1576.
- Robins, H., Li, Y., and Padgett, R. W. (2005). Incorporating structure to predict microRNA targets. *Proc Natl Acad Sci U S A* 102, 4006-4009.
- Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic RAS provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88, 593-602.
- Suh, M. R., Lee, Y., Kim, J. Y., Kim, S. K., Moon, S. H., Lee, J. Y., Cha, K. Y., Chung, H. M., Yoon, H. S., Moon, S. Y., et al. (2004). Human embryonic stem cells express a unique set of microRNAs. *Dev Biol* 270, 488-498.
- Takahashi, Y., Miyoshi, Y., Takahata, C., Irahara, N., Taguchi, T., Tamaki, Y., and Noguchi, S. (2005). Downregulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. *Clin Cancer Res* 11, 1380-1385.
- Tarapore, P., and Fukasawa, K. (2002). Loss of p53 and centrosome hyperamplification. *Oncogene* 21, 6234-6240.
- Toji, S., Yabuta, N., Hosomi, T., Nishihara, S., Kobayashi, T., Suzuki, S., Tamai, K., and Nojima, H. (2004). The centrosomal protein Lats2 is a phosphorylation target of Aurora A kinase. *Genes Cells* 9, 383-397.
- Voorhoeve, P. M., and Agami, R. (2003). The tumor-suppressive functions of the human INK4A locus. *Cancer Cell* 4, 311-319.
- Weinert, T. (1998). DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. *Cell* 94, 555-558.
- Westbrook, T. F., Martin, E. S., Schlabach, M. R., Leng, Y., Liang, A. C., Feng, B., Zhao, J. J., Roberts, T. M., Mandel, G., Hannon, G. J., et al. (2005). A genetic screen for candidate tumor suppressors identifies REST. *Cell* 121, 837-848.
- Xu, T., Wang, W., Zhang, S., Stewart, R. A., and Yu, W. (1995). Identifying tumor suppressors in genetic mosaics: the *Drosophila* lats gene encodes a putative protein kinase. *Development* 121, 1053-1063.
- Zamore, P. D., and Haley, B. (2005). Ribo-gnome: the big world of small RNAs. *Science* 309, 1519-1524.

Supplementary data

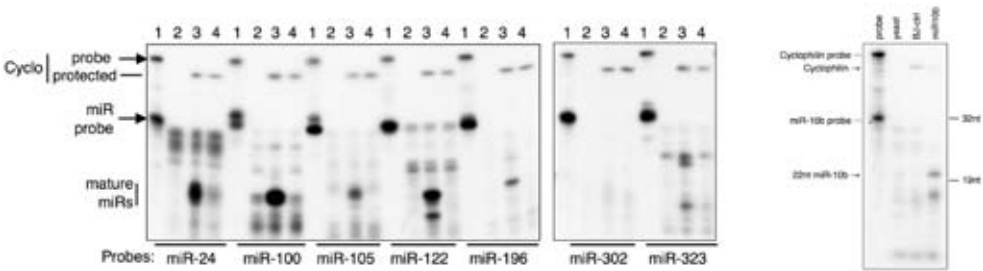


Figure S1. Expression from miR-Vec. RPA analysis (1=probe input, 2=yeast RNA control) of the BJ-ET cells expressing a relevant (3) or a control (4) miR-Vec.

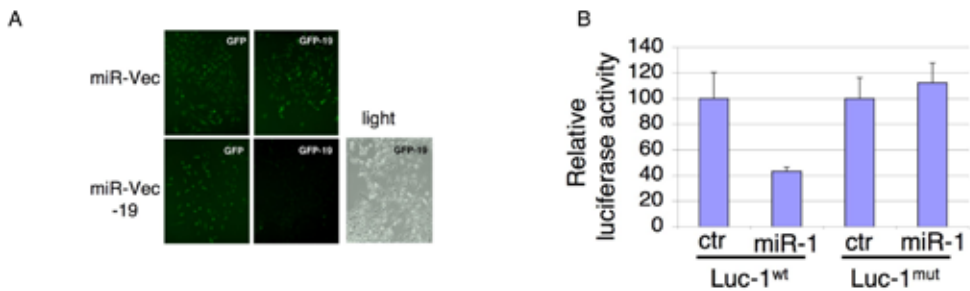


Figure S2. Testing the functionality of miR-Vec. (A) GFP was tagged at its 3' end with a sequence that is complementary to mature miR-19 (GFP-19). HeLa cells (lacking endogenous miR-19) were transfected with either miR-Vec-19 or miR-Vec-Ctrl together with either GFP-19 or GFP control. After 24 hours, GFP containing cells were visualized with fluorescence microscopy. (B) HeLa cells were transfected with the indicated plasmids together with a control Renilla vector. Firefly luciferase activity was measured and normalized to Renilla activity. Error bars show standard variation of three independent transfections.

MSCV Forward	GCCTTTAAACTTAACTTGGTACCGAGC	
MSCV Reverse	CATTCCCCCTTTTCTGGGAGAC	
miR-	oligo for RPA probe:	
24	TACACACTGGCTCAGTTCCAGGAAACAGGAGCCTGTCTC	
34	GTTTCTTTGGCAGTGTCTTAGCTGGTGTGTGTGCTGTCTC	
100	TTGCCACAAACCCGTAGATCCGAACCTGTGTGCTGTCTC	
105	ATCTGTGTCAAAATCTCCAGACTCTCTGTGTGTCTGTCTC	
122	AGAGCTGTGAGTGTGACAAATGGTGTGTGTGTCTGTCTC	
149	CCCGAGCTCTGGTCCGTGTCTTCACTCCCGTCTGTCTC	
155	TTAAATGCTAAATCTGTATAGGGGTTTTGGCTCCCTGTCTC	
196	AATTAGTAGTGTTCATGTGTGTGGGCTGGGCTGTCTC	
302	AAAGAAGTAAGTCTTCCATGTGTGTGGTGTGGCTGTCTC	
323	TTATGGGACCAATACAGCTGTGACCTTTTGGCTGTCTC	
371	GGTGAAGTCCGCCATCTTTTGGTGTACCTGTCTC	
372	CAAGTGAAGTCTCTCGACATTTGAGCTGCACCTGTCTC	
373	GTACTGGGAAGTCTCTCGATTTTGGGTTGCCCTGTCTC	
oligo for mutating Mir372	372mutfwd	ATTCTGATGTCCAAAGTGGAGGCAAGCTTGACATTTGAGCTCACCG
oligo for mutating Mir373	373mutfwd	CTTTTGTCTGTACTGGGGTTAACTGCGATTTTGGGTTGCCCTG
for luciferase constructs:	pGL3-SP FW	ctag gagcaaggtcaataaatacacaagcttggGACCTCCATATGACGGTg
	pGL3-SP Re	ctagcACCGTCATATGgGACGTCCaaacatttgtgattttattacttctgtc
pGL3-SP-372	FW	ggagcgtccaaatgtcgcagcactttga
	Re	cgggtcaaatgtcgcagcatttgagctccacgt
pGL3-SP-373	FW	tgacaccccaaatcgaagcacttoga
	Re	cgggtcgaatgtcttogatatttgggggtgtcaacgt
primers for LATS2 QPCR		
set1	caggatcgagccagcagatg	
	ccgcacaaatctgtctatc	
set2	GCGACCTCTGGGATGATGTG	
	TTGGAGTCCCCACCACTGAA	
set3	cagactggcagcagcagcat	
	gtcactgggggttggcatgag	
ACTB fwd	CTGGCACCGACACAAAT	
ACTB rev	GGGCCGACTGTCATACT	
CDK4 fwd	CCCTGGTGTGTTACACTCTGGTACCGAGC	
CDK4 rev	GTCACTCTCTGGAGGCAGCCCAACAGG	
cloning of LATS2 3'UTR to pGL3-SP	FW	gcggaagctctga atggggggcaggaccccccaaccactgc
	Re	Cgcacoggt cyttattgcacagagatttctcatcaatgttcttcag
mutating two 372/3 sites in LATS2 3'UTR (in red the mutation)	Re mut2	gttttttccata gcactt attcacatttgaagaagcctaaaacgaagacattgtgagaatctctgtgcaataag
	Re mut2+1	Gtttgaaga gagcact tatttgttatccatttttctactaatatagggttaacttgcacaaatcatgctctgttttttctacattgtattttaccata catatg
targeting LATS2 sequences for knockdown constructs against LATS2		
LATS2#1	AGCTTCAGGAGCAGCAAC	
LATS2#2	CAAGCATCTGAGCACGCA	
LATS2#3	CTCTGTGACTGTGGAGTG	

Supplementary data S3. List of primers. A list of the primers used in our study to clone human miRNAs to miR-Vec, the primers used to recover the inserts, primers used to generate miR-372&373 mutations, the primers used for the RPA analysis, primers used for Q-RT-PCR, primers used for LATS2 knockdown shRNA vectors and primers used for cloning the LATS2 3'UTR wt and mutant.

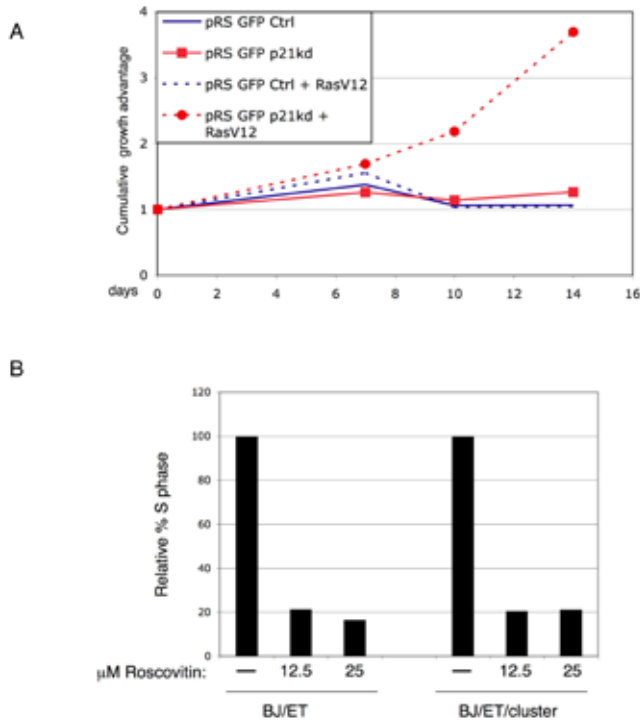


Figure S4. (A) YFP-competition assay in BJ-ET cells as in Figure 3B with a p21^{kd} shRNA expressing vector or empty control vector. (B) BJ-ET cells expressing the miR-371-3 cluster or empty vector control were incubated overnight with the indicated concentrations of roscovitin and relative S phase was determined as in Figure 4C.

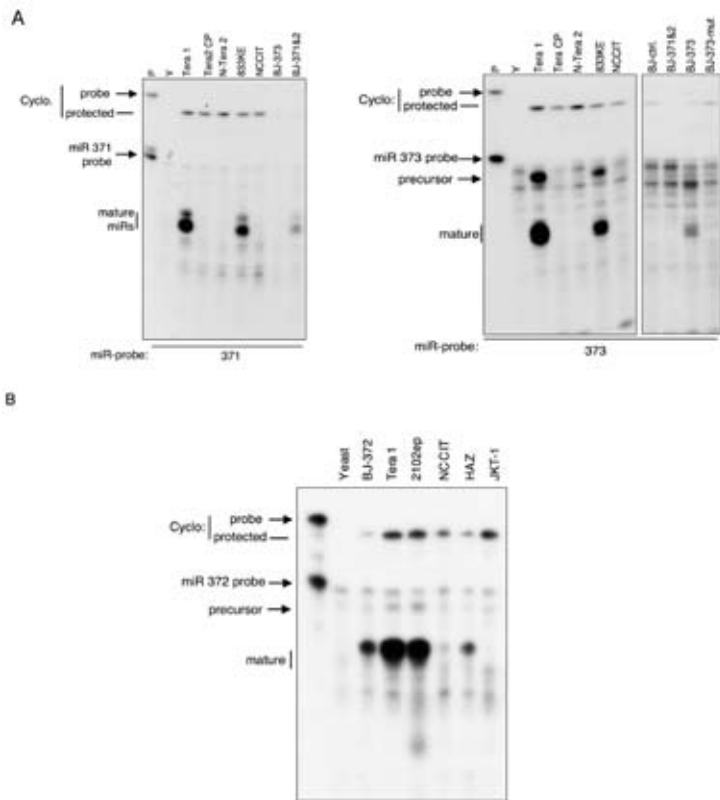


Figure S5. Expression of miR-371, miR-372 and miR-373 in TGCT cell lines. (A) We used RPA to detect miR-371, miR-372 and miR-373 expression in several TGCT cell lines, and BJ-ET cells transduced with the indicated miR-Vecs for comparison. (B) RPA analysis of miR-372 in additional TGCT cell lines.

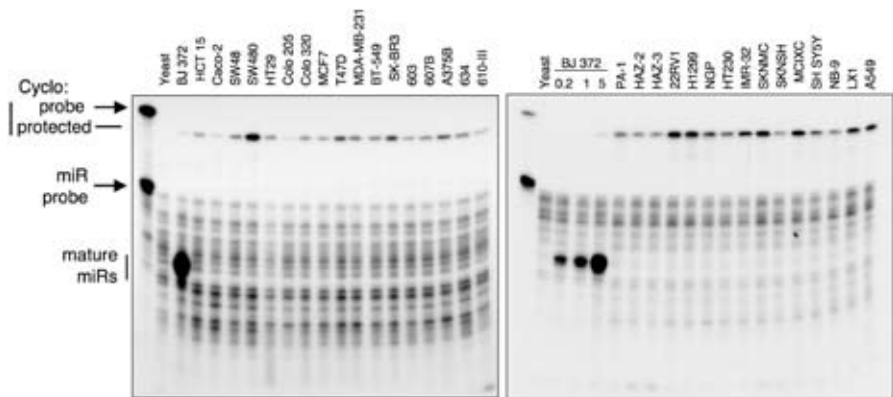


Figure S6. Lack of expression of miR-372 in somatic cell lines. RPA analysis of indicated cell lines for the expression of miR-372. 10 μ g of RNA was used. As comparison RNA from BJ-ET expressing miR-Vec-372 was titrated in the indicated amounts to control RNA.

SEMINOMA		miRNA 372:	exon 5-8	miRNA 302
	pathology-nr		p53	
SE-1	L05-158	++		
SE-2	L05-119	++	WT	-
SE-3	L05-114	++	WT	-
SE-4	L05-106	++		-
SE-5	L05-89	++	WT	+
SE-6	L05-74	++		+
SE-7	L05-49	++		+/-
SE-8	L05-43	++		
SE-9	L05-32	++		
SE-10	L04-235	++		
SE-11	L04-197	++		
SE-12	L04-175	++		
SE-13	L04-174	++		
SE-14	L04-163	++		
SE-15	L04-152	++		
SE-16	L04-105	neg	WT	-
SE-17	L04-89	+	WT	
SE-18	L04-48	+	WT	
SE-19	L03-250	+		
SE-20	L03-234	neg	mut exon 8	-
SE-21	L03-230	++		
SE-22	L02-141	+	WT	
SE-24	L01-139	neg	WT	-
SE-25	L00-109	nd		
SE-26	T94-9831	pos	WT	
SE-27	T93-2207	pos	WT	
SE-28	L03-19	neg	mut exon 5	-
SE-29	L99-108	pos	WT	
SE-30	L98-15	pos	WT	
SE-31	L99-82	pos	WT	
SE-32	T97-3179	pos	WT	
SE-33	T95-1087	pos	WT	
SE-34	L05-164	pos		

NON-SEMINOMA		pathology-nr	components			
NS-1	L05-109	EC, Te, YS	++			
NS-2	L05-99	EC, Te, YS	++			-
NS-3	L05-81	EC, Te, YS	++	WT		+/-
NS-4	L05-79	EC, Te, YS	+	WT		+
NS-5	L05-59	EC, Te, CH	++			
NS-7	L05-53	EC, Te, YS	++			
NS-10	L05-27	CH	-			-
NS-11	L05-18	EC, CH	-	WT		-
NS-12	L04-108	EC, Te, YS	++			
NS-13	L04-77	EC	++	WT		-
NS-14	L02-56	EC, YS	+			
NS-15	L03-215	EC, YS	+			
NS-16	L03-197	EC, YS	+			
NS-17	T94-1013	YS	+/-	WT		
NS-18	T95-888	YS	++			-
NS-22	L02-130	EC	-	WT		-
NS-23	T92-5207	EC	+			
NS-24	T96-2795	EC	+			
NS-25	L02-120	EC, Te, YS, CH	+/-			
NS-26	L05-155	EC	+			

SPERMATOCYTAIR-SEMINOMA		pathology-nr	
SS-1	T96-642		-
SS-3	T94-7593		-
SS-4	T94-8444		-
SS-5	L03-242		-

NORMAL TESTIS		
testis-1		-
testis-2		-
testis-3		-
testis-5		-

Supplementary data S7. Summary of the expression of miR-372, miR-302 and p53 status in primary material.

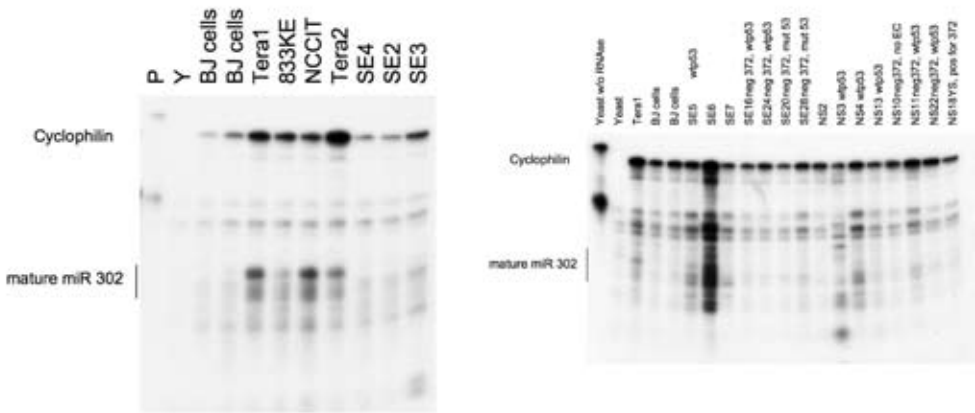


Figure S8. Expression of miR-302 in TGCT cell lines and primary tumor material. We used RPA to detect miR-302 expression in several TGCT cell lines. The RPA probe was designed such that it detects all five miR-302a-d species (Supplementary data S3).

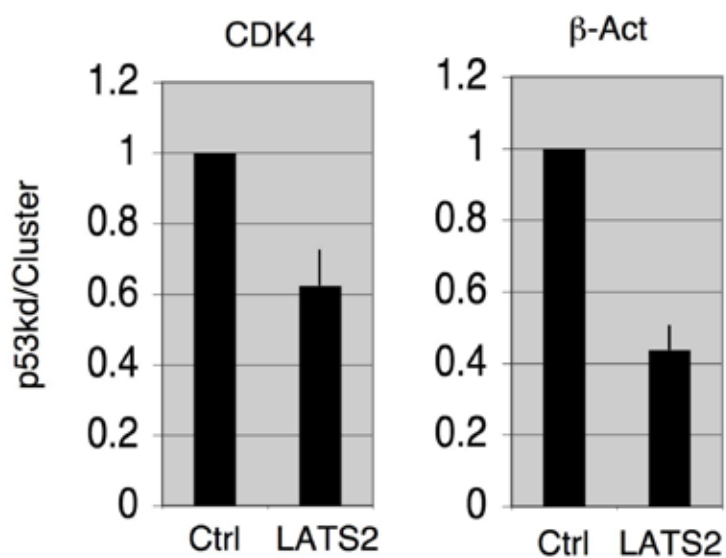


Figure S9. RNA extracted from the same cell populations as in Figure 5A, was used for Q-RT-PCR. SD of LATS2 represents independent experiments performed with three different primer sets. The level of LATS2 RNA was either compared with CDK4 expression or β -actin.

Chapter 4

Regulation of the p27^{Kip1} tumor suppressor by miRNA-221 and miRNA-222 promotes cancer cell proliferation

EMBO Journal (2007)

Regulation of the p27^{Kip1} tumor suppressor by miRNA-221 and miRNA-222 promotes cancer cell proliferation

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microRNAs (miRNAs) are potent posttranscriptional regulators of protein coding genes. Patterns of misexpression of miRNAs in cancer suggest key functions of miRNAs in tumorigenesis. However, current bioinformatics tools do not fully support the identification and characterization of the mode of action of such miRNAs. Here we used a novel functional genetic approach and identified miR-221 and miR-222 (miR-221&222) as potent regulators of p27^{Kip1}, a cell-cycle inhibitor and tumor suppressor. Using miRNA-inhibitors we demonstrate that certain cancer cell lines require high activity of miR-221&222 to maintain low p27^{Kip1} levels and continuous proliferation. Interestingly, high levels of miR-221&222 appear in glioblastomas and correlate with low levels of p27^{Kip1} protein. Thus, deregulated expression of miR-221&222 promote cancerous growth by inhibiting the expression of p27^{Kip1}.

Introduction

The p27^{Kip1} gene is a member of the Cip/Kip family of cyclin dependent kinase inhibitors that function to negatively control cell-cycle progression (recently reviewed in (Koff, 2006)). It binds to CDK2 and cyclin E complexes to prevent cell-cycle progression from G1 to S phase. p27^{Kip1} also acts as a tumor suppressor and its expression is often disrupted in human cancers. Studies in mice have shown that loss of p27^{Kip1} increases tumor incidence and tumor growth rate in either specific genetic backgrounds or when mice are challenged with carcinogens (Fero et al., 1998). Decreased p27^{Kip1} levels have been correlated with tumor aggressiveness and poor patient survival (Loda et al., 1997; Lu et al., 1999; Migita et al., 2002; Mineta et al., 1999; Ponce-Castaneda et al., 1995; Porter et al., 1997).

Although p27^{Kip1} is characterized as a tumor suppressor, inactivating point mutations with loss of heterozygosity are rarely observed in human cancer. Therefore, the low levels of p27^{Kip1} protein observed in many aggressive types of cancer are likely to be mediated by other mechanisms (Ponce-Castaneda et al., 1995). The abundance of p27^{Kip1} protein is largely controlled through a variety of posttranscriptional regulatory mechanisms (Alessandrini et al., 1997; Chu

et al., 2007; Grimmier et al., 2007; Kardinal et al., 2006), among which are sequestration by cyclin D/CDK4 complexes, accelerated protein destruction and cytoplasmic retention (Koff, 2006). In certain types of cancers, such as colorectal cancer, high expression levels of Skp2 and Cks1, p27^{Kip1} specific ubiquitin ligase subunits, were strongly associated with low p27^{Kip1} expression and aggressive tumor behavior (Hershko and Shapira, 2006). However, several studies have indicated that the genes controlling the stability of p27^{Kip1} protein might not always account for its lower expression in cancer and that p27^{Kip1} can also be regulated at the level of translation (Chilosi et al., 2000; Hengst and Reed, 1996; Millard et al., 1997).

miRNAs are a class of small noncoding RNAs that function to control gene expression through association with the 3'UnTranslated Region (3'UTR) of protein coding genes and subsequent induction of translation inhibition, which can also be associated with transcript destabilization (Bagga et al., 2005; Giraldez et al., 2006; Wu et al., 2006). miRNAs have been found to be implicated in a large variety of cellular processes and their aberrant expression has been linked to disease (Kloosterman and Plasterk, 2006). Recently, research has uncovered both the tumor sup-

pressive and oncogenic potential of a number of miRNAs, underscoring their importance in human cancer (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; He et al., 2005b; Kent and Mendell, 2006; Lu et al., 2005; Mayr et al., 2007). In particular, we have constructed and used a library of miRNA expressing vectors (miR-Lib, see Voorhoeve et al., 2006) to identify the oncogenic potential of the miR-372 family using a functional genetic approach (Voorhoeve et al., 2006). As p27^{Kip1} is mostly controlled at the posttranscriptional level and miRNAs are potent regulators of gene expression, we hypothesized a role for miRNAs in the contribution of cancer progression through the suppression of p27^{Kip1} expression.

Results

miR-221 and miR-222 are potent suppressors of p27^{Kip1} expression

To identify miRNAs that control the expression of p27^{Kip1} through its 3'UTR we constructed a retroviral sensor vector containing the GFP coding region upstream of the 3'UTR of p27^{Kip1} (p27Sen; Figure 1A). We transduced p27Sen into HeLa cells and expanded a single clone expressing GFP-p27-3'UTR (Figure 1B, data not shown). Subse-

quently, all miRNA expressing vectors from our miRNA expression library were individually transduced and drug selected to obtain resistant growing cells, each containing one unique integrated miR-Vec. Around 90% of vectors gave stable clones. These cells were mixed into one pool and after 2 weeks of culturing, flow cytometry analysis revealed only a slight lower overall GFP signal in the miR-Lib transduced cells when compared with the untransduced population (Figure 1C). This effect was most likely due to nonspecific promoter competition between miR-Vec and the sensor vector. Subsequently, from the total miR-Lib-transduced population we sorted out the low level GFP-expressing cells (less than half of the fluorescence peak signal), extracted genomic DNA and compared the abundance of miR-Vec inserts between low and total GFP-expressing populations in a barcode experiment using miR-Array (Figure 1D, see Voorhoeve et al., 2006) for details). This analysis identified miR-Vec constructs that were enriched in the low GFP-expressing cells (Figure 1E). miR-221 vector gave the most pronounced and reproducible effect.

To verify and quantify the effect of each miRNA

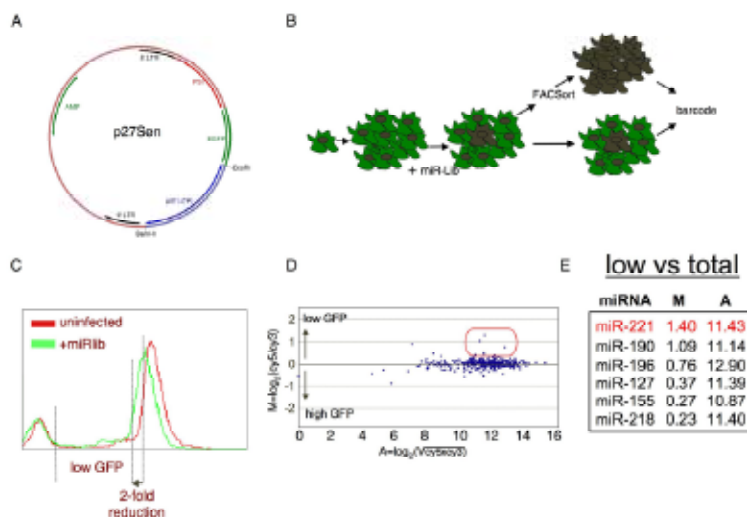


Figure 1. A genetic screen to identify miRNA-suppressors of p27^{Kip1}. (A) A schematic representation of p27Sen, a retroviral vector for stable expression of GFP under the control of p27-3'UTR. (B) A clonal population of GFP-p27-3'UTR expressing cells was subjected to transduction with the entire miRNA library (miR-Lib) in a single well format, selected and then mixed for further analysis. Two independent experiments were performed. Subsequently, low expressing GFP cells were sorted and the abundance of miRNA inserts was compared to the total population using miR-Array. (C) A GFP-expression profile of control and miR-Lib transduced cells. The region marked 'low GFP' was sorted out of the entire population of cells. (D) A representative MA plot showing the signal and change of signal of each miRNA insert. (E) The top 6 hits found enriched in the low GFP-expressing cells.

vector in the list of enriched miRNA vectors, we subcloned the p27-3'UTR downstream of luciferase. Analysis of transiently transfected cells revealed that only the miR-221 expressing vector significantly suppressed p27-3'UTR activity around 2.5 fold (Figure 2A). To further substantiate the specificity of p27-3'UTR mediated suppression by miR-221, we stably introduced it into cells containing p27Sen or control-Sen vectors. Consistent with the results above, we found that miR-221 expression suppressed GFP expression in p27Sen-transduced cells by 2.5 fold (Figure 2B). As the expression of GFP in the control-Sen-transduced cells remained unchanged, we concluded that miR-221 is a potential regulator of the 3'UTR of p27.

We next examined the expression of miR-221 and its effect on endogenous p27^{Kip1} expression. By RNase protection assay (RPA), we detected low miR-221 level in HeLa cells and found potent expression of miR-221 from its vector (Figure 2C). By immunostaining we found the endogenous p27^{Kip1} protein level to be 2.5 fold lower in HeLa cells stably expressing miR-221, compared with control cells (Figure 2D). Intriguingly, by quantitative RT-PCR we found that the mRNA level of p27 remained unaltered in the miR-221 transduced cells, indicating that miR-221 controls p27^{Kip1} translation but not mRNA stability (Figure 2E). To rule out any miR-221 mediated effect on p27 protein stability, a cyclohexamide experiment was performed (Figures 2F and S3). Although miR-221 expressing HeLa cells have lower p27 protein levels, the half-life of p27 was comparable to control cells. Therefore, we conclude that miR-221 expression does not change the rate of p27 decay. p27^{Kip1} protein level changes during cell-cycle progression, accumulating when cells progress through G1 and sharply decreasing just before cells enter S phase (Kaldis, 2007). Additionally, p27^{Kip1} protein levels rise when cells exit cell-cycle to G0, and decreases when cells enter the cell-cycle again (Kaldis, 2007). These alterations in p27^{Kip1} levels are mainly caused by regulation at the protein degradation level (Alessandrini et al., 1997; Chu et al., 2007; Grimmmler et al., 2007; Kardinal et al., 2006). To examine the effect of miR-221 on p27^{Kip1} levels during the cell-cycle, we blocked cells in mitosis and then released them to enter G1 and S phases. Immunoblot analysis revealed that while a global reduction in p27^{Kip1} was observed in miR-221 cells compared with control, still, a similar relative increase in p27 levels during G1 and decrease just before S phase were seen in both cell types (Figure S1A). As expected

from p27 function in G1, its lower levels in miR-221-expressing cells resulted in a faster entry of cells into S phase, while entering into G1 was unaffected (Figure S1B). Similarly, the exit of cells from the cell-cycle and the relative accumulation of p27 during this process were not affected by miR-221 in primary human cells (data not shown). Thus, we identified miR-221 as a suppressor of endogenous p27^{Kip1} expression.

miR-221 is part of a gene cluster also expressing miR-222, a close homologue of miR-221. Both miRNAs share an identical seed sequence and are predicted (by PicTar and TargetScanS) to bind to p27-3'UTR at two sites (Figure 3A). Our library already contained a vector designed to express miR-222. However, due to a low virus titer, this vector was among the 10% of unsuccessful stable clones in our screen (data not shown). To examine the effectiveness of miR-222 as well as the specificity of both miR-221 and miR-222, we constructed seed mutated miR-221 and miR-222 vectors as well as a cluster vector. RPA analysis verified the respective expression and lack of expression of wildtype and mutated miR-221 and miR-222 constructs (data not shown). As expected from their seed sequence identity, luciferase reporter assays revealed a similar suppressive activity of miR-222 towards p27-3'UTR as miR-221 (Figure 3B). Importantly, mutating the seed sequence of miR-221 and miR-222 completely abolished their suppressive activity, indicating that the expression of the miRNAs is responsible for p27 suppression. In line with these experiments, the introduction of a cluster-containing vector that directs the expression of both miRNAs gave a similar suppressive activity (Figure 3C).

The 3'UTR of p27 contains two predicted miR-221 and 222 target sequences. We have mutated these predicted sites in order to examine their requirement for miR-221&222 function using luciferase reporter assays (Figure 3A). We found that the mutated p27-3'UTR-DM was completely refractory to the miR-221 and miR-222 suppressive effect (Figure 3C). As each single mutant retained at least some sensitivity to miR-221&222 (data not shown), this result indicates that both predicted sites are required for miR-221&222 effects. In summary, we have identified and verified miR-221&222 as potent suppressors of p27^{Kip1} expression.

miR-221&222 activity is required for cancer cell proliferation

Intriguingly, three papers reporting the differential expression of miRNAs in primary glioblasto-

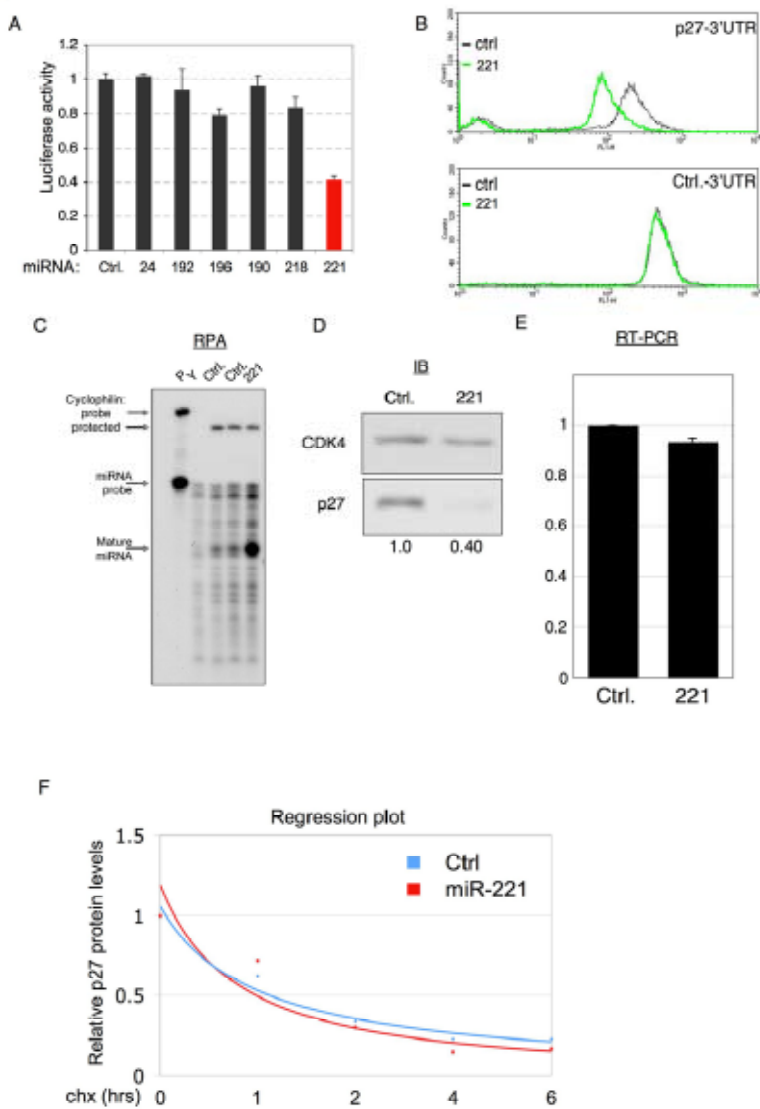


Figure 2. miR-221 inhibits translation of p27^{Kip1}. (A) Luciferase reporter experiments were performed with Firefly-luciferase-p27-3'UTR, control Renilla-luciferase and the indicated miRNA constructs. The luciferase ratio between the Firefly and Renilla of the control sample was adjusted to 1. A summary of three independent experiments is shown. (B) A stable p27Sen-HeLa cell line was transduced with miR-221 and control expressing vectors, and drug selected for a week. Polyclonal cell populations were analyzed by flow cytometry one week later. (C) HeLa cells were transduced with control or miR-221-expressing vectors and drug selected for one week. Subsequently, total RNA was extracted from the stable cells and RPA was performed with miR-221 and control cyclophilin probes. P is a lane of the probes without RNase treatment, Y is a lane where yeast RNA was used as control. (D) An immunoblot analysis with p27 and CDK4 antibodies on the same cell populations as in B. Quantification was performed using Tina 2.0 software. (E) Q-RT-PCR was performed on the same RNA extracts used in C. (F) HeLa cells stably expressing control or miR-221 vectors were treated with 100 µg/ml cyclohexamide. At the indicated timepoints, whole cell extracts were made, and analyzed by immunoblot with p27 and control tubulin antibodies. Band intensities were quantified using Tina 2.0 software, and the resulting p27-tubulin ratios plotted in a regression plot.

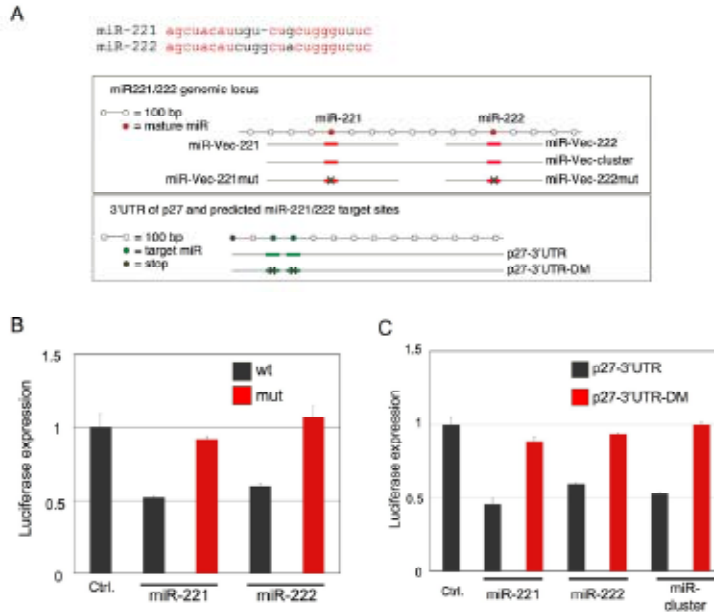


Figure 3. Specificity of p27^{Kip1} inhibition by miRNA-221 and miRNA-222. (A) A schematic representation showing the mature miR-221 and miR-222 sequences (in red identical sequences). In addition, the genomic locus of miR-221&222 cluster with the inserts used in the miRNA vectors. Red boxes represent miRNA precursor positions. Also, the 3'UTR of p27^{Kip1} is drawn. The green boxes represent the two predicted miR-221&222 targeting sequences (as predicted by Pictar and TargetScanS software). (B) Luciferase reporter experiments were performed as in Figure 2A. 221SM and 222SM are constructs in which the seed sequence of the miRNAs was altered. (C) Luciferase reporter experiments were performed as in Figure 2A. p27-3'UTR-DM (double mutant) is a construct where both predicted miR-221&222 targeting sequences were modified (see experimental procedures). (B+C) The histograms show a summary of the results of three independent experiments.

mas, papillary thyroid carcinomas and pancreas tumors, describe the upregulation of miR-221 as part of a cancer signature (Ciafre et al., 2005; He et al., 2005a; Lee et al., 2007; Pallante et al., 2006). In accordance with their findings, our data suggests that these miR-221&222-expressing tumors might depend on p27^{Kip1} downregulation for their survival. To examine this issue we generated antagomiR-221&222 molecules, antisense RNA oligos containing a cholesterol molecule at their 5' end that are 2'-O-methylated at every nucleotide (Figure 4A, see also experimental procedures for details). These molecules were previously shown to be capable of passing through cellular membranes to inhibit miRNA action by sequestering it from its targets (Krutzfeldt et al., 2005). To test antagomiR function, we added antagomiR-222 to the culture medium of cells that were cotransfected 24 hours earlier with luciferase-p27-3'UTR and either miR-222 or control expressing constructs (Figure 4B). Figure 4C shows that while the expected reduction (~2 fold) in luciferase expression was seen when miR-222

was cotransfected with p27-3'UTR, the luciferase level remained as high as in the control cells in the presence of antagomiR-222, indicating a complete block of miR-222 activity. To confirm specificity, we also added antagomiR-222 to cells transfected with constructs expressing miR-372 and its target luciferase-LATS2-3'UTR (see (Voorhoeve et al., 2006)). In this case, miR-372 remained fully active in the presence of the antagomiR, indicating the specific inhibitory effect of antagomiR-222 on miR-222 (Figure 4C). Similar results were obtained with antagomiR-221 (data not shown). To support these data we also tested the effect of antagomiR-221 on HeLa cells that have stable expression of miR-221. Figure 4D shows that the addition of antagomiR-221 to cells containing miR-221 restored p27^{Kip1} protein level to almost its normal level. As a result of antagomiR-221&222 administration, we detected lower levels of each of the mature miRNAs (Figure S5). Thus, these results establish the specific and powerful inhibitory effect exerted by antagomiR-221 and antagomiR-222 on their

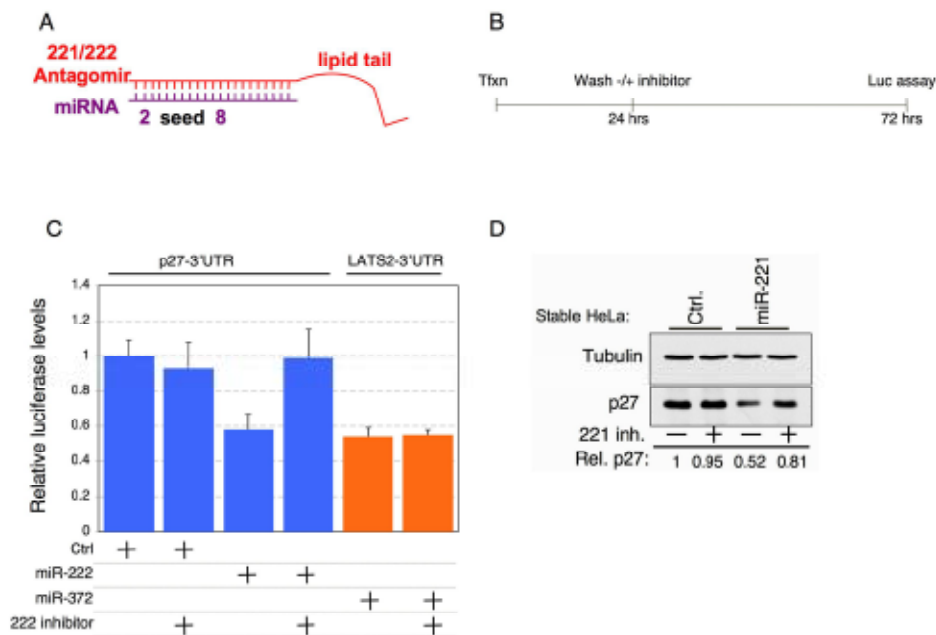


Figure 4. Inactivation of miR-221&222 with corresponding antagonomiRs. (A) Drawing showing the design of antagomiR-221 and antagomiR-222. (B+C) MCF-7 cells were transfected with the indicated luciferase reporter constructs, as done in Figure 2A. Twenty four hours following transfection antagomiR-222 (25 μ g) was added to the culture medium. Luciferase activity was measured 48 hours later. Results show a summary of three independent experiments. (D) Stable control and miR-221-expressing HeLa cell populations were treated with antagomiR-221 for a period of 24 hours. Whole cell extracts were made and analyzed by immunoblot with p27 and control tubulin antibodies. Bands were quantified using Tina 2.0 software.

corresponding miRNAs. Equally important, these observations also demonstrate the tight interaction between miR-221&222 activity and p27^{Kip1} gene expression.

Using antagomiR-221&222 we set out to test the relationship between miR-221&222, p27^{Kip1} and cell proliferation. We first examined several breast and glioblastoma cancer cell lines for miR-221&222 expression (Figure 5A). To explore the effect of miR-221&222 antagonomiRs on cellular proliferation we chose two cell lines that showed endogenous expression of miR-221&222 (U87 and MDA-MB-231), and two negative cell lines (MCF-7 and HeLa). Interestingly, treatment of U87 and MDA-MB-231 with both antagomiR-221&222 resulted in a clear proliferation arrest phenotype, which was accompanied by a significant reduction in detectable miRNA levels (Figure S5). However, no significant effect on proliferation was observed in treated MCF-7 and HeLa cell lines (Figure 5B). We verified and quantified the proliferation block using a 3T3 cell growth protocol (Figure 5C). Here, we observed that while treated HeLa cells and MCF-7 cells

continued to proliferate indistinguishably from mock treated cells, both U87 and MDA-MB-231 cells ceased proliferating almost completely and very rapidly following treatment. The arrest observed in U87 and MDA-MB-231 required simultaneous addition of both antagonomiRs, as the addition of only one antagonomiR (221 or 222) or a control antagonomiR designed to inhibit miR-372 was not sufficient to affect cellular growth (data not shown and Figure 5C). This indicates a functional overlap between miR-221 and miR-222 in controlling proliferation. Flow cytometry analysis showed that the antagomiR-221&222-induced proliferation arrest observed in the miR-221&222-expressing cell lines was due to a block in G1, which is consistent with the role of p27^{Kip1} in controlling progression through G1 phase of the cell-cycle. As observed above, miR-221&222 are regulators of p27^{Kip1}. Interestingly, when p27^{Kip1} levels were examined by immunostaining, only the cells containing miR-221&222 (U87 and MDA-MB-231) showed increased levels of the cell-cycle inhibitor p27^{Kip1} after administration of the antagonomiRs (Figure 5E). Furthermore, we

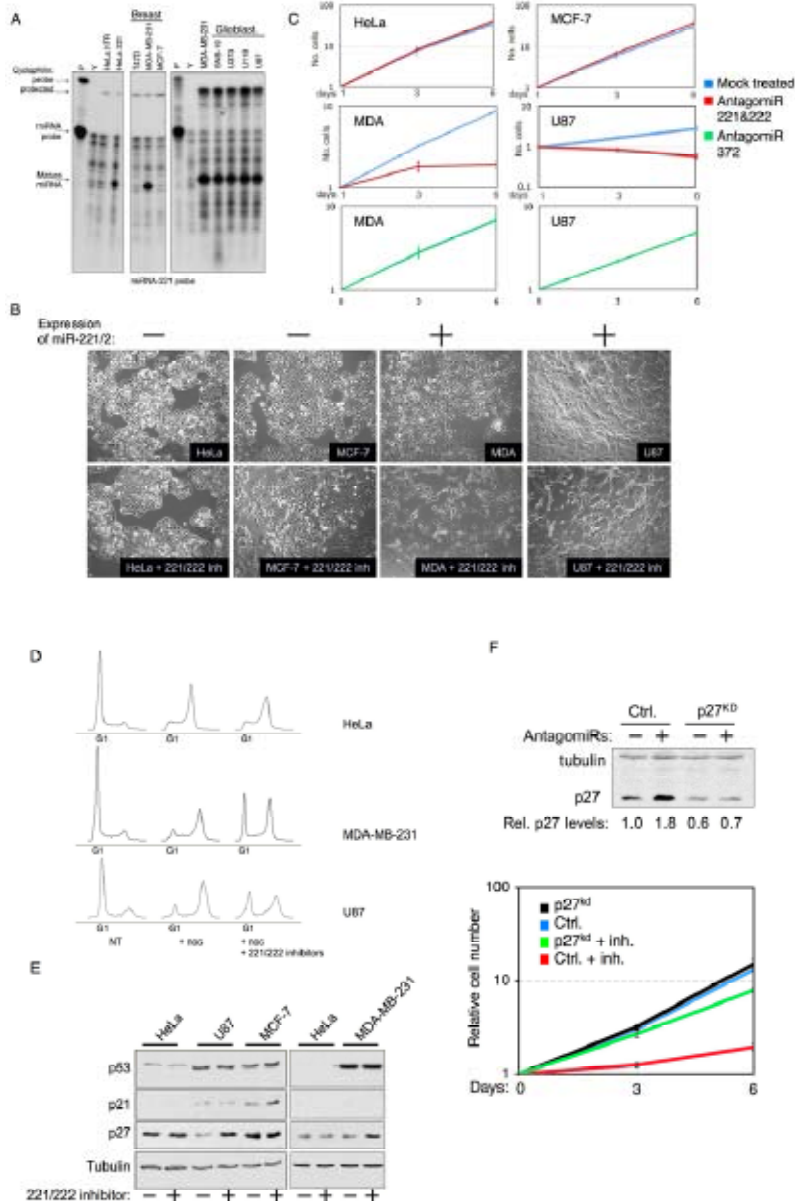


Figure 5. Oncogenic addition of certain cancer cell lines to miR-221&222 activity. (A) RPA was performed with miR-221 probe using total RNA from the indicated cell lines. Sizes of probes and mature miR-221 are indicated. (B) The indicated cancer cell lines were grown in the presence or absence of a mix of antagomiR-221 and antagomiR-222 oligos. Pictures were made 4 days after treatment. (C) The growth of the indicated cancer cell lines, either exposed or not to antagomiR treatment, was measured using a 3T3 protocol. (D) The indicated cell lines were either treated or not (NT = nontreated) with a mix of antagomiR-221 and antagomiR-222 oligos. 48 hours later, cells were split and nocodazole was added (+noc). Flow cytometry analysis was performed 24 hours later. (E) Cells were treated as in Figure 5B, whole cell lysate was extracted 3 days after treatment and subjected to immunoblot analysis with antibodies to detect the indicated proteins. (F) U87 cells were transfected with either control or a p27 shRNA-expressing construct. Efficiency of transfection was at least 80%, as determined by GFP (data not shown). After 24 hours, cell populations were treated with a mix of antagomiR-221 and antagomiR-222 and proliferation was determined as in panel C. An immunoblot analysis for p27^{Kip1} and control tubulin proteins was performed on wildtype and p27^{KD} transfected U87 cells. Bands were quantified using Tina 2.0 software.

found that regulation of p27 by miR-221&222 is a general feature, as inhibition of endogenously expressed miR-221&222 in BJ human primary fibroblasts by antagomiRs also resulted in elevation of p27 protein levels (Figure S4). The effect on p27^{Kip1} was specific since no change was observed in p21^{Cip1}, a stress associated cell-cycle inhibitor and p27's closest homologue, or in p53, a major tumor suppressor gene. Moreover, the induction of p27 by antagomiRs-221&222 was direct, as this was observed as early as 4 hours following treatment and preceded any detectable changes in cell-cycle profile (Figure S2). To examine whether the inhibition of p27^{Kip1} is essential for the effect of miR-221&222 on cell proliferation, we studied U87 cells treated with antagomiR-221&222 when p27 levels were suppressed using an shRNA vector targeting p27^{Kip1} (p27^{kd}). Figure 5F shows that while the proliferation of control U87 cells was efficiently blocked when miR-221&222 activity was inhibited, p27^{kd} cells continued to proliferate to a comparable ex-

periment as untreated cells (green line). No significant effect of p27^{kd} was seen in the mock treated cells (compare black and blue lines). These results demonstrate the causal relationship between miR-221&222 and cell proliferation. In certain cancer cell lines, the expression of miR-221&222 is required for maintaining low p27^{Kip1} protein levels and continuous cellular growth.

Next, we searched for evidence for this mechanism in human cancer. It was recently shown that high level of miR-221&222 is part of a signature for poor prognosis in glioblastomas (Ciafre et al., 2005). We therefore compared the expression levels of miR-221&222 and p27^{Kip1} in grade 3 tumors and peripheral tissues from 5 glioblastoma patients. Using quantitative RT-PCR, five tumors (#1-5) showed miR-221&222 expression that was significantly higher in the core tumor region when compared with the peripheral normal region (Figure 6A). As controls we used U87 and HeLa cells, which are miR-221&222 positive and negative cell lines respectively, as well as HeLa-

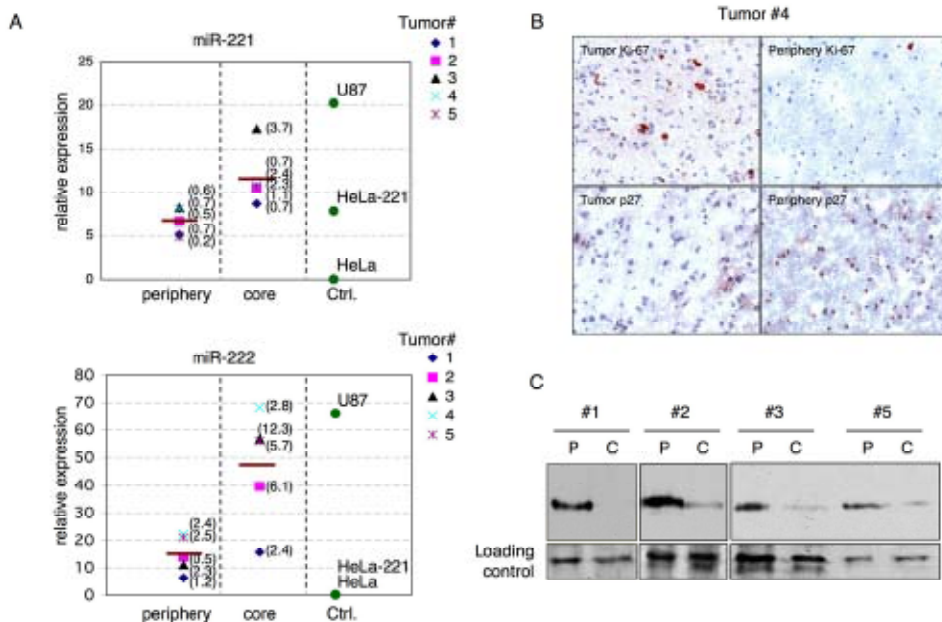


Figure 6. Deregulated miR-221 and miR-222 expression in glioblastomas correlate with p27^{Kip1} levels. (A) Q-RT-PCR for the expression of miR-221 and miR-222 was performed on RNA extracted from 5 frozen tumor tissues and 5 frozen peripheral tissues (0.5 cm from core tumor). As controls, we used the miR-221&222 negative HeLa cells, HeLa with miR-Vec-221 (HeLa-221) and U87, which are positive for both miRNAs. In brackets the standard deviation of three experiments. (B) Core and periphery material of tumor #4 was stained brown with Ki-67 and p27 antibodies. Nuclei were stained blue. (C) Whole cell extracts were made from core (C) and peripheral (P) material of tumors #1, 2, 3 and 5. Twenty micrograms of each sample was loaded on gel and subjected to either immunostaining with p27 antibody or silverstaining to show equal loading.

221, a cell line that was engineered to express miR-221 only. Interestingly, the elevated level of miR-221&222 in tumors #1, 2, 3, 4 and 5 was correlated with reduced expression of p27^{Kip1}. By immunohistochemistry, p27^{Kip1} staining was high and nuclear in peripheral tissue, whereas only a very weak staining was seen in the core of the tumor (Figure 6B). As control, we stained for Ki-67, a marker for cell proliferation expressed in many tumors. Additionally, using immunoblot analysis of whole cell extracts, we detected lower levels of p27^{Kip1} protein in the core of tumors #1, 2, 3 and 5 compared to their peripheral controls (Figure 6C). These results suggest that in a large proportion of glioblastoma tumors, elevated levels of miR-221&222 are correlated with low levels of its target p27^{Kip1}. Altogether, our results propose a mechanism by which deregulated expression of miR-221&222 promote cancerous growth by inhibiting the expression of p27^{Kip1} in human tumors.

Discussion

Perhaps the main challenge in the identification and characterization of miRNA function is the gain of a comprehensive understanding of their effect on the expression of target mRNAs and the resulting outcome on relevant cellular pathways and phenotypes. Up until now, only a handful of comprehensive studies on miRNA function have been done. One major reason for this is the prominent effect of miRNAs on protein translation, an effect that still cannot be quantified genome-wide. A second is that each cellular phenotype exhibits a different sensitivity to changes in protein levels and pathway activity, due to complex and robust network connections. This individual protein-to-phenotype behavior prompted us to test the effectiveness and activity of miRNAs on a single gene of interest in an unbiased manner. We therefore designed a novel genetic screen to experimentally identify potent miRNA effectors for a single gene. We exploited a miRNA expression library to select for miRNAs that target p27^{Kip1}, a gene whose tumor suppressor activity correlates with its cellular protein level. In contrast to genes such as p53 and pRb, whose complete loss is required to inactivate the pathway, the tumor suppressor activity of p27^{Kip1} is haplo-insufficient. Our results uncover miR-221 and miR-222 as endogenous regulators of p27^{Kip1} and demonstrate the importance of its genetic interaction for the proliferation of certain cancer cells. Notably, this genetic screening method can be used for the identification and characterization of miRNAs targeting

other tumor suppressors and oncogenes.

miR-221&222 and p27^{Kip1}

From around 400 miRNAs in our library we have identified one family that significantly controls p27^{Kip1} expression. As many predicted p27^{Kip1}-targeting miRNAs showed no significant effect on p27^{Kip1} protein level and since no other miRNA vector was reproducibly enriched in our screen, this suggests that targeting p27^{Kip1} is specific to the miR-221 family. However, since endogenously expressed miRNAs might obscure effects on the reporter gene induced by our miR-Vectors, it does not rule out that other miRNAs may target p27. Moreover, additional miRNAs regulating p27 might not be detected by the developed method due to either insensitivity to detect weak regulation of the miRNA towards the target, or inefficient processing of the overexpressed miRNA. Still, the finding that p27 is regulated by miR-221&222 proves that this method is a powerful tool for identifying target specific miRNAs.

An additional interesting point is how many other relevant targets do miR-221&222 have in our cell system? Previously, it was shown that c-kit is a target of miR-221&222 (He et al., 2005a). However, c-kit expression could not be detected in the cell lines we used. At the moment there are no genome-wide methods that are sensitive and quantitative enough to identify miRNA effects at the protein level. Thus, how many additional targets miR-221&222 have in our cell system and which of them is required for their oncogenic function are interesting questions that remain to be explored.

Relationship between miRNAs and mRNA

Using target prediction algorithms and large-scale mRNA expression arrays the current view of miRNA function suggests that miRNAs possess the potential to affect the expression of hundreds of target genes. Our results indicate a highly specific and influential effect of very few miRNAs on a protein-coding gene of interest. These views are not contradictory as very few miRNA targets, once downregulated, contain the capacity to influence a certain phenotype of interest in a given cellular environment. Secondly, we should not exclude the possibility that many predicted miRNA targets are indifferent to miRNA activity simply because their gene expression is robust due to negative feedback loops. Thus, we speculate that a given phenotype can be affected by the activity of at least some miRNAs through the suppression of only few targets.

Oncogenic addiction of several cancer cell lines to miR-221&222

Based on our data, we can conclude that regulation of p27 by miR-221&222 is a common phenomenon, as p27 protein levels are regulated by these miRNAs in both cancerous and primary cells. Therefore, we would predict that the up-regulated expression of the miR-221&222 cluster in human tumors would decrease p27 levels and as such will correlate with poor prognosis. Indeed, aberrant expression of miR-221&222 was observed in poor prognosis miRNA signatures in tumors such as papillary thyroid carcinoma (He et al., 2005a; Pallante et al., 2006), pancreatic adenocarcinoma (Lee et al., 2006), and glioblastoma (Ciafre et al., 2005). Our results show that persistent expression of miR-221&222 is required for the abnormal cellular proliferation of at least some tumor cell lines, in particular glioblastoma. We find that in high proportion of glioblastoma cancers, high level of miR-221&222 in the tumor is associated with lower level of p27^{Kip1}. In combination with our tissue culture results, our data suggest that miR-221&222 function as oncogenes by controlling cell-cycle progression through inhibition of p27^{Kip1}. The requirement of miR-221&222 for tumor survival may suggest that it might be possible to use antagomiR-221&222 as a form of cancer therapy.

Materials and methods

Constructs and inhibitors

Genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen) according to the manufacturers instructions.

p27Sen-GFP was constructed by deleting the MCS, PGK-promoter and blasticidin resistance marker from pMSCV-Blast, followed by insertion of GFP-MCS from pEGFP-C1 (Clontech). The 3'UTR of p27 was synthesized from genomic DNA using forward GCGAATTCctaaACAGCTCGAATTAAGAATATGTTTCC (which includes an in-frame stop codon for GFP) and reverse GCGGATCCGCTATGGAAGTTTCTTTATTGATTACTTAATGTG primers. The resulting PCR product was cloned downstream of the GFP gene.

p27Sen-Luc was made by cloning the 3'UTR of p27 from the GFP sensor vector into pGL3 (Promega) downstream of the luciferase gene. For creating pGL3-p27-3'UTR-DM (double mutant), a PCR was done using the following primer: CCCCAAAGTTTATGTGGGATCCAAAAGG-TAAAACTATATACACAGGTAGTACAATGAAGCAAATAAGGAAAAACCTAATTGCATAATGGGATCCCCAACGCTTTTAGAGGC. In red are the BamHI sites that substituted the two seed

sequences.

The miR-Vec-221 and miR-Vec-222 and MSCV-hTR constructs were made as described previously (Voorhoeve et al., 2006; Kedde et al., 2006).

The miR-Vec-221 and miR-Vec-222 seed mutants were made in the same manner as described above for creating the double mutated p27 3'UTR. In this case, the 221/222 seed sequences were replaced by HindIII sites (underlined).

221:GAAACCCAGCAGACAAAGCTTTGTTGCCTAACGAAC

222:GAGACCCAGTAGCCAGAAGCTTTGCTGATTACGAAAG

Sequences of the miR-221 and miR-222 antagomiRs (Dharmacon):

221:ChI-AGCCUGAAACCCAGCAGACAAU-GUAGCUGUUGCC

222:ChI-CAUCAGAGACCCAGUAGC-CAGAUGUAGCUGUGA

The p27^{Kd} was designed to target the sequence gtacgagtggcaagaggtg (Le et al., 2003) and was made as described in (Brummelkamp et al., 2002).

RNase protection assay (RPA)

RPA was performed using mirVana miRNA probe construction and detection kits (Ambion) according to the manufacturers instructions. 2.5 µg of total RNA was used per reaction. The primer sequence of the 221 RPA probe was GCAACAGC-TACATTGTCTGCTGGGTTTCAGGCTctgtctc

The antisense cyclophilin probe contained nucleotides 46-149 of accession # BC013915 (Voorhoeve et al., 2006).

Western blot analysis and antibodies

Proteins were analyzed by SDS-PAGE and western blotting using Immobilon-P transfer membrane (Millipore). Prior to staining, unspecific sites were blocked in 5% nonfat milk at RT for 1 hour. Antibodies used were p21 (sc-6246, Santa Cruz Biotechnology), p53 (sc-126, Santa Cruz Biotechnology), p27 (K25020, BD Transduction Laboratories), CDK4 (sc601, Santa Cruz Biotechnology) and tubulin (YL1/2, ECACC). Secondary antibodies were goat-anti-mouse-HRP or swine-anti-rabbit-HRP (DakoCytomation). Proteins were detected using ECL (Amersham Biosciences) and film (Kodak).

Luciferase assay

MCF-7 cells were transfected using PEI (Polysciences, Inc). For reporter assays, cells were cultured in 24 well plates and transfected with

5 ng pGL3-p27-3'UTR (or DM), 5 ng Renilla, and 0.5 µg of the miR-Vec of interest (WT or SM). Luciferase activity was measured 72 hours after transfection using the Dual-luciferase reporter assay system (Promega).

Tissue procurement

Tissue samples were obtained after informed consent from adult patients diagnosed with glioblastoma *de novo*, freshly resected during surgery and immediately frozen in liquid nitrogen for subsequent total RNA extraction. In order to yield a very specific, case by case matching pair of tumor and control sample, for each patient we resected a central tumor area (core), surgically and histopathologically recognized as frankly tumoral, and one or more samples from a peripheral glial area (periphery), at an average distance of 0.5 cm from the border of the enhanced tumor, which did not show any evidence of tumor presence, by macroscopical surgeons evaluation.

Tissue lysates were prepared with ELB lysis buffer (0.1% NP40, 125 mM NaCl, 50 mM HEPES (pH 7.4) supplemented with protease inhibitor cocktail set (Roche Diagnostics GmbH). 20 µg of total protein was used to detect p27 levels.

Cell culture

HeLa, MCF-7, MDA-MB-231 and U87 were cultured in Dulbecco's modified Eagle's medium (41966 Invitrogen) supplemented with 10% FCS and antibiotics (complete medium). For miR-221&222 inhibitor treatment, cells were grown to 50% confluency and the tissue culture medium was replaced with medium containing either 25 µM (24 wells) or 75 µM (6 wells) of each of the inhibitors.

All miRNA transfection and virus harvesting steps were carried out on a Hamilton ML STAR with 96-channel and 8-channel pipetting systems, (Hamilton Bonaduz AG, Bonaduz, Switzerland). Protocols were developed at the NKI using Hamilton STAR Software 3.2. Plates were incubated in a Cytomat 2C450 automated tissue-culture plate incubator (Thermo Electron Corporation, Asheville, NC, USA). After transfection, the medium was removed using Biotek ELX405 Select plate washer (Biotek Instruments Inc., Winooski, VT, USA). The methods were completely automated; a Hamilton SWAP robotic arm was used to transfer plates between the various instruments.

Immunoperoxidase Staining

Serial frozen sections (5 µm) were fixed in acetone for 10 min. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen perox-

ide/100% methanol for 20 min. Sections were washed in phosphate-buffered saline (PBS) and blocked with 4% BSA in PBS for 30 minutes. Primary antibodies were diluted (p27 (BD Transduction Laboratories) 1:500, Ki-67 (Dako) 1:4,000) in PBS containing 1% BSA, and 100 µl was added to each section. Incubation was done for 2 hours at room temperature in a humidified chamber. The slides were then washed in PBS. For p27, sections were incubated with biotinylated goat-anti-mouse secondary antibody (Dako) at 1:50 in PBS containing 1% BSA for 30 min at room temperature. After washes in PBS-Tween, ABC biotinylation complex (1:200, Dako) was added for 1 hour at room temperature. Then, AEC (Sigma) was added to the slide for 20 minutes. For Ki-67, sections were incubated with envision goat-anti-rabbit (1:200, Dako) for 30 minutes at room temperature. Then, AEC (Sigma) was added to the slide for 20 minutes.

3T3 cell growth protocol

3 x 10⁴ cells were plated in triplicates in 24 well plates and propagated in complete medium with or without 221&222 inhibitors (2.5-25 µg) for a week. After 3 days, cells were counted and replated at 30,000 cells to grow for an additional 3 days

RT-PCR and real-time TaqMan PCR

Total RNA was extracted from HeLa cells stably expressing either miR-221 or a control vector using TRIzol reagent (Life Technologies) according to manufacturers instructions. RNA was resuspended in DEPC treated H₂O. Synthesis of cDNA with Superscript III reverse transcriptase (Invitrogen) was primed with oligo(dT). Primers for p27 (Set 1: Fwd AGCGGAGCAATGCGCAGG and Rev TCTTCTGAGGCCAGGCTTCT Set 2: Fwd ACGATTCTTCTACTCAAAACAAAAGAGC and Rev ATTTGGGGAACCGTCTGAAA) and beta-actin (Fwd CCTGGCACCCAGCACAAT and Rev GGGCCGGACTCGTCATACT) were designed to amplify 100-200 bp fragments. Analyses were carried out using SYBR Green PCR master mix (Applied Biosystems) and ABI Prism 7000 (Amersham-Pharmacia). Results were normalized with respect to beta-actin expression. Ct values for gene expression were calculated according to the delta delta Ct method.

TaqMan® microRNA assays (Applied Biosystems) that include RT primers and TaqMan probes were used to quantify the expression of mature miRNA-221 (AB: 4373077) and miRNA-222 (AB: 4373076) in both tissue samples and cell lines. The mean Ct was determined from

triplicate PCRs. Gene expression was calculated relative to 18S rRNA (AB: 4333760F) and multiplied by 10^4 to simplify data presentation.

Flow cytometry

GFP detection

For the validation of miRNA hits, HeLa GFP-p27-3'UTR cells were made to stably express these miRNAs. Each of the cell lines was then analyzed for the expression of GFP using FACScan (Becton Dickinson).

GFP sorting

The separation of low GFP expressing miR-Lib containing cells was done by cell sorting using the FACSria cell sorter from Becton Dickinson.

Cell-cycle profile analysis

For the cell-cycle experiments, cells were captured in mitosis using 0.25 $\mu\text{g}/\text{ml}$ nocodazole.

Cells were trypsinized, collected by centrifugation, and resuspended in PBS containing 0.6% NP-40, 50 $\mu\text{g}/\text{ml}$ RNaseA and 50 $\mu\text{g}/\text{ml}$ propidium iodide for 10 minutes. Subsequently, cells were analyzed using FACScan (Becton Dickinson). In each assay, 10,000-100,000 cells were collected by FACScan and analyzed with Cell Quest software (Becton Dickinson)

Acknowledgments

We thank Ron Kerkhoven and Mike Heimerikx for technical assistance in using the array facility, Anita Pfauth and Frank van Diepen in assisting with flow cytometry and Roderick Beijersbergen for setting the high throughput-screening facility. This work was supported by grants from the Dutch Cancer Society (KWF) and the European young investigator program (EURYI).

References

Alessandrini, A., Chiaur, D.S. and Pagano, M. (1997) Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation. *Leukemia*, 11, 342-345.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R. and Pasquinelli, A.E. (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*, 122, 553-563.

Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell*, 2, 243-247.

Calin, G.A. and Croce, C.M. (2006) MicroRNA signatures in human cancers. *Nat Rev Cancer*, 6, 857-866.

Chilosi, M., Chiarle, R., Lestani, M., Menestrina, F., Montagna, L., Ambrosetti, A., Prolla, G., Pizzolo, G., Doglioni, C., Piva, R., Pagano, M. and Inghirami, G. (2000) Low expression of p27 and low proliferation index do not correlate in hairy cell leukaemia. *Br J Haematol*, 111, 263-271.

Chu, I., Sun, J., Arnaout, A., Kahn, H., Hanna, W., Narod, S., Sun, P., Tan, C.K., Hengst, L. and Slingerland, J. (2007) p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. *Cell*, 128, 281-294.

Ciafre, S.A., Galardi, S., Mangiola, A., Ferracin, M., Liu, C.G., Sabatino, G., Negrini, M., Maira, G., Croce, C.M. and Farace, M.G. (2005) Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem Biophys Res Commun*, 334, 1351-1358.

Esquela-Kerscher, A. and Slack, F.J. (2006) Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*, 6, 259-269.

Fero, M.L., Randel, E., Gurley, K.E., Roberts, J.M. and Kemp, C.J. (1998) The murine gene p27^{Kip1} is haplo-insufficient for tumor suppression. *Nature*, 396, 177-180.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J. and Schier, A.F. (2006) Zebrafish miR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*, 312, 75-79.

Grimmler, M., Wang, Y., Mund, T., Cilensek, Z., Keidel, E.M., Waddell, M.B., Jakel, H., Kullmann, M., Kriwacki, R.W. and Hengst, L. (2007) Cdk-inhibitory activity and stability of p27^{Kip1} are directly regulated by oncogenic tyrosine kinases. *Cell*, 128, 269-280.

He, H., Jazdzewski, K., Li, W., Liyanarachchi, S., Nagy, R., Volinia, S., Calin, G.A., Liu, C.G., Franssila, K., Suster, S., Kloos, R.T., Croce, C.M. and de la Chapelle, A. (2005a) The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*, 102, 19075-19080.

He, L., Thomson, J.M., Hemann, M.T., Hernandez-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J. and Hammond, S.M. (2005b) A microRNA polycistron as a potential human oncogene. *Nature*, 435, 828-833.

Hengst, L. and Reed, S.I. (1996) Translational

control of p27^{Kip1} accumulation during the cell-cycle. *Science*, 271, 1861-1864.

Hershko, D.D. and Shapira, M. (2006) Prognostic role of p27^{Kip1} deregulation in colorectal cancer. *Cancer*, 107, 668-675.

Kaldis, P. (2007) Another piece of the p27^{Kip1} puzzle. *Cell*, 128, 241-244.

Kardinal, C., Dangers, M., Kardinal, A., Koch, A., Brandt, D.T., Tamura, T. and Welte, K. (2006) Tyrosine phosphorylation modulates binding preference to cyclin-dependent kinases and subcellular localization of p27^{Kip1} in the acute promyelocytic leukemia cell line NB4. *Blood*, 107, 1133-1140.

Kedde, M., le Sage, C., Duursma, A., Zlotorynski, E., van Leeuwen, B., Nijkamp, W., Beijersbergen, R. and Agami, R. (2006) Telomerase-independent Regulation of ATR by Human Telomerase RNA. *J Biol Chem*, 281, 40503-40514.

Kent, O.A. and Mendell, J.T. (2006) A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. *Oncogene*, 25, 6188-6196.

Kloosterman, W.P. and Plasterk, R.H. (2006) The diverse functions of microRNAs in animal development and disease. *Dev Cell*, 11, 441-450.

Koff, A. (2006) How to decrease p27^{Kip1} levels during tumor development. *Cancer Cell*, 9, 75-76.

Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K.G., Tuschl, T., Manoharan, M. and Stoffel, M. (2005) Silencing of microRNAs *in vivo* with 'antagomirs'. *Nature*, 438, 685-689.

Le, X.F., Claret, F.X., Lammayot, A., Tian, L., Deshpande, D., LaPushin, R., Tari, A.M. and Bast, R.C., Jr. (2003) The role of cyclin-dependent kinase inhibitor p27^{Kip1} in anti-HER2 antibody-induced G1 cell-cycle arrest and tumor growth inhibition. *J Biol Chem*, 278, 23441-23450.

Lee, E.J., Gusev, Y., Jiang, J., Nuovo, G.J., Lerner, M.R., Frankel, W.L., Morgan, D.L., Postier, R.G., Brackett, D.J. and Schmittgen, T.D. (2006) Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*.

Lee, E.J., Gusev, Y., Jiang, J., Nuovo, G.J., Lerner, M.R., Frankel, W.L., Morgan, D.L., Postier, R.G., Brackett, D.J. and Schmittgen, T.D. (2007)

Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*, 120, 1046-1054.

Loda, M., Cukor, B., Tam, S.W., Lavin, P., Fiorentino, M., Draetta, G.F., Jessup, J.M. and Pagano, M. (1997) Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med*, 3, 231-234.

Lu, C.D., Morita, S., Ishibashi, T., Hara, H., Isozaki, H. and Tanigawa, N. (1999) Loss of p27^{Kip1} expression independently predicts poor prognosis for patients with resectable pancreatic adenocarcinoma. *Cancer*, 85, 1250-1260.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R. and Golub, T.R. (2005) microRNA expression profiles classify human cancers. *Nature*, 435, 834-838.

Mayr, C., Hemann, M.T. and Bartel, D.P. (2007) Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science*, 315, 1576-1579.

Migita, T., Oda, Y., Naito, S. and Tsuneyoshi, M. (2002) Low expression of p27^{Kip1} is associated with tumor size and poor prognosis in patients with renal cell carcinoma. *Cancer*, 94, 973-979.

Millard, S.S., Yan, J.S., Nguyen, H., Pagano, M., Kiyokawa, H. and Koff, A. (1997) Enhanced ribosomal association of p27^{Kip1} mRNA is a mechanism contributing to accumulation during growth arrest. *J Biol Chem*, 272, 7093-7098.

Mineta, H., Miura, K., Suzuki, I., Takebayashi, S., Amano, H., Araki, K., Harada, H., Ichimura, K., Wennerberg, J.P. and Dictor, M.R. (1999) Low p27 expression correlates with poor prognosis for patients with oral tongue squamous cell carcinoma. *Cancer*, 85, 1011-1017.

Pallante, P., Visone, R., Ferracin, M., Ferraro, A., Berlingieri, M.T., Troncone, G., Chiappetta, G., Liu, C.G., Santoro, M., Negrini, M., Croce, C.M. and Fusco, A. (2006) microRNA deregulation in human thyroid papillary carcinomas. *Endocr Relat Cancer*, 13, 497-508.

Ponce-Castaneda, M.V., Lee, M.H., Latres, E., Polyak, K., Lacombe, L., Montgomery, K., Mathew, S., Krauter, K., Sheinfeld, J. and Mas-

sague, J. (1995) p27^{Kip1}: chromosomal mapping to 12p12-12p13.1 and absence of mutations in human tumors. *Cancer Res*, 55, 1211-1214.

Porter, P.L., Malone, K.E., Heagerty, P.J., Alexander, G.M., Gatti, L.A., Firpo, E.J., Daling, J.R. and Roberts, J.M. (1997) Expression of cell-cycle regulators p27^{Kip1} and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med*, 3, 222-225.

Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A.J., Stoop, H., Nagel, R., Liu, Y.P., van Duijse, J., Drost, J., Griekspoor, A., Zlotorynski, E., Yabuta, N., De Vita, G., Nojima, H., Looijenga, L.H. and Agami, R. (2006) A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell*, 124, 1169-1181.

Wu, L., Fan, J. and Belasco, J.G. (2006) microRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A*, 103, 4034-4039.

Supplementary data

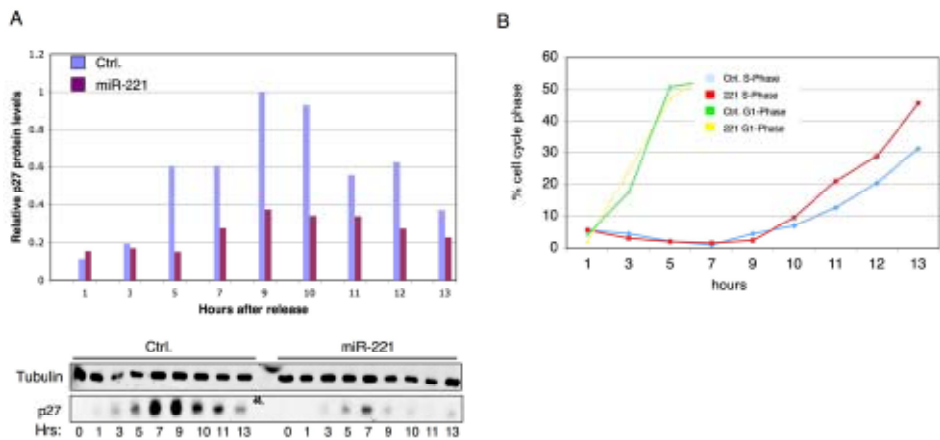


Figure S1. The effects of miR-221 on p27 levels during cell-cycle progression. (A) HeLa cells stably expressing miR-221 or control vector were synchronized in mitosis by a double thymidine treatment followed by nocodazole. Following release from nocodazole, whole cell lysates were prepared at the indicated timepoints and subjected to immunoblotting analysis using p27 and tubulin antibodies. Bands were quantified using Tina 2.0 software. (B) At the same timepoints, cell-cycle profiles were determined using BRDU-PI staining.

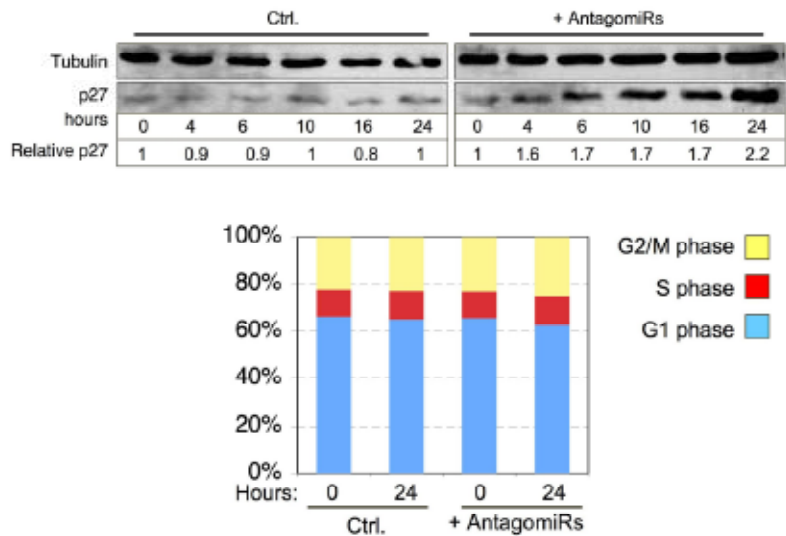


Figure S2. Elevation of p27 protein levels by antagomiR-221&222 precedes changes in cell-cycle profiles. U87 cell were treated with antagomiR-221&222 and cells were collected for immunoblot and cell-cycle analyses at the indicated timepoints.

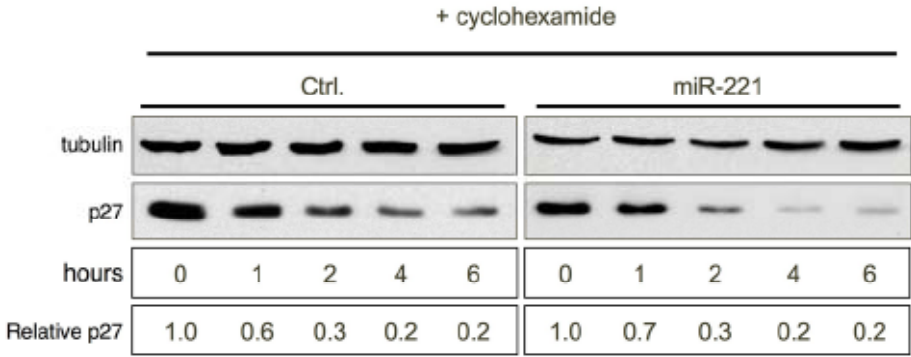


Figure S3. The effects of miR-221 on p27 protein stability. HeLa control and HeLa miR-221 cells were treated with 100 µg/ml cyclohexamide. At the indicated timepoints, whole cell lysates were prepared and subjected to immunoblotting analysis using p27 and tubulin antibodies. Bands were quantified using Tina 2.0 software.

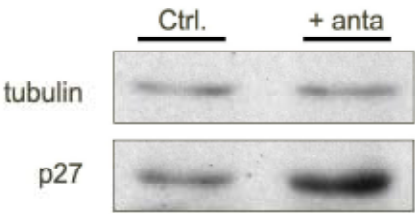


Figure S4. Effects of antagomiRs-221&222 on endogenous p27 levels of primary human fibroblasts. Human BJ primary fibroblasts were treated or not with antagomiRs-221&222. After 48 hours, whole cell lysates were prepared and subjected to immunoblotting analysis using p27 and tubulin antibodies.

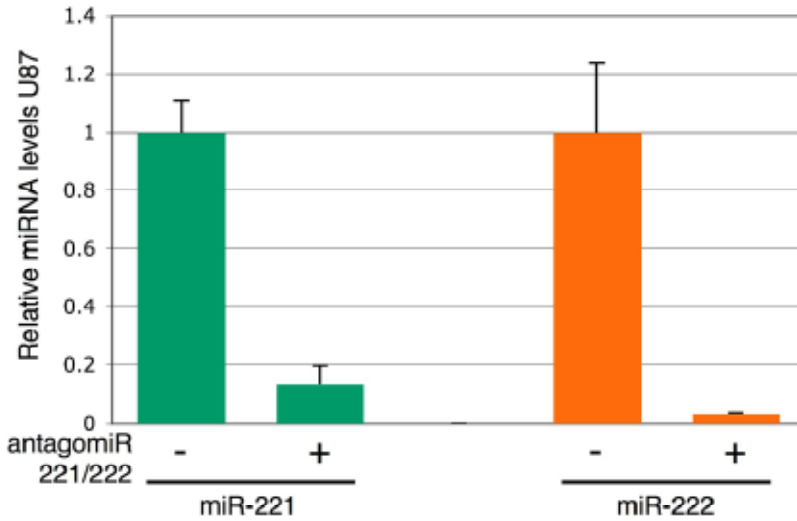


Figure S5. Reduction of miR-221 and miR-222 levels upon antagomiR treatment. U87 cells were treated with either antagomiR-221, antagomiR-222, or left untreated. After 24 hours, RNA was extracted, and the miRNA levels were determined using miR-221 and miR-222 specific TaqMan probes, as detailed in the materials and methods. The mean Ct was determined from triplicate PCRs. Gene expression was calculated relative to 18S rRNA and multiplied by 10^4 to simplify data presentation.

Chapter 5

Telomerase-independent regulation of ATR by human telomerase RNA

Journal of Biological Chemistry (2006)

Telomerase-independent regulation of ATR by human telomerase RNA

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The human telomerase RNA (hTR), together with the telomerase reverse transcriptase, hTERT, constitute the core components of telomerase that is essential for telomere maintenance. While hTR is ubiquitously expressed, hTERT is normally restricted to germ cells and certain stem cells, but both are often deregulated during tumorigenesis. Here, we investigated the effects of changes in hTR cellular levels. Surprisingly, while inhibition of hTR expression triggers a rapid, telomerase-independent, growth arrest associated with p53 and CHK1 activation, its increased expression neutralizes activation of these pathways in response to genotoxic stress. These hTR effects are mediated through ATR and are sufficiently strong to impair ATR-mediated DNA-damage checkpoint responses. Furthermore, in response to low UV radiation, which activates ATR, endogenous hTR levels increase irrespective of telomerase status. Thus, we uncovered a novel, telomerase-independent, function of hTR that restrains ATR activity and participates in the recovery of cells from UV radiation.

Introduction

Most human somatic cells have a limited replicative lifespan when propagated *in vitro* which is due to their inability to maintain chromosome ends, the telomeres (Cong et al., 2002; Hayflick and Moorhead, 1961). Telomeres are specialized DNA-protein structures that preserve the integrity of the ends of the chromosomes and the stability of the genome (Cong et al., 2002). However, with each cell division telomeres are shortened and once one or more erode to a certain critical point, the shortening induces a DNA damage checkpoint, thereby posing a barrier to continued cell growth, and therefore, to cancer (d'Adda di Fagagna et al., 2003; Harley et al., 1990; Hermann et al., 2001; Herbig et al., 2004; Stewart et al., 2003; Takai et al., 2003). Cells that escape replicative senescence by inactivating critical cell-cycle checkpoint genes, such as p53, continue to erode their telomeres, eventually reaching a point that is called crisis (Shay et al., 1991). The only way to escape crisis is to ensure that telomeres are maintained. Most cells do this by reactivating telomerase, but alternative mechanisms (ALT), involving recombination, are also observed (Bryan et al., 1995; Kim et al., 1994; Shay and Bacchetti, 1997).

Telomerase, or TERT, is a ribonucleoprotein

that copies a short RNA template into telomeric DNA, thereby maintaining eukaryotic chromosome ends and preventing replicative senescence (Greider and Blackburn, 1985; Lingner et al., 1997). Telomerase activity is low or absent in normal human cells but is high during development, in certain stem cells, germ cells and 80-90% of human cancers and immortalized human cell lines (Kim et al., 1994; Masutomi et al., 2003; Shay and Bacchetti, 1997; Wright et al., 1996). In fact, telomerase expression is sufficient for immortalization of primary human cells, rendering them insensitive to replicative senescence and crisis (Counter et al., 1992).

In humans, the core components of the telomerase complex are the protein catalytic subunit hTERT and the telomerase RNA subunit hTR (Feng et al., 1995; Lingner et al., 1997). hTR is transcribed by RNA polymerase II and is 3' processed to generate a 451 nucleotides long mature transcript. Its secondary structure is very well conserved with telomerase RNAs from several vertebrate species, indicating an important role for RNA structure in telomerase function (Chen et al., 2000). Several proteins have been described to bind to hTR and these are involved in hTR stability, maturation, accumulation, and functional assembly of the telomerase ribonu-

cleoprotein complex (Cong et al., 2002). Certain mutations or deletions in hTR lead to a rare skin and bone marrow failure syndrome called dyskeratosis congenita which is believed to be caused by defective telomere maintenance in stem cells (Mitchell et al., 1999; Vulliamy et al., 2001). As expression of hTR has been found to be essential for telomere maintenance in human disease and also for telomere length maintenance in mouse models, it is expected to be upregulated in cancer cells (Chiang et al., 2004). Indeed, several studies have shown that hTR upregulation is an early event in tumorigenesis and that hTR levels correlate better to tumor grade than telomerase activity or hTERT expression (Brown et al., 1997; Dome et al., 2005; Maitra et al., 1999; Morales et al., 1998; Rushing et al., 1997; Soder et al., 1997; Yashima et al., 1997; Yashima et al., 1998; Yi et al., 1999). Upregulation of hTR was also found to be an early event in mouse models of tumorigenesis. Here, telomerase RNA levels did not parallel the amount of telomerase activity detected (Blasco et al., 1996; Broccoli et al., 1996). These studies show that even in tumors that lacked telomerase activity, telomerase RNA was upregulated. Therefore, hTR may have functions that are separable from its role in telomerase activity (Blasco et al., 1996). Contrasting these observations are mice in which mouse telomerase RNA (mTR) was deleted from the germline. These mice are viable for 6 generations until telomeres have completely eroded (Blasco et al., 1997). However, the first generation of these mice, which still have long telomeres, have less skin tumors than wildtype mice following skin chemical carcinogenesis, indicating some telomerase independent effects of mTR (Gonzalez-Suarez et al., 2000). This argues that most mTR function is dependent on telomerase. However, as germ line gene knockout may allow for compensating events to occur while acute inhibition in somatic cells may not (Sage et al., 2003), inhibition of telomerase RNA by RNAi in cancer cell lines may expose its telomerase-independent functions.

Telomeres are intimately linked with DNA damage responses. Dysfunctional telomeres are recognized as damaged DNA and directly associate with many DNA damage response proteins (d'Adda di Fagagna et al., 2004). Moreover, the main cellular transducers of DNA damage, ATM and, to a lesser extent, ATR, have been shown to play an important role in telomere homeostasis (Takata et al., 2004; Verdun et al., 2005). ATM and ATR are phosphatidylinositol-3-kinase like protein kinases (PIKKs) that coordinate the repair,

cell-cycle checkpoint, and apoptotic responses to DNA damage (Sancar et al., 2004). Loss of ATM causes telomere decapping and shortening in every organism investigated thus far and TRF2, a telomeric DNA binding protein, has been shown to bind and inhibit ATM activation (d'Adda di Fagagna et al., 2004; Karlseder et al., 2004). Probing a telomeric function for ATR in mammalian cells has not been possible since ATR is essential for cell viability (Brown and Baltimore, 2003). Interestingly, a recent report has shown that in *Arabidopsis* ATR is required for maintenance of telomeric DNA (Vespa et al., 2005). Thus, PIKKs function in telomere homeostasis and telomeres with their telomere-associated factors that influence PIKKs activity.

Telomerase and telomeres are attractive targets for cancer therapies and have been extensively explored to this end (Cong et al., 2002). Inhibition of telomerase by antisense strategies or a dominant negative hTERT protein leads to the expected telomere shortening although the growth inhibition induced by this mechanism requires a long lag period due to the number of cell divisions required for telomeres to become substantially shortened to induce growth arrest (Hahn et al., 1999; Herbert et al., 1999). Recently, some studies have reported rapid cytotoxic responses of cancer cell lines in response to low levels of hTERT and hTR. A novel telomere-independent growth-inhibitory response pathway was proposed (Li et al., 2005; Li et al., 2004). Here, we investigated the effects exerted by hTR on the cellular growth and checkpoint controls, and uncovered a novel, telomerase-independent, function of hTR to counterbalance the activity of endogenous ATR.

Results

Inhibition of hTR expression induces a rapid cellular growth arrest

To inhibit hTR expression we designed shRNA constructs to target sequences in different regions of the hTR molecule. We aimed our constructs to three distinct single-stranded (loops) regions of the hTR molecule, as predicted by its very strong and conserved secondary structure (Chen et al., 2000) (Figure 1A). To assess the efficacy of inhibition of hTR expression, we transfected MCF-7 breast carcinoma cells with the shRNA constructs or a vector control. We used RNase protection assays (RPA) with RNA probes partially complementary to hTR or cyclophilin control to assess hTR RNA levels in cell extracts (Figure 1B). Quantitative analysis revealed that hTR^{kd}#1 and hTR^{kd}#2 reduced the levels of endogenous

hTR to 19% and 67%, respectively, whereas no hTR reduction was seen using hTR^{kd}#3. U2OS osteosarcoma cells, which lack expression of hTR and hTERT but maintain their telomeres by ALT (Bryan et al., 1997; Scheel et al., 2001), were used as a control. Indeed, no hTR expression was observed in U2OS cells, which could be re-expressed through transfection of a CMV-hTR plasmid (Figure 1B). We also verified the extent of hTR knockdown in nuclear and cytoplasmic extracts to exclude knockdown of hTR only in cytoplasmic fractions (Figures S1 and S2). We found that hTR resides mainly in the nucleus and upon expression of hTR^{kd}#1, hTR levels in both nuclear and cytoplasmic fractions were dramatically decreased. To assess the effects of inhibition of hTR on telomerase activity we performed a TRAP (Telomere Repeat Amplification Protocol) assay with extracts of MCF-7 cells transfected with the hTR^{kd} constructs or control plasmid. As

expected we found almost no telomerase activity in hTR^{kd}#1 transfected cells, hTR^{kd}#2 gave a moderate reduction, whereas hTR^{kd}#3 did not inhibit telomerase activity.

To investigate the effect of hTR knockdown on cell growth, we transfected MCF-7 cells with the three hTR^{kd} constructs and analyzed them two days later by flow cytometry. Surprisingly, the introduction of hTR^{kd}#1 shRNA-vector induced a cell-cycle arrest at both G1 and G2 (Figure 1D) whereas the hTR^{kd}#2 arrest was reduced and -#3 had no effect (data not shown). To verify this result we tested additional hTR-targeting siRNAs with RPA and flow cytometric analysis in MCF-7 cells (Figures S3 and S4). Transfection of these siRNAs suppressed the expression of hTR and elicited a comparable rapid growth arrest as hTR^{kd}#1. Consistent with these results, the long-term survival of hTR^{kd}#1-transfected MCF-7 cells (Figure 1E) and virally transduced hTR^{kd}#1 MCF-7

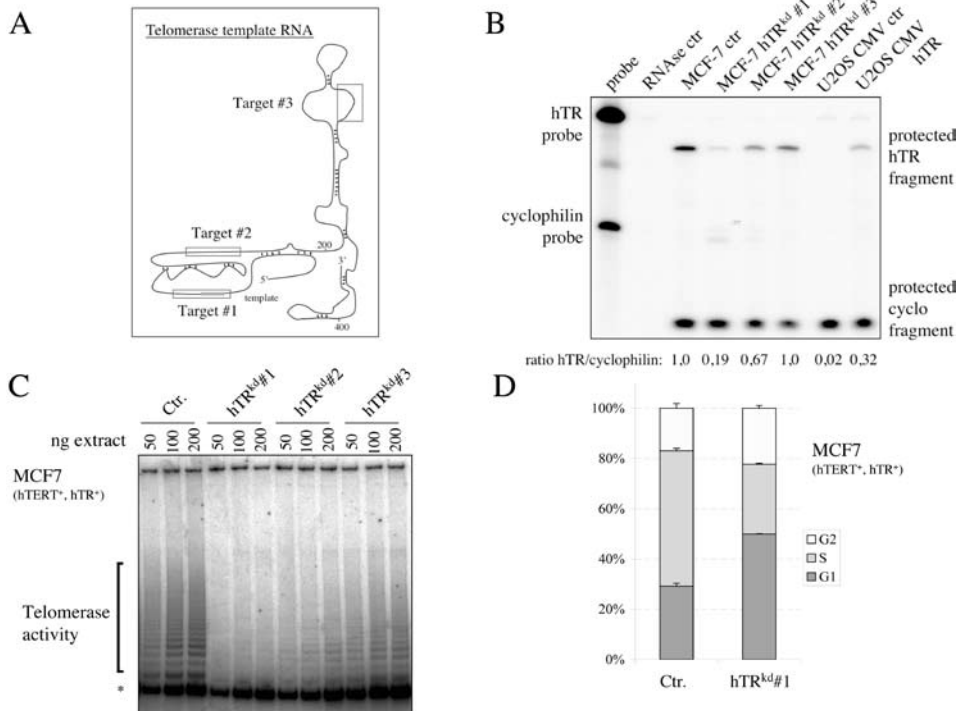


Figure 1. Reduction of telomerase activity and rapid growth inhibition by reduction of hTR expression. (A) Schematic representation of the hTR RNA. Boxed regions correspond to targeted sequences by shRNAs 1, 2 and 3, the template sequence is shown in a closed box. (B) RPA was used to detect the levels of hTR and cyclophilin control. The full length probes and protected fragments are indicated, 10% of the input probe was loaded on the gel. Quantification was performed by densitometry. (C) A TRAP assay was performed to detect telomerase activity in extracts of MCF-7 cells transfected with the indicated constructs. (D) MCF-7 cells were transfected with hTR^{kd}#1 or vector constructs and subjected to flow cytometric analysis. The percentage of cells in G1, S and G2 phases are shown. SD is from 3 independent experiments.

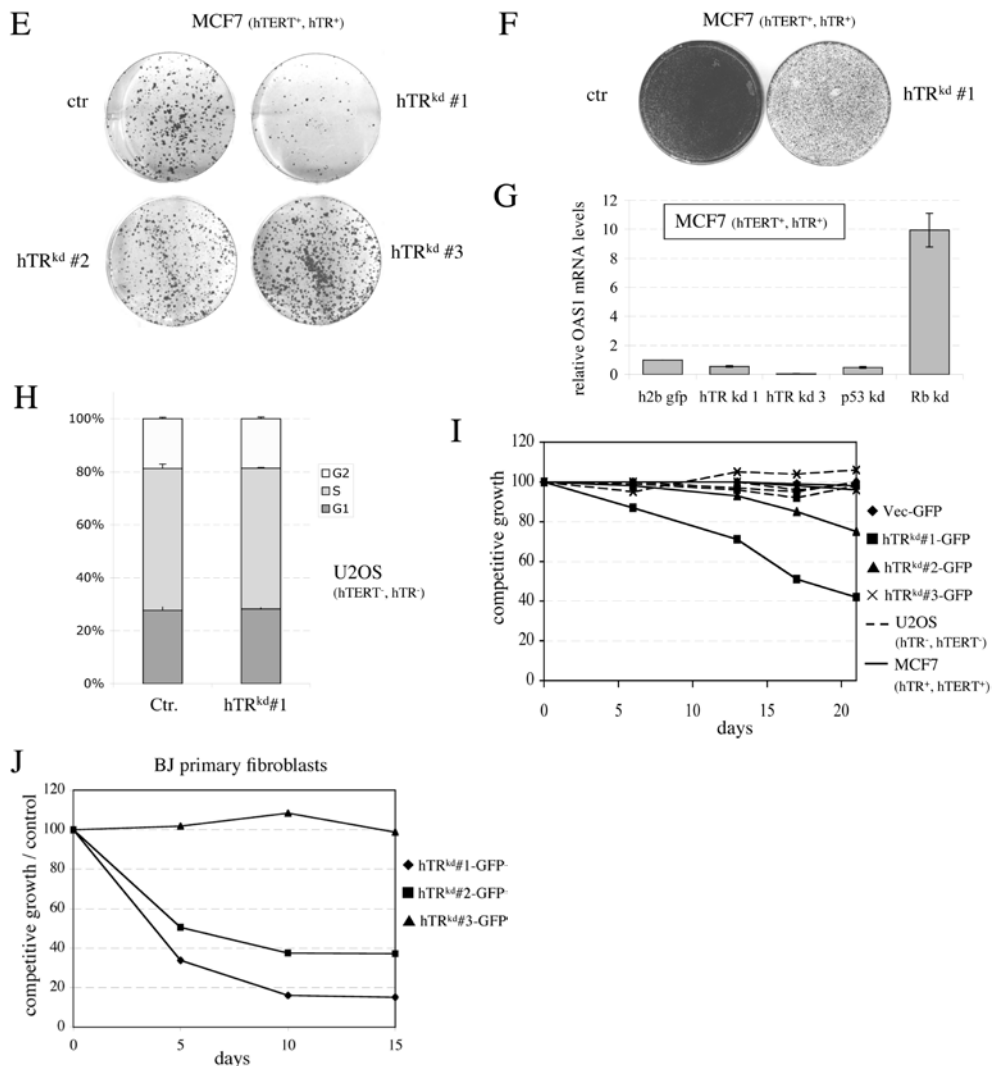


Figure 1. Reduction of telomerase activity and rapid growth inhibition by reduction of hTR expression (continued). (E) A colony growth assay of MCF-7 cells transfected with the hTR knockdown constructs or a vector control. Cells were selected with hygromycin for 10 days and stained with coomassie. (F) Colony growth assay of virally transduced MCF-7 cells. (G) MCF-7 cells were transfected with indicated constructs and RNA was extracted after 3 days. OAS1 (2'5'-oligoadenylate synthetase) mRNA levels were measured by Q-RT-PCR, shown relative to β -actin mRNA. SD is from 3 independent experiments. (H) Flow cytometry as in panel D only that U2OS cells were used. (I) Competitive growth assay in which U2OS and MCF-7 cells were transduced with indicated pRS-GFP constructs. Fluorescence was monitored by flow cytometry at the indicated timepoints after transduction. (J) Competitive growth assay performed on transduced primary BJ fibroblasts as in panel I.

cells (Figure 1F) was markedly impaired. The observed inhibition of proliferation by hTR^{kd}#1 was not a consequence of nonrelevant toxicity as this vector induced no interferon response (Figure 1G). Furthermore, no antiproliferative response

was seen in U2OS cells, as these express no hTR (Figure 1B). Both by flow cytometry as well as by competitive growth assays, hTR^{kd}#1 was not toxic to U2OS cells whereas highly toxic in MCF-7 cells (Figures 1H and I). hTR^{kd}#2 inhibited

growth of MCF-7 cells to intermediate levels, reflecting its capacity to knockdown hTR. These results are further strengthened by the use of additional siRNA reagents targeting hTR (Figures S2 and S3). This shows that the growth inhibition triggered by the knockdown of hTR in MCF-7 cells is dependent on the level of knockdown of hTR. The same antiproliferative effect of hTR^{kd}#1 was also obtained in other human cell lines, such as HaCaT immortalized keratinocytes, HeLa cervical carcinoma cells and T47D mammary carcinoma cells (Figure S5). Altogether, these results show that inhibition of hTR expression by shRNA elicits a rapid antiproliferative response in human cells.

Due to the continuous rapid proliferation of cancer cell lines and emergence of critically short telomeres, the inhibition of hTR expression may induce a stress response. To examine this, we studied hTR inhibition in young primary human cells (passage 35), that express hTR and contain sufficiently long telomeres to maintain proliferation for 30-40 additional passages. Also in these cells, suppression of hTR inhibited cell growth depending on the level of hTR knockdown (Figure 1J). Altogether, our results indicate that in-

hibition of hTR expression induces a cell-cycle arrest, which is not caused by critically short telomeres.

Cell-cycle arrest induced by inhibition of hTR expression requires p53 and CHK1

To elucidate the response of cells to reduced hTR levels we monitored p53 levels, a tumor suppressor and a major transducer of cell-cycle arrest in response to oncogenic and genotoxic stresses. We transfected MCF-7 cells with p53^{kd} (Brummelkamp et al., 2002a), and hTR^{kd} or control constructs and found p53 protein levels to be three times higher in the hTR^{kd}#1 transfected cells compared to controls (Figure 2A). This suggests that the growth arrest observed in hTR^{kd} cells involves p53 activation. To test this directly, we performed competitive growth assays using MCF-7-p53^{kd} and MCF-7 control cells that were virally transduced with GFP-hTR^{kd}#1 or GFP-control vector. Figure 2B shows that loss of p53 expression, which completely abrogates a DNA damage response (Brummelkamp et al., 2002a), only partially rescued the arrest induced by loss of hTR. A similar result was obtained in TIG3 primary fibroblasts (Figure S6).

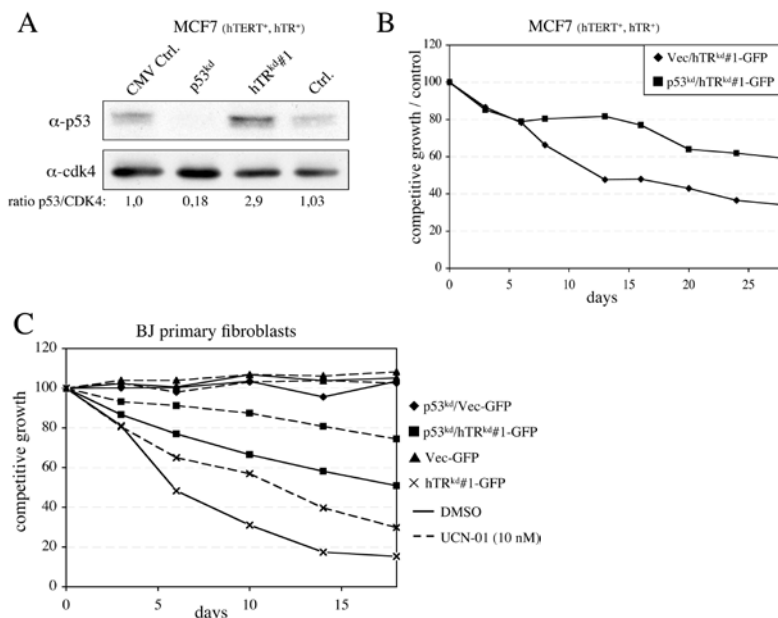


Figure 2. Chk1 and p53 are required for induction of the hTR^{kd}-mediated cell-cycle arrest. (A) MCF-7 cells were transfected with indicated constructs and subjected to immunoblot analysis to detect p53 and CDK4 control. Band intensity was calculated by densitometry. (B) MCF-7 cells were transduced with p53^{kd} or control vector, drug selected and transduced with pRS-GFP control and GFP-hTR^{kd}#1. Competitive growth assays were performed as described in Figure 11. (C) Competitive growth assays with BJ cells as in panel 2B. Cells were either treated with UCN-01 (10 nM, dashed lines) or with vehicle (DMSO, solid lines).

Activation of p53 can be a result of activation of the ATM and ATR kinases, which in turn activate CHK2 and CHK1, respectively (Sancar et al., 2004). To examine whether the cell-cycle arrest induced by the loss of hTR depends on the combination of p53 and CHK1, we performed a GFP-competition assay with BJ and BJ-p53^{kd} cells in the presence of the CHK1 inhibitor UCN-01 or vehicle as control (Busby et al., 2000; Graves et al., 2000). Figure 2C shows that hTR^{kd}#1-induced toxicity was almost completely abrogated when both p53 expression and CHK1 activity were inhibited. Similar results were obtained in MCF-7 cells (data not shown). These results indicate that loss of hTR activates both p53 and CHK1 to elicit a rapid cell-cycle arrest, indicating the involvement of ATR in this process.

hTR inhibits ATR activity

Our results suggest an inverse correlation between hTR levels and ATR activity. Reduction in hTR levels induces p53 and CHK1, two main substrates of ATR. To examine this further, we asked whether the presence of hTR inhibits ATR activity. ATR is activated by DNA damage assaults such as UV radiation, thereby inducing the phosphorylation of p53^{S15} and CHK1^{S317}, respectively (Heffernan et al., 2002; Tibbetts et al., 1999; Zhao and Piwnicka-Worms, 2001). To test the role of hTR in this process we used U2OS cells, which express no hTR, and ectopically expressed hTR (Figure 1A). Importantly, the levels of expressed hTR in U2OS cells were lower than the endogenous levels observed in MCF-7 cells and could not complement for telomerase activity due to the lack of hTERT expression in these cells (data not shown). We irradiated hTR expressing and control cells with UV and followed the phosphorylation kinetics of CHK1^{S317} and p53^{S15} in time. Interestingly, upon radiation, the phosphorylations of both CHK1^{S317} and p53^{S15} were severely attenuated in U2OS cells expressing hTR (Figure 3A). In addition, p53 stability was not increased upon radiation in hTR expressing cells as compared to control cells.

Since U2OS cells express neither hTR nor hTERT, it seems that hTR inhibits ATR in an hTERT independent manner. To examine this issue we aimed at testing the effects of hTR knockdown in a telomerase negative cell line. Therefore, we used GM847 human fibroblasts, these cells elongate telomeres by the ALT mechanism, and express hTR, but not hTERT (Bryan et al., 1995). We transfected cells with the hTR^{kd}#1 construct, treated the cells with UV radiation (3 J/m²) and monitored phosphorylation of CHK1^{S317} and

p53^{S15} using immunoblot analysis. We found that reduction in endogenous hTR levels triggered CHK1^{S317} and p53^{S15} phosphorylation, as well as p53 stabilization, indicating ATR activation (Figure 3B, time 0'). To exclude off-target effects of hTR knockdown we tested additional siRNAs that inhibit hTR expression. We found also these knockdowns to elicit a rapid cell-cycle arrest and to trigger CHK1^{S317} and p53^{S15} phosphorylation, and p53 stabilization (Figures S3, S4 and S7). This strongly suggests that inhibition of hTR causes the reduction in cell proliferation capacity and activation of DNA damage checkpoints. Interestingly, hTR depletion activated the DNA damage response almost to full extent as treating hTR^{kd} cells with UV resulted in only a slight activation of p53 and CHK1 compared to control cells (Figure 3B). Altogether, these results suggest that endogenous levels of hTR are functioning to balance ATR activity, a function that is telomerase-independent.

To study the effects of hTR on ATR kinase activity in more detail, we used an ATR construct with a TAP (tandem affinity purification) tag containing a TEV protease cleavage site (Rigaut et al., 1999). We transfected HEK293 cells with TAP-ATR or TAP control, purified them from extracts by immunoprecipitation (IP) using IgG beads (Figure 3C, lanes B), and released using TEV protease (lanes S). We studied effects of hTR on ATR kinase activity using GST-coupled p53 (amino acids 1-101) as a substrate. Figure 3D shows that our purified ATR could phosphorylate p53, as expected (lane ctrl). When hTR is incubated with ATR a clear and potent decrease in ATR activity is observed (lane hTR). As control we used a truncated hTR RNA encompassing the first 211 nucleotides of hTR (lane hTR 211). This truncated hTR inhibited ATR activity *in vitro* to a lesser extent than the full length hTR, indicating the specificity of the hTR effect. Additionally, since hTR contains a H/ACA box (Chen et al., 2000; Cong et al., 2002), we tested related H/ACA snoRNAs for their ability to influence ATR kinase activity. We found that these RNAs hardly affect ATR kinase activity, indicating that the inhibition of ATR by hTR is specific (Figure S8). As HEK293 cells express hTR, we reasoned that addition of RNase should increase ATR activity. Indeed, when RNase was added to ATR, or to ATR and hTR, kinase activity was relatively increased. These results indicate that ATR kinase activity is inhibited by hTR *in vitro*.

To further examine the effects of hTR on ATR kinase activity we performed kinase assays with TAP-ATR in U2OS cells that lack hTR expression.

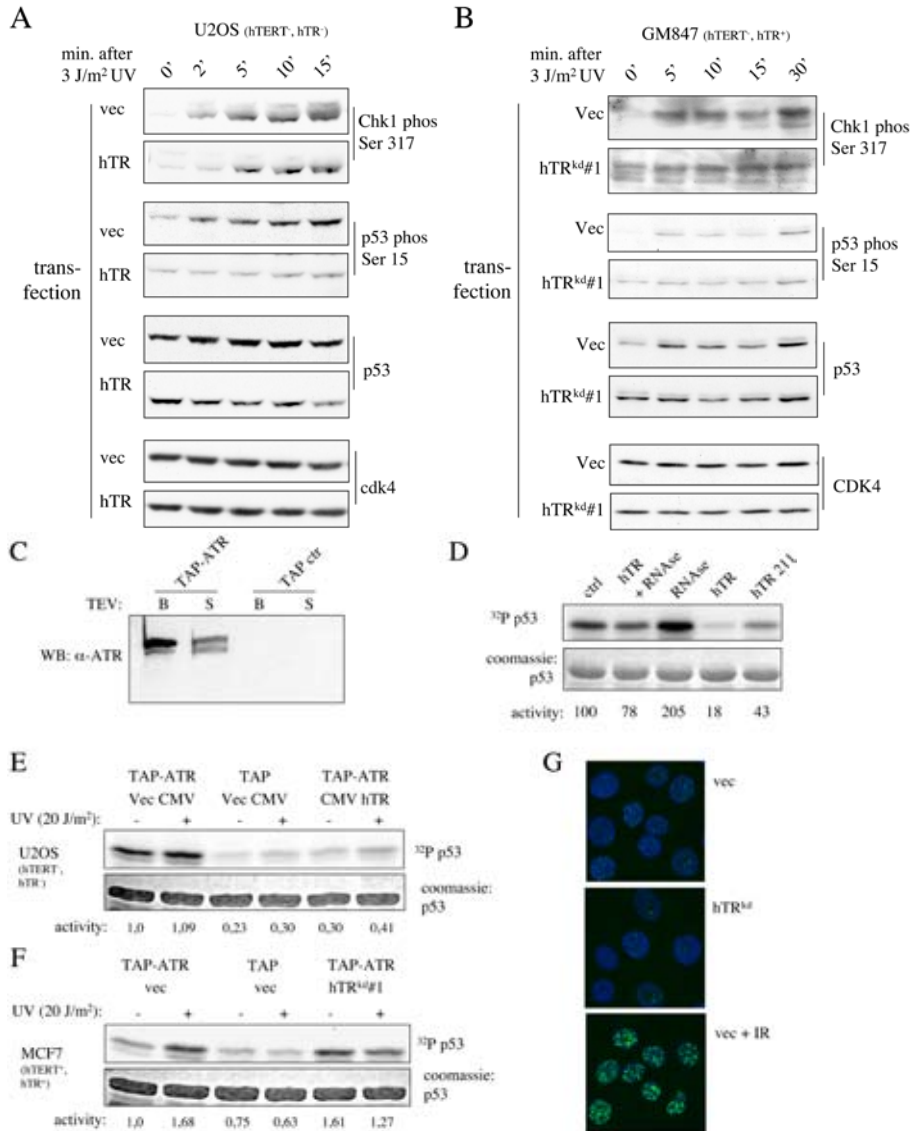


Figure 3. hTR inhibits ATR kinase activity. (A) U2OS cells were transfected with hTR^{kd} or control vector, irradiated after 3 days with 3 J/m² UV and harvested at the indicated timepoints. Whole cell extracts were immunoblotted to detect CHK1^{S317} phosphorylation, p53^{S15} phosphorylation, p53, and CDK4 as a loading control. (B) GM847 fibroblasts were transfected with hTR^{kd} or control and treated as in panel A. (C) HEK293 cells were transfected with TAP-ctr or TAP-ATR and IP-ed with IgG. TAP-ATR was cleaved with TEV protease and beads (B) and supernatant (S) were immunoblotted to detect ATR. (D) Kinase assay performed with IP and TEV cleaved TAP-ATR from HEK293 cells with GST-p53 (residues 1-101) as a substrate. Cleaved ATR was split and hTR, RNase, and mock were added prior to kinase reaction. Samples were separated by 10% SDS-PAGE and stained with coomassie blue to detect GST-p53 protein and autoradiography was performed to detect kinase activity. Band intensities were measured by densitometry. (E) U2OS cells were transfected with the indicated constructs and kinase assays were performed as in panel 3B. (F) MCF-7 cells were transfected with the indicated constructs and kinase assays were performed as in panel 3B. (G) Immunofluorescence images of MCF-7 cells transfected with hTR^{kd}#1, vector or control irradiated cells (5Gy IR), stained with γ-H2AX antibody. Nuclei are stained with DAPI (blue). Pictures were made with 200x magnification. Vec = vector.

Cells were transfected with TAP-ATR or TAP control plasmid and cotransfected with CMV-hTR or control plasmids. Three days after transfection, cells were either left untreated or UV irradiated to activate ATR. Subsequently, ATR was purified and subjected to a kinase assay. Figure 3E shows that exogenous ATR is active in mock treated U2OS cells and is slightly activated when cells are irradiated (lanes 1 and 2). However, when hTR is coexpressed with TAP-ATR, ATR activity is markedly decreased, almost to the level of the control transfections (lanes 5 and 6). Next, we performed a similar experiment using MCF-7 cells that, unlike U2OS cells, express hTR. Figure 3F shows ATR activation upon UV radiation (lanes 1-4). However, reduction in hTR levels induced ATR activity already in the untreated cells (lane 5), which was not further activated by UV at this timepoint after treatment (lane 6). As shown above, ATR signalling is activated when hTR is depleted. Our results so far suggest that the effect of hTR on ATR activity is direct. ATR is normally activated by DNA damage, upon which

it activates p53 and CHK1 but also phosphorylates histone H2AX (Sancar et al., 2004). Phosphorylated H2AX (γ -H2AX) colocalizes with other factors, such as p53BP1, MRE11 and SMC1, to foci marking sites of DNA damage, thereby facilitating the assembly of checkpoint and DNA repair factors (Sancar et al., 2004). Such foci are also found at dysfunctional telomeres (d'Adda di Fagagna et al., 2003; d'Adda di Fagagna et al., 2004). To test whether hTR depletion acts directly through ATR or indirectly via induction of DNA damage we stained hTR^{kd} cells for γ -H2AX foci. MCF-7 cells were transfected with hTR^{kd} or vector control, or irradiated as positive control. While ionizing radiation (IR) generated γ -H2AX foci, no increase in foci formation above control was observed in cells expressing hTR^{kd} shRNA, or hTR^{kd} siRNA (not shown) (Figure 3G). A similar result was obtained previously with 53BP1 foci in LOX cells (Li et al., 2005). Thus, we found no evidence of DNA damage in cells depleted of hTR supporting the notion that the effects of hTR on ATR are direct. Altogether, our results show that

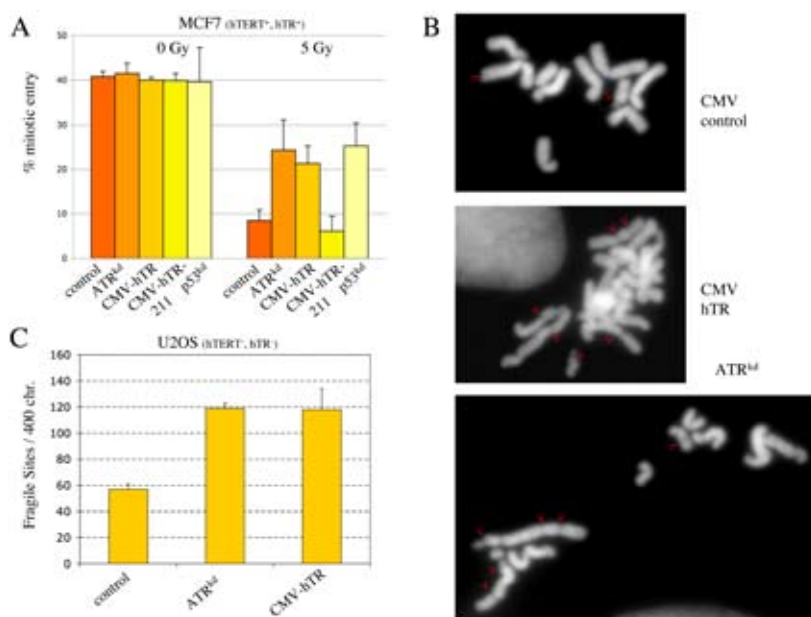


Figure 4. hTR partially abrogates the G2/M checkpoint and enhances the expression of fragile sites. (A) MCF-7 cells were transfected with the indicated constructs irradiated with 5 Gy IR or mock treated and 3 days later incubated with nocodazole. Twenty four hours later, cells were fixed, stained with Hoechst and mitotic entry was scored. SD from three independent experiments is shown. (B) The expression of fragile sites in U2OS cells transfected with the indicated constructs. Pictures were made with 1,000x magnification. (C) Quantification of the expression of fragile sites in U2OS cells as shown in Figure 4B. The number of fragile sites expressed in 400 chromosomes is shown, data are representative of three independent experiments.

the expression of hTR influences ATR kinase activity *in vivo*.

Partial abrogation of the G2/M checkpoint and enhanced appearance of fragile sites by hTR expression

ATR has been shown to be involved in the radiation-induced G2/M phase checkpoint in eukaryotic cells as it prevents mitotic entry of cells mainly in the late phase of the response to ionizing radiation (IR) and cooperates with ATM in the early phase of the response (Brown and Baltimore, 2003). Since our results show that hTR inhibits ATR, we studied the effects of hTR expression on the G2/M checkpoint. We monitored mitotic entry of MCF-7 cells in response to IR as a measure for the number of cells able to arrest in G2/M. We transfected MCF-7 cells with CMV-hTR and as controls ATR^{kd}, p53^{kd} or CMV control

constructs (for validation of the ATR^{kd} constructs, see Figure S9). Three days later we irradiated cells with 5 Gy IR and treated the cells with nocodazole to inhibit progression of the cell-cycle in mitosis. Cells were then fixed, stained with Hoechst and mitotic cells were counted (Figure 4A). While we found no difference in the accumulation of mitotic cells in unirradiated controls, hTR overexpression, ATR^{kd} and p53^{kd}, showed a partial override of the G2/M arrest. In contrast, cells expressing a truncated form of hTR (hTR211) behaved as control cells. Notably, hTR levels are only moderately increased in MCF-7 cells upon expression of CMV-hTR (a 35% increase, Figure S10). Altogether, these results indicate that an increase in hTR levels impairs ATR-mediated DNA damage responses in a manner similar to those induced by loss of ATR.

To further investigate whether hTR expression

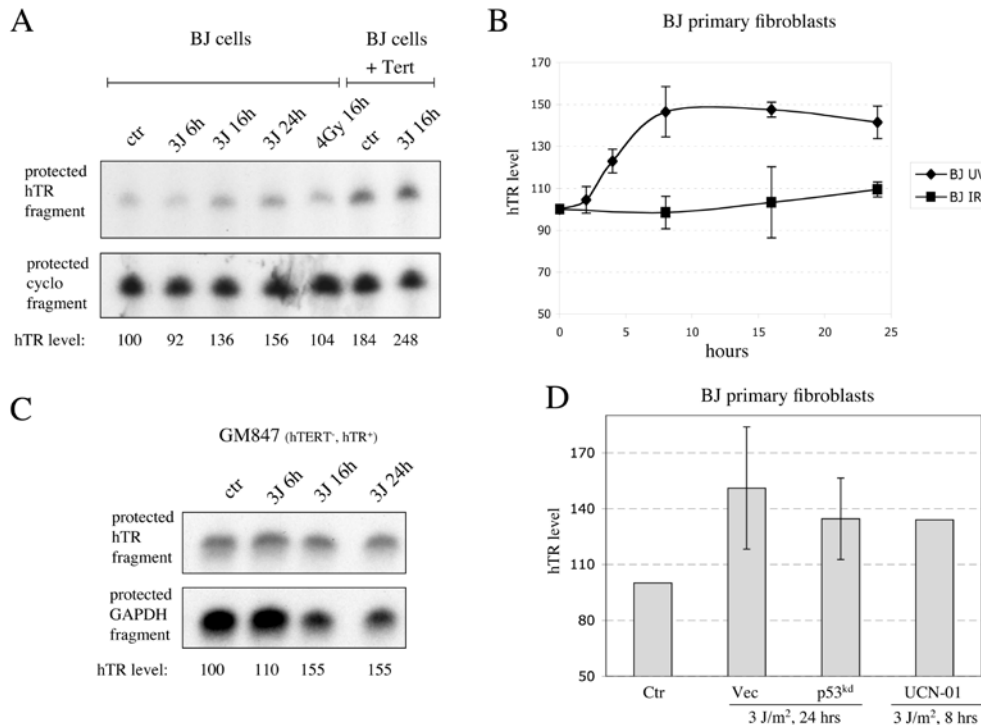


Figure 5. Upregulation of hTR levels following UV radiation. (A) RPA performed on extracts from BJ cells and BJ cells immortalized with hTERT irradiated with 3 J/m² UV or 4 Gy ionizing radiation. Cells were harvested after irradiation at indicated timepoints and quantification was performed by densitometry. (B) Quantification of RPAs on extracts of BJ cells irradiated with either 3 J/m² UV or 4Gy ionizing radiation. Samples were harvested at indicated timepoints after irradiation. SD is from three independent experiments, band intensities were quantified by densitometry. (C) RPA performed on extracts of GM847 cells irradiated with 3 J/m² UV as in panel 5A. (D) Quantification of RPAs on extracts of the indicated cells irradiated with 3 J/m² UV. UCN-01 was added to a final concentration of 100 nM, 1 hour prior to irradiation. SD is from three independent experiments.

perturbs ATR function, we studied fragile site stability in U2OS cells. Common fragile sites are specific chromosomal loci that appear as constrictions, gaps or breaks on metaphase chromosomes from cells that have been exposed to partial inhibition of DNA replication (Schwartz et al., 2006). Addition of low levels of the DNA polymerase inhibitor aphidicolin to cells in culture has been shown to induce fragile sites. ATR recognizes stalled and collapsed DNA replication forks, activates their repair and restarts replication. Fragile sites are believed to be expressed when stalled forks escape the ATR replication surveillance (Casper et al., 2002). We transfected U2OS cells with CMV-hTR, ATR^{kd} or CMV ctrl, and treated the cells for 24 hours with aphidicolin. Chromosomes were fixed, stained and dropped onto slides to count chromosomes and fragile sites. Upon inhibition of replication, we found fragile sites instability in control cells, which was exacerbated by either loss of ATR or expression of hTR (Figure 4B). Quantification of the frequency of fragile site-expression showed a twofold increase in the number of fragile sites in the hTR-expressing cells as well as in the ATR^{kd} cells (Figure 4C). As maintaining low levels of fragile site expression are a prime function of ATR, our results indicate that hTR inhibits ATR activity. Altogether, these results show that hTR inhibits ATR activity to a level sufficient to impair different ATR-mediated DNA-damage checkpoint responses, implying a novel function for hTR.

hTR upregulation following UV radiation

We next set out to elucidate the function of the inhibitory effect of hTR on ATR. To this end, we investigated whether stimuli that activate ATR also influence the expression of hTR. We treated primary BJ fibroblasts and BJ cells overexpressing hTERT (BJ-ET) with 3 J/m² UV light and extracted RNA at several timepoints after radiation. Subsequently, we subjected these RNA extracts to RPA with an hTR probe and cyclophilin or GAPDH as controls. Figure 5A shows that following UV radiation, but not IR (4 Gy), hTR levels increase in parent BJ cells. In BJ-ET cells, as published previously (Yi et al., 1999), higher levels of hTR are detected in untreated cells. Still, a clear upregulation of hTR levels following UV treatment is detected (Figure 5A). The increase in hTR level following UV radiation was not due to a cell-cycle arrest of the cells, as hTR levels did not increase in IR-treated cells (Figures 5A and 5B). Next, we characterized the response of hTR to UV. First, we examined dependency on hTERT by using GM847 cells that express hTR but not

hTERT. Also in these cells hTR was upregulated in response to UV radiation (Figure 5C), indicating that the upregulation of hTR is independent of hTERT. Second, we investigated whether the upregulation of hTR is dependent upon p53 or CHK1 activity by using BJ-p53^{kd} cells and cells treated with UCN-01. In these experiments, hTR was still upregulated in response to UV radiation when compared to control timepoints, indicating that p53 and CHK1 are not involved in the upregulation of hTR in response to UV radiation (Figure 5D). Collectively, we uncovered that hTR is specifically upregulated in response to low levels of UV radiation. This upregulation is not observed in response to ionizing radiation and is independent of p53, CHK1 and hTERT.

Discussion

We have identified a causative genetic interaction between the human telomerase RNA (hTR) and the checkpoint kinase ATR. Ectopic expression of hTR inhibits ATR while reduction in hTR levels stimulates ATR activity. This interaction is independent of telomerase activity and telomere length as it was observed in cells lacking hTERT, and in young primary human cells with telomeres long enough to allow proliferation for at least 20 to 30 passages. Interestingly, the effect of hTR on ATR activity was strong enough to influence cellular pathways. Inhibition of hTR expression elicited a p53/CHK1-dependent cell-cycle arrest in the absence of apparent DNA damage while increased expression of hTR caused defects in ATR dependent checkpoints, such as override of the G2/M arrest in response to DNA damage and the enhanced induction of fragile sites. These effects are specific since the hTR knockdowns do not elicit any interferon response, phenotypically depend on the endogenous expression of hTR and the effects were observed with mild (less than 50% above endogenous) overexpression of hTR. Notably, such a mild increase in hTR expression is also observed when cells are UV irradiated, indicating that such an increase is sufficient to impair ATR-dependent checkpoints (Figure 5B). An important observation is that both the inhibition of ATR activity by hTR and the upregulation of hTR by UV damage are independent of hTERT. Activation of ATR in response to UV is very rapid, occurring within minutes (Figures 3A and B), whereas the increase in hTR levels takes several hours and is comparable to the increase seen in MCF-7 cells following hTR overexpression resulting in checkpoint suppression (Figures 5B and S10). These kinetics are consistent with the idea that cell-cycle inhibition has to be rapid while the

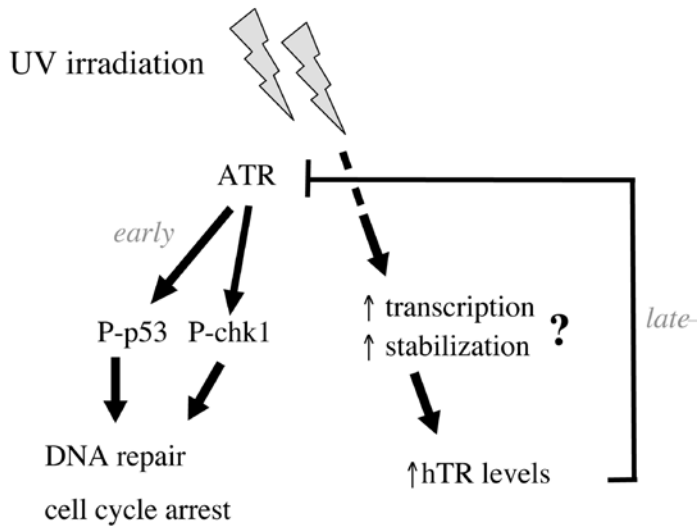


Figure 6. Model for the hTR mediated negative feedback loop on ATR activity in response to UV. Low levels of UV radiation activate the ATR kinase early in the UV response, which phosphorylates downstream targets, among which p53 and Chk1, leading to a cell-cycle arrest and induction of DNA repair. Independently, hTR levels are increased by a yet unknown mechanism. These increased hTR levels inhibit ATR at a later stage.

recovery takes several hours, depending on the extent of damage. Based on these findings, our results imply a model where upregulation of hTR in response to UV constitutes a feedback loop bringing down ATR activity to reinstate cell-cycle progression (Figure 6).

Our results show that reduction in endogenous hTR levels leads to ATR activation without the induction of apparent DNA damage. These results prompted us to investigate whether ATR and hTR interact directly. Interestingly, homologues of ATR in yeast and *Arabidopsis* are involved in telomere metabolism, and in yeast Mec1p (ATR homolog) associates with telomeres (Ritchie et al., 1999; Takata et al., 2004; Takata et al., 2005; Vespa et al., 2005). We were unable to demonstrate a direct interaction of ATR and hTR. Thus, our further experiments should elucidate by which mechanism hTR influences ATR activity. ATR was previously shown to be involved in signaling pathways activated by replication arrest and DNA damage assault in cells in S and G2 phases (Brown and Baltimore, 2003; Heffernan et al., 2002; Shechter et al., 2004). Common fragile sites are known to be hotspots susceptible to chromosomal breakage, rearrangement and deletion and have been implicated in the genomic instability frequently observed in cancer (Schwartz et al., 2006). ATR is involved in maintaining the stability of fragile sites, as its inhibition leads to an increase in the expression of fragile sites (Casper et al., 2002).

Our findings demonstrate that hTR expression induces the appearance of fragile sites to an extent similar to ATR^{kd}, thus suggesting that an increase in hTR levels can lead to genomic instability. Supporting this conclusion is our observation that an increase in hTR levels weakens the G2/M arrest, a checkpoint also controlled by ATR. These results also suggest that hTR expression can lead to reduced fidelity of the ATR-dependent checkpoints and therefore to increased genomic instability.

Recently, the group of Elizabeth Blackburn has shown that reduction in hTR levels in cancer cells elicits a rapid antiproliferative response (Li et al., 2005). However, when comparing hTR knockdown in HCT116 cells to p53-null HCT116 cells they conclude that the growth inhibitory response is p53-independent. Our results, on the other hand, indicate a partial dependency on p53 function. One obvious reason for this discrepancy can be that Li et al. based their conclusion on results obtained with two HCT116 cell lines with very different growth rates, the p53-null cell line grows an order of magnitude slower than the wildtype cell line, making a comparison between growth rates very difficult. In contrast, in our study we used primary human BJ and TIG3 fibroblasts as well as MCF-7 breast carcinoma cells, and directly compared them with corresponding p53 knockdown cells. Furthermore, our results clearly indicate that the regulation of ATR by hTR

is sufficient to affect cellular pathways.

Our results seem to contrast the findings that mTR^{-/-} mice have no obvious phenotype in the first generations (Blasco et al., 1997). The fact that first generation mTR^{-/-} mice are less prone to developing skin tumors upon chemical carcinogenesis can be explained by mild activation of some DNA damage checkpoints induced by loss of mTR (Gonzalez-Suarez et al., 2000). Whether this effect is mediated through ATR remains to be elucidated. Additionally, there are several differences between mouse and human telomere homeostasis that complicate extending findings from mouse models to the human setting. First, murine cells have extremely long and hypervariable telomeres and telomerase activity is detectable in most somatic tissues (Kipling and Cooke, 1990; Prowse and Greider, 1995). Second, although telomerase is activated and mTR is upregulated *in vivo* in several mouse tumor models, it appears not to be required for growth during the cell divisions necessary for tumor formation, suggesting that mTR and/or telomerase have additional functions to telomere extension (Blasco et al., 1997; Blasco et al., 1996; Broccoli et al., 1996). In addition, telomere dysfunction in mice appears to be solely dependent on p53, whereas in human cells the pRb pathway is also activated (Chin et al., 1999; Smogorzewska and de Lange, 2002). Third, while RNAi causes a fast reduction in hTR levels, knockout of telomerase RNA in germ line cells may allow for compensation events to occur (Sage et al., 2003). Thus, our results may indicate for either a difference in telomerase RNA biology between mice and men or to differences in methods used. Further experiments with murine cells are required to establish whether mTR also regulates ATR.

Last, our results may provide an explanation why in the vast majority of somatic human cells, hTR is ubiquitously expressed whereas both hTERT and telomerase activity are mostly absent. hTR regulates DNA damage pathways in a telomerase and hTERT-independent manner. Our results, thus, can explain observations suggesting that hTR plays a role in the initiation of tumorigenicity and that it is frequently upregulated in human cancer cell lines (Blasco et al., 1996; Broccoli et al., 1996; Brown et al., 1997; Dome et al., 2005; Maitra et al., 1999; Morales et al., 1998; Rushing et al., 1997; Soder et al., 1997; Yashima et al., 1997; Yashima et al., 1998; Yi et al., 1999).

Materials and methods

Materials and antibodies

UV radiation was performed with a stratalinker

(Stratagene), and ionizing irradiation was performed with a 2 X 415-Ci ¹³⁷Cs source. Prior to UV radiation, medium was kept aside and cells were washed with phosphate buffered saline (PBS). Antibodies used in this study were directed against cdk4 (C-22), p53 (DO1) (Santa Cruz), phospho-S15 p53, phospho-S317 CHK1, phospho-T68 CHK2 (Cell Signalling), Flag (M2, Sigma), γ -H2AX (S139, Upstate) and ATR (ab2905, Abcam).

Constructs

hTR knockdown constructs were cloned in pSuper, pRetroSuper(pRS)-Hygro (Brummelkamp et al., 2002b), and pRS-GFP (Voorhoeve and Agami, 2003), sequences used were: hTR^{kd}#1: GTCTAACCCCTAACTGAGAAGG, -#2: CCGTTCATTCTAGAGCAAAC, -#3: GAGTTGGGCTCTGTGACCC. The CMV-hTR expression construct was cloned by PCR in pcDNA3.1 vector (Invitrogen) to contain 149 nt downstream of the full length hTR for proper processing, H/ACA box snoRNA constructs were also cloned into this vector. pRS-p53^{kd} and pRS-Rb were described previously (Voorhoeve and Agami, 2003). ATR^{kd} sequences used were: #1: GACGGTGTGCTCATGCGGC, #2: CCTGATGGAGTGGCCGGAG, we used a combination of the two constructs cloned in pSuper. We cloned the ATR cDNA from the pBJ5.1-Flag-ATR (a kind gift from Professor Steve Jackson) vector into the pZome1N vector (Cellzome) downstream of the TAP tag (Rigaut et al., 1999). The GST-p53 (amino acid 1-101) construct was cloned by PCR into pGEX-1N (Amrad). pTRI-GAPDH and -cyclophilin constructs for RPA were from Ambion, the hTR RPA vector was cloned by PCR into pTRI (Ambion) from nucleotide 105-370 in reverse orientation.

Cell culture, retroviral transduction and transfection

All cells described were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum in 5% CO₂ at 37°C. BJ cells were transduced at population doubling 35. The generation of BJ-TERT cells has been described previously (Voorhoeve and Agami, 2003), GM847 cells were a kind gift from Professor Batsheva Kerem. MCF-7, U2OS and TIG3 cell lines expressing the ecotropic receptor were infected with ecotropic retroviral supernatants as previously described (Brummelkamp et al., 2002b) to generate polyclonal pools of cells. Except for kinase assays, transfection was done by electroporation as described previously (Agami and Bernards, 2000). For kinase assays, HEK293, U2OS and MCF-7

cells were transiently transfected using calcium-phosphate precipitation.

Western blotting, cell-cycle profile analysis and competitive growth assays

For western blot analysis, whole-cell extracts were prepared and separated on 10% SDS-PAGE gels, ATR immunoblots were separated on 5% SDS-PAGE gels, and transferred to Immobilon-P membranes (Milipore). Western blots were developed with Supersignal (Pierce), and densitometric quantification of western blots was performed with Aida 3.40 software (Raytek, Sheffield, UK). Cell-cycle profile analysis was performed as described before by Duursma et al. (Duursma and Agami, 2005). For competitive growth assays, cells were infected with pRS-GFP-hTR^{kd} or pRS-GFP retrovirus and allowed to recover for 4 days, the initial percentage of GFP-positive cells varied between 15 and 50%. The cells were analysed by flow cytometry with the Cell Quest program (Beckton Dickinson).

Telomerase activity assays, quantitative RT-PCR, and RNase protection assays

MCF-7 extracts were assayed for telomerase activity using a PCR-based telomeric repeat amplification protocol (TRAP) assay (Kim and Wu, 1997). For Q-RT-PCR, cDNA was transcribed using Superscript III (Invitrogen) with random hexamers following manufacturer's instructions. Q-RT-PCR was performed with a SYBRgreen master mix (Applied Biosystems), and the samples were amplified and analysed by an ABI-prism 7000 sequence detection system (Applied Biosystems). Primers for OAS1 were described before (Scherr et al., 2005) and Ct values were normalized for β -actin. RPAs were performed using the HybSpeed RPA and MAXIsript kits from Ambion according to manufacturers instructions. We used 5–8 μ g of RNA per reaction. *In vitro* transcription of pTRI-hTR yielded an RNA of 300 nt, 265 nt of which are complementary to hTR.

IP-kinase assays

To determine ATR activity, TAP-ATR was immunoprecipitated from extracts of transfected HEK293, MCF-7 or U2OS cells using rabbit IgG sepharose (Sigma). Prior to immunoprecipitation, cells were UV irradiated with either 80 or 20 J/m² or mock treated. Beads were washed 3 times with ELB lysis buffer and ATR was cleaved from the beads by addition of recombinant TEV protease (Invitrogen) for 2 hrs at 8°C according to manufacturers instructions. The substrate, GST-p53 (1–101) bound to glutathione beads (Amer-

sham), was washed three times with kinase buffer (20 mM HEPES [pH 7.5], 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 1mM DTT, 0.5 mM NaOAc, and 2 mM β -Glycerophosphate). Reactions were carried out in a kinase buffer at a volume of 50 μ l with cleaved ATR, 10 μ g GST-p53 (1–101), 10 μ Ci [γ -³²P]-ATP (500 mCi/mmol, Amersham) at 30°C for 15 min. In samples with RNase A, 0.5 μ g was added to the kinase reaction prior to the addition of the substrate, we added 1 μ g of *in vitro* transcribed hTR, or H/ACA snoRNA to indicated samples which was produced from linearized CMV-hTR/snoRNA plasmid with a MAXIsript *in vitro* transcription kit (Ambion) according to manufacturers instructions. The beads with GST-p53 (1–101) were then extensively washed and subjected to 10% SDS-PAGE, the gel was dried, stained with coomassie and exposed to a phospho imager screen for quantification on a Basereader 3000 (Fuji) with Aida 3.40 software (Raytek, Sheffield, UK).

Immunofluorescence, mitotic entry and fragile site assays

MCF-7 cells were transfected by electroporation (>90% efficiency) with indicated constructs. Cells were washed with PBS, fixed and permeabilized in 4% formaldehyde and 0.2% Triton X-100, and washed with PBS containing 0.05% saponin. Slides were blocked with 10% normal goat serum in PBS with 0.05% saponin. Cells were stained with antibody directed against phosphorylated H2AX, FITC-conjugated goat-anti-mouse antibodies were used as secondary antibodies. Images were recorded with a Leica TCS SP2-AOBS (Leica Microsystems, Heidelberg, Germany) confocal system. For counting mitotic entry, after 72 hrs, transfected MCF-7 cells were treated with 5 Gy IR or mock treated and incubated with nocodazole (0.25 μ g/ml) for 24 hrs. Cells were fixed with 3.7% formaldehyde, permeabilized for 5 min with 0.1% triton X-100 (Sigma) and stained with Hoechst. Mitotic cells were scored double blind with a Zeiss RS III microscope. For each sample, 600 cells were counted. For fragile site assays, cells were grown on coverslips, and common fragile sites were induced by growing the cells in M-199 medium in the presence of 0.4 μ M aphidicolin and 0.5% ethanol for 24 h prior to the fixation of chromosomes by standard procedures. Images were obtained with a Zeiss Axiovert 100 TV inverted microscope controlled by SmartCapture2 software. For each sample, 400 chromosomes were counted double blind.

Acknowledgments

We thank all members of the Agami lab for stimulating discussions and Steve Jackson and Batsheva Kerem for reagents. This work was supported by grants from the Dutch Cancer Society (KWF) to M.K. and R.A. and by the EURL award to R.A.

References

Agami, R. and Bernards, R. (2000) Distinct initiation and maintenance mechanisms cooperate to induce G1 cell-cycle arrest in response to DNA damage. *Cell*, 102, 55-66.

Blasco, M.A., Lee, H.W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A. and Greider, C.W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell*, 91, 25-34.

Blasco, M.A., Rizen, M., Greider, C.W. and Hanahan, D. (1996) Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nat Genet*, 12, 200-204.

Broccoli, D., Godley, L.A., Donehower, L.A., Varmus, H.E. and de Lange, T. (1996) Telomerase activation in mouse mammary tumors: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. *Mol Cell Biol*, 16, 3765-3772.

Brown, D.F., Gazdar, A.F., White, C.L., 3rd, Yoshima, K., Shay, J.W. and Rushing, E.J. (1997) Human telomerase RNA expression and MIB-1 (Ki-67) proliferation index distinguish hemangioblastomas from metastatic renal cell carcinomas. *J Neuropathol Exp Neurol*, 56, 1349-1355.

Brown, E.J. and Baltimore, D. (2003) Essential and dispensable roles of ATR in cell-cycle arrest and genome maintenance. *Genes Dev*, 17, 615-628.

Brummelkamp, T.R., Bernards, R. and Agami, R. (2002a) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, 296, 550-553.

Brummelkamp, T.R., Bernards, R. and Agami, R. (2002b) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell*, 2, 243-247.

Bryan, T.M., Englezou, A., Dalla-Pozza, L., Dunham, M.A. and Reddel, R.R. (1997) Evidence for an alternative mechanism for maintaining telo-

mere length in human tumors and tumor-derived cell lines. *Nat Med*, 3, 1271-1274.

Bryan, T.M., Englezou, A., Gupta, J., Bacchetti, S. and Reddel, R.R. (1995) Telomere elongation in immortal human cells without detectable telomerase activity. *Embo J*, 14, 4240-4248.

Busby, E.C., Leistriz, D.F., Abraham, R.T., Karnitz, L.M. and Sarkaria, J.N. (2000) The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hCHK1. *Cancer Res*, 60, 2108-2112.

Casper, A.M., Nghiem, P., Arlt, M.F. and Glover, T.W. (2002) ATR regulates fragile site stability. *Cell*, 111, 779-789.

Chen, J.L., Blasco, M.A. and Greider, C.W. (2000) Secondary structure of vertebrate telomerase RNA. *Cell*, 100, 503-514.

Chiang, Y.J., Hemann, M.T., Hathcock, K.S., Tessarollo, L., Feigenbaum, L., Hahn, W.C. and Hodes, R.J. (2004) Expression of telomerase RNA template, but not telomerase reverse transcriptase, is limiting for telomere length maintenance *in vivo*. *Mol Cell Biol*, 24, 7024-7031.

Chin, L., Artandi, S.E., Shen, Q., Tam, A., Lee, S.L., Gottlieb, G.J., Greider, C.W. and DePinho, R.A. (1999) p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell*, 97, 527-538.

Cong, Y.S., Wright, W.E. and Shay, J.W. (2002) Human telomerase and its regulation. *Microbiol Mol Biol Rev*, 66, 407-425, table of contents.

Counter, C.M., Ailion, A.A., LeFeuvre, C.E., Stewart, N.G., Greider, C.W., Harley, C.B. and Bacchetti, S. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *Embo J*, 11, 1921-1929.

d'Adda di Fagagna, F., Reaper, P.M., Clay-Farace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P. and Jackson, S.P. (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature*, 426, 194-198.

d'Adda di Fagagna, F., Teo, S.-H. and Jackson, S.P. (2004) Functional links between telomeres and proteins of the DNA-damage response. *Genes Dev*, 18, 1781-1799.

- Dome, J.S., Bockhold, C.A., Li, S.M., Baker, S.D., Green, D.M., Perlman, E.J., Hill, D.A. and Breslow, N.E. (2005) High telomerase RNA expression level is an adverse prognostic factor for favorable-histology Wilms' tumor. *J Clin Oncol*, 23, 9138-9145.
- Duursma, A. and Agami, R. (2005) p53-Dependent regulation of Cdc6 protein stability controls cellular proliferation. *Mol Cell Biol*, 25, 6937-6947.
- Feng, J., Funk, W.D., Wang, S.S., Weinrich, S.L., Avilion, A.A., Chiu, C.P., Adams, R.R., Chang, E., Allsopp, R.C., Yu, J. and et al. (1995) The RNA component of human telomerase. *Science*, 269, 1236-1241.
- Gonzalez-Suarez, E., Samper, E., Flores, J.M. and Blasco, M.A. (2000) Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. *Nat Genet*, 26, 114-117.
- Graves, P.R., Yu, L., Schwarz, J.K., Gales, J., Sausville, E.A., O'Connor, P.M. and Piwnica-Worms, H. (2000) The CHK1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. *J Biol Chem*, 275, 5600-5605.
- Greider, C.W. and Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell*, 43, 405-413.
- Hahn, W.C., Stewart, S.A., Brooks, M.W., York, S.G., Eaton, E., Kurachi, A., Beijersbergen, R.L., Knoll, J.H., Meyerson, M. and Weinberg, R.A. (1999) Inhibition of telomerase limits the growth of human cancer cells. *Nat Med*, 5, 1164-1170.
- Harley, C.B., Futcher, A.B. and Greider, C.W. (1990) Telomeres shorten during ageing of human fibroblasts. *Nature*, 345, 458-460.
- Hayflick, L. and Moorhead, P.S. (1961) The serial cultivation of human diploid cell strains. *Exp Cell Res*, 25, 585-621.
- Heffernan, T.P., Simpson, D.A., Frank, A.R., Heinloth, A.N., Paules, R.S., Cordeiro-Stone, M. and Kaufmann, W.K. (2002) An ATR- and CHK1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol Cell Biol*, 22, 8552-8561.
- Hemann, M.T., Strong, M.A., Hao, L.Y. and Greider, C.W. (2001) The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell*, 107, 67-77.
- Herbert, B., Pitts, A.E., Baker, S.I., Hamilton, S.E., Wright, W.E., Shay, J.W. and Corey, D.R. (1999) Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci U S A*, 96, 14276-14281.
- Herbig, U., Jobling, W.A., Chen, B.P., Chen, D.J. and Sedivy, J.M. (2004) Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21^{Cip1}, but not p16INK4a. *Mol Cell*, 14, 501-513.
- Karlseder, J., Hoke, K., Mirzoeva, O.K., Bakkenist, C., Kastan, M.B., Petrini, J.H. and de Lange, T. (2004) The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. *PLoS Biol*, 2, E240.
- Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L. and Shay, J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, 266, 2011-2015.
- Kim, N.W. and Wu, F. (1997) Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res*, 25, 2595-2597.
- Kipling, D. and Cooke, H.J. (1990) Hypervariable ultra-long telomeres in mice. *Nature*, 347, 400-402.
- Li, S., Crothers, J., Haqq, C.M. and Blackburn, E.H. (2005) Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. *J Biol Chem*, 280, 23709-23717.
- Li, S., Rosenberg, J.E., Donjacour, A.A., Botchkina, I.L., Hom, Y.K., Cunha, G.R. and Blackburn, E.H. (2004) Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. *Cancer Res*, 64, 4833-4840.
- Lingner, J., Hughes, T.R., Shevchenko, A., Mann, M., Lundblad, V. and Cech, T.R. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*, 276, 561-567.

- Maitra, A., Yashima, K., Rath, A., Timmons, C.F., Rogers, B.B., Shay, J.W. and Gazdar, A.F. (1999) The RNA component of telomerase as a marker of biologic potential and clinical outcome in childhood neuroblastoma tumors. *Cancer*, 85, 741-749.
- Masutomi, K., Yu, E.Y., Khurts, S., Ben-Porath, I., Currier, J.L., Metz, G.B., Brooks, M.W., Kaneko, S., Murakami, S., DeCaprio, J.A., Weinberg, R.A., Stewart, S.A. and Hahn, W.C. (2003) Telomerase maintains telomere structure in normal human cells. *Cell*, 114, 241-253.
- Mitchell, J.R., Wood, E. and Collins, K. (1999) A telomerase component is defective in the human disease dyskeratosis congenita. *Nature*, 402, 551-555.
- Morales, C.P., Lee, E.L. and Shay, J.W. (1998) *In situ* hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. *Cancer*, 83, 652-659.
- Prowse, K.R. and Greider, C.W. (1995) Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc Natl Acad Sci U S A*, 92, 4818-4822.
- Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol*, 17, 1030-1032.
- Ritchie, K.B., Mallory, J.C. and Petes, T.D. (1999) Interactions of TLC1 (which encodes the RNA subunit of telomerase), TEL1, and MEC1 in regulating telomere length in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol*, 19, 6065-6075.
- Rushing, E.J., Yashima, K., Brown, D.F., White, C.L., 3rd, Shay, J.W., Risser, R.C. and Gazdar, A.F. (1997) Expression of telomerase RNA component correlates with the MIB-1 proliferation index in ependymomas. *J Neuropathol Exp Neurol*, 56, 1142-1146.
- Sage, J., Miller, A.L., Perez-Mancera, P.A., Wysocki, J.M. and Jacks, T. (2003) Acute mutation of retinoblastoma gene function is sufficient for cell-cycle re-entry. *Nature*, 424, 223-228.
- Sancar, A., Lindsey-Boltz, L.A., Unsal-Kacmaz, K. and Linn, S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem*, 73, 39-85.
- Scheel, C., Schaefer, K.L., Jauch, A., Keller, M., Wai, D., Brinkschmidt, C., van Valen, F., Boecker, W., Dockhorn-Dworiczak, B. and Poremba, C. (2001) Alternative lengthening of telomeres is associated with chromosomal instability in osteosarcomas. *Oncogene*, 20, 3835-3844.
- Scherr, M., Battmer, K., Schultheis, B., Ganser, A. and Eder, M. (2005) Stable RNA interference (RNAi) as an option for anti-bcr-abl therapy. *Gene Ther*, 12, 12-21.
- Schwartz, M., Zlotorynski, E. and Kerem, B. (2006) The molecular basis of common and rare fragile sites. *Cancer Lett*, 232, 13-26.
- Shay, J.W. and Bacchetti, S. (1997) A survey of telomerase activity in human cancer. *Eur J Cancer*, 33, 787-791.
- Shay, J.W., Pereira-Smith, O.M. and Wright, W.E. (1991) A role for both Rb and p53 in the regulation of human cellular senescence. *Exp Cell Res*, 196, 33-39.
- Shechter, D., Costanzo, V. and Gautier, J. (2004) ATR and ATM regulate the timing of DNA replication origin firing. *Nat Cell Biol*, 6, 648-655.
- Smogorzewska, A. and de Lange, T. (2002) Different telomere damage signaling pathways in human and mouse cells. *Embo J*, 21, 4338-4348.
- Soder, A.I., Hoare, S.F., Muir, S., Going, J.J., Parkinson, E.K. and Keith, W.N. (1997) Amplification, increased dosage and *in situ* expression of the telomerase RNA gene in human cancer. *Oncogene*, 14, 1013-1021.
- Stewart, S.A., Ben-Porath, I., Carey, V.J., O'Connor, B.F., Hahn, W.C. and Weinberg, R.A. (2003) Erosion of the telomeric single-strand overhang at replicative senescence. *Nat Genet*, 33, 492-496.
- Takai, H., Smogorzewska, A. and de Lange, T. (2003) DNA damage foci at dysfunctional telomeres. *Curr Biol*, 13, 1549-1556.
- Takata, H., Kanoh, Y., Gunge, N., Shirahige, K. and Matsuura, A. (2004) Reciprocal association of the budding yeast ATM-related proteins Tel1 and Mec1 with telomeres *in vivo*. *Mol Cell*, 14, 515-522.
- Takata, H., Tanaka, Y. and Matsuura, A. (2005)

Late S phase-specific recruitment of Mre11 complex triggers hierarchical assembly of telomere replication proteins in *Saccharomyces cerevisiae*. *Mol Cell*, 17, 573-583.

Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C. and Abraham, R.T. (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev*, 13, 152-157.

Verdun, R.E., Crabbe, L., Haggblom, C. and Karlseder, J. (2005) Functional human telomeres are recognized as DNA damage in G2 of the cell-cycle. *Mol Cell*, 20, 551-561.

Vespa, L., Couvillion, M., Spangler, E. and Shippen, D.E. (2005) ATM and ATR make distinct contributions to chromosome end protection and the maintenance of telomeric DNA in *Arabidopsis*. *Genes Dev*, 19, 2111-2115.

Voorhoeve, P.M. and Agami, R. (2003) The tumor-suppressive functions of the human INK4A locus. *Cancer Cell*, 4, 311-319.

Vulliamy, T., Marrone, A., Goldman, F., Dearlove, A., Bessler, M., Mason, P.J. and Dokal, I. (2001) The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature*, 413, 432-435.

Wright, W.E., Piatyszek, M.A., Rainey, W.E., Byrd, W. and Shay, J.W. (1996) Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet*, 18, 173-179.

Yashima, K., Litzky, L.A., Kaiser, L., Rogers, T., Lam, S., Wistuba, II, Milchgrub, S., Srivastava, S., Piatyszek, M.A., Shay, J.W. and Gazdar, A.F. (1997) Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. *Cancer Res*, 57, 2373-2377.

Yashima, K., Milchgrub, S., Gollahon, L.S., Maitra, A., Saboorian, M.H., Shay, J.W. and Gazdar, A.F. (1998) Telomerase enzyme activity and RNA expression during the multistage pathogenesis of breast carcinoma. *Clin Cancer Res*, 4, 229-234.

Yi, X., Tesmer, V.M., Savre-Train, I., Shay, J.W. and Wright, W.E. (1999) Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. *Mol Cell Biol*, 19, 3989-3997.

Zhao, H. and Piwnica-Worms, H. (2001) ATR-mediated checkpoint pathways regulate phosphor-

ylation and activation of human CHK1. *Mol Cell Biol*, 21, 4129-4139.

Supplementary data

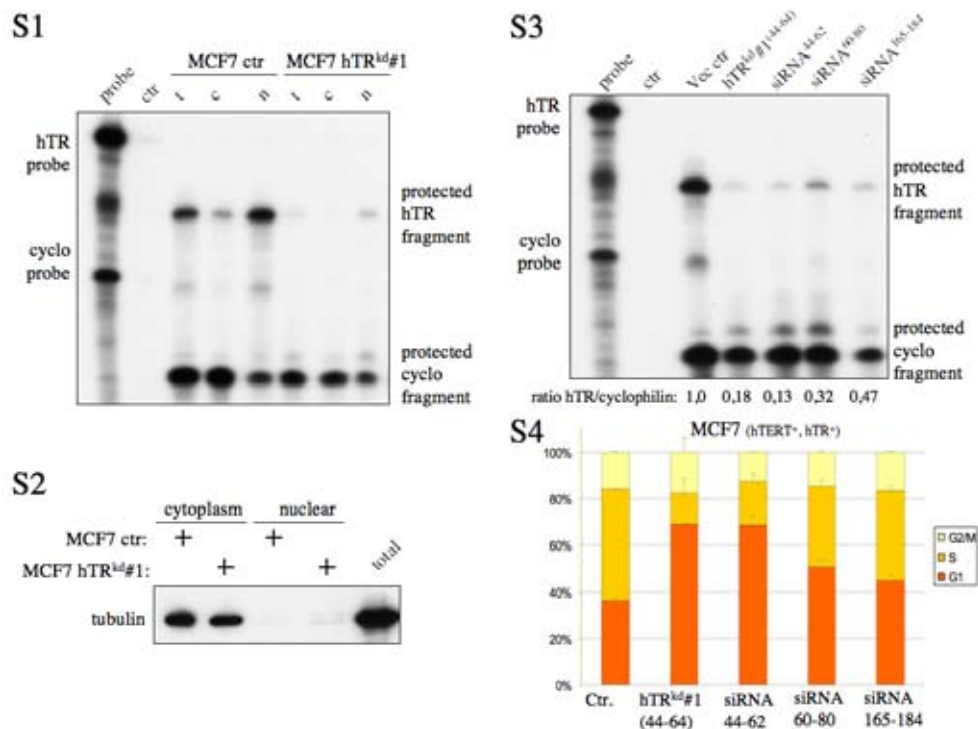


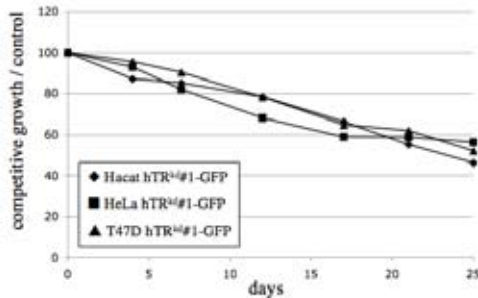
Figure S1. hTR knockdown in MCF-7 cytoplasmic and nuclear extracts. RPA performed on equal amounts of cytosolic and nuclear extracts from MCF-7 cells transfected with either pS-hTR^{kd#1} or control pS (t=total lysate; c=cytoplasmic extract; n=nuclear extract). Cells were harvested 4 days after transfection, cellular fractionation was performed using NE-PER kit (Pierce) according to manufacturers instructions.

Figure S2. A control immunoblot performed with 50 µg of cytosolic and nuclear protein extracts obtained from the same cell populations as described in Figure S1.

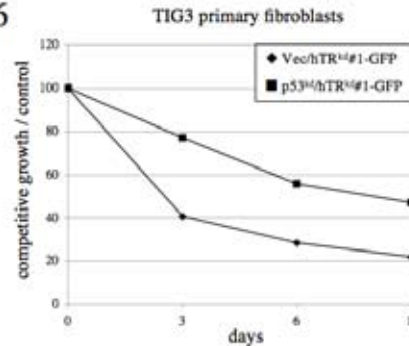
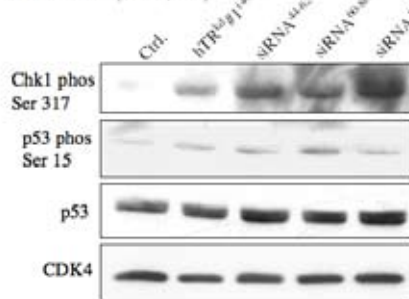
Figure S3. Multiple siRNAs inhibit hTR expression. RPA performed on extracts from MCF-7 cells transfected with pS, hTR^{kd#1}, and three different siRNAs targeting the hTR regions indicated (the siRNAs targeting nucleotides 44-62 and 165-184 of hTR were the same as used by Li et al., 2005). Cells were harvested 4 days after transfection. Quantification was performed by densitometry.

Figure S4. Inhibition of hTR expression triggers rapid growth inhibition. MCF-7 cells were transfected with hTR^{kd#1}, control constructs and indicated siRNAs and subjected to flow cytometric analysis. The percentages of cells in G1, S and G2 phases are shown. SD is from 3 independent experiments.

S5



S6

S7 GM847 (hTERT⁺, hTR⁺)

S8

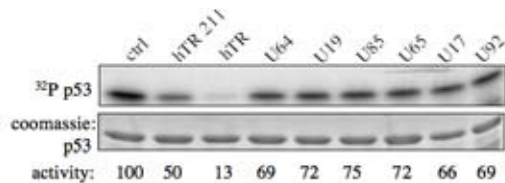


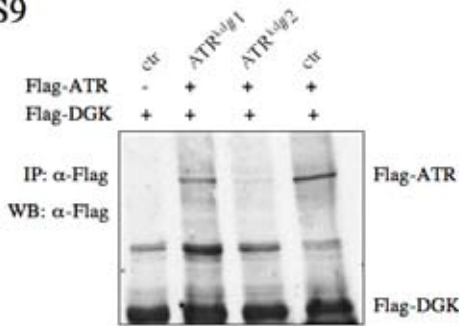
Figure S5. Rapid growth inhibition by inhibition of hTR expression. Competitive growth assay of HaCaT, HeLa, and T47D cell lines. Cells were transduced with the hTR^{kd}#1GFP construct and fluorescence was monitored by flow cytometry at the indicated timepoints after transduction.

Figure S6. Induction of the cell-cycle arrest mediated by hTR^{kd} in TIG3 fibroblasts is partially dependent on p53. TIG3 cells were transduced with p53^{kd} or control vector, drug selected and subsequently transduced with GFP-hTR^{kd}#1, or pRS-GFP control. Competitive growth assays were performed as described in Figure S4.

Figure S7. Inhibition of hTR expression triggers activation of ATR downstream targets. GM847 fibroblasts were transfected with hTR^{kd}#1, control pS and the indicated siRNAs. Whole cell extracts were immunoblotted to detect CHK1^{S317} phosphorylation, p53^{S15} phosphorylation, p53, and CDK4 as a loading control.

Figure S8. Inhibition of ATR kinase activity is specific for hTR. Kinase assay performed with IP and TEV cleaved TAP-ATR from HEK293 cells with GST-p53 (residues 1-101) as a substrate. Cleaved ATR was split and mock, hTR, hTR 211, and indicated H/ACA snoRNAs were added prior to kinase reaction. Samples were separated by 10% SDS-PAGE and stained with coomassie blue to detect GST-p53 protein, and autoradiography was performed to detect kinase activity. Band intensities were measured by densitometry.

S9



S10

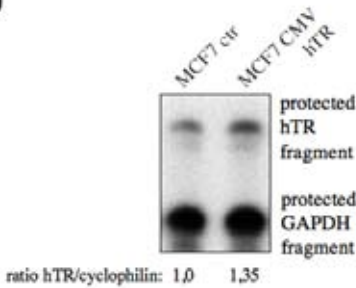


Figure S9. Validation of ATR^{kd} constructs. Indicated ATR^{kd} constructs were cotransfected with Flag-ATR and Flag-DGK as internal control. Flag immunoprecipitation was performed 3 days after transfection and IPs were immunoblotted for Flag.

Figure S10. hTR overexpression in MCF-7 cells. RPA performed on extracts from MCF-7 cells transfected with CMV-hTR, or CMV control constructs, cells were harvested 4 days after transfection. Quantification was performed by densitometry.

Chapter 6

microRNA Summary & Discussion

microRNA Summary & Discussion

Novel approaches to uncover microRNA function

To circumvent the dependency on prediction models, we developed a microRNA-screen-based assay to establish links between cellular phenotypes and microRNAs (miRNAs). To this end, a miRNA expression library (miR-Lib) was built consisting of 300 annotated miRNAs and around 100 candidate miRNAs. These miRNA 'minigenes' were cloned from genomic DNA of human cells and inserted in a specially engineered retroviral expression vector. This vector, named miR-Vec, is a modified murine stem cell virus (pMSCV) vector, with the expression of the miRNA minigenes under the control of the CMV pol II promoter. The vast majority of naturally transcribed miRNA genes are known to be expressed by pol II promoters. In parallel with the miRNA library, a corresponding microarray was developed (miR-Array) containing DNA spots of the miRNA minigenes from the library. By combining the miRNA library and microarray tools, we designed and performed three distinct functional genetic approaches to identify cancerous microRNAs, as detailed in the following sections.

Growth-dependent functional approach: identification and characterization of miR-372&373

In cell-growth-dependent screens one scores for miRNAs whose expression changes the growth capacity of cells once subjected to a growth inhibiting treatment. A microRNA may confer resistance to cells by interfering with the pathway that is required to induce or maintain the growth arrest, resulting in the continued proliferation of cells expressing this particular miRNA. As a consequence, the resisting cells simply outgrow the growth-arrested cells. To identify the miRNA that causes the resistance, the relative abundance of each of the miR-Vec inserts between treated (growth inhibited) and untreated cells is compared by PCRing the minigenes from both cell populations, labelling with red and green and hybridizing to a microarray. miRNAs that confer resistance towards the treatment are enriched in the treated population while the signal of naive miRNAs remains unchanged.

In **chapter 3**, this genetic screening method led

to the identification of miR-372 and miR-373 (miR-372&373), whose expression in BJ primary fibroblasts overcomes the induction of cellular senescence upon oncogenic stress. We demonstrated that miR-372&373 expression could substitute for the loss of p53 in a transformation model, which indicated that these miRNAs protect cells from oncogene-induced senescence despite having functional p53. We also show that miR-372&373 prevent senescence-associated cell-cycle arrest by maintaining high CDK2 activity. Furthermore, we identified LATS2 as a target of miR-372&373, and showed that suppression of LATS2 is sufficient to overcome the induction of cellular senescence upon oncogenic stress. LATS2 is known to inhibit CDK2 activity, and is required to inhibit oncogene mediated transformation (Li et al., 2003; McPherson et al., 2004). This fits with a model in which miR-372&373 sustain CDK2 activity via the suppression of LATS2 expression. Finally, we found that the coincidental expression of miR-372&373 and wildtype p53 holds true for cancer, although it is confined to testicular germ cell tumors. Part of the examined TGCTs expressed both miRNAs and wildtype p53, whereas TGCTs not expressing the miRNAs, either had low p53 levels or mutated p53. In summary, we have devised miR-Lib and miR-Array as novel tools in the search for miRNA function. As a proof of principle, we applied these tools to a growth dependent screen aimed at finding miRNAs that confer resistance to senescence. We identified the oncogenic function of miR-372&373, and suggested their involvement in TGCTs.

A number of questions still remain. For instance, knocking down LATS2 partially prevents oncogenic RAS^{V12}-induced senescence. In contrast, miR-372&373, the miRNAs that target LATS2, allow full resistance to oncogenic stress signals and show full blown transformation. This indicates that besides LATS2, other targets of miR-372&373 might be responsible for preventing senescence. What other targets that participate in senescence are regulated by miR-372&373? Most likely, these targets, and possibly many other targets of other miRNAs picked up in similar functional screens, are regulated at the level of translation, which masks the miRNAs inhibitory effect at the RNA level, and makes is very difficult

to find targets. Currently, mRNA expression arrays are used to identify miRNA targets, however this method relies on differences in mRNA levels, and as such only identifies targets that have been subjected to degradation (mRNA decay) by the miRNA. It is very likely that those miR-372&373 target genes are regulated by translational repression, something that can only be detected at the protein level. This underscores the need for a high-throughput method that can identify miRNA-induced alterations in protein production. As long as this method is lacking, it is still hard to understand the full impact of a certain miRNA on gene expression. Nevertheless the potential of miR-372&373 was identified.

An important issue is the way in which miR-372&373 expression is regulated. During the course of sperm development, miRNA expression is silenced. Although there is no evidence suggesting this switch-like mechanism is required for normal sperm development, we believe that sustained miR-372&373 expression is harmful, as it desensitizes cells to the emergence of oncogenes.

A clue as to how miR-372&373 might be silenced came from a recent report (Lujambio et al., 2007). miR-373 is situated in close proximity to a CpG island, which suggests that the mechanism involved in properly silencing miR-372&373 depends on epigenetics. This was confirmed by Lujambio et al (Lujambio et al., 2007), who screened for differential miRNA expression in cells engineered with DNMT knockdown constructs. As a result of methyltransferase inhibition, methylation at CpG islands were lost in time, and miRNAs that are normally silenced by methylation, were found to be expressed, one of which was miR-373. Despite these findings, some issues need to be resolved. For example, are the miRNAs silenced by means of methylation during normal sperm development. Is methylation used to silence the miRNAs in somatic cells? And finally, is maintenance or restoration of the expression of miR-372&373, in tumors such as TGCTs, caused by methylation problems? Further investigation is required to elucidate how miR-372&373 expression is suppressed, and whether this mechanism is interfered with in the process of tumorigenesis.

Growth-independent screening: identification and characterization of miR-221&222

In chapter 4, we developed a direct target approach to search for miRNA function. This particular screen relies on a reporter-tagged target,

the 3'UTR of a gene of interest, that acts as bait for microRNAs. When miRNAs affect the target, the reporter changes. Therefore, cells with lower levels of the reporter, and thus lower levels of the target, express a miRNA that regulates the target. We then sorted the low reporter expressing cells from the total population of cells, and compared the relative abundance of the miR-Vec inserts between these two populations. miRNAs that affect the target are more abundant in cells expressing low levels of the reporter relative to the total population of reporter expressing cells, and thus could be identified using our miR-Ar-ray.

The above-mentioned method was used to screen for miRNAs regulating the cyclin dependent kinase inhibitor p27^{Kip1}. Previous findings make p27^{Kip1} an appealing target for miRNAs. A publication by the lab of Hatfield already hinted the involvement of miRNAs in the regulation of the *Drosophila* p27^{Kip1} orthologue dacapo (Hatfield et al., 2005). The work describes the mutation of Dicer-1, important for the production of miRNAs, in germline cyst production. Without miRNAs to aid in the process of germline cell proliferation, Dicer-1 mutant cells are delayed in their progression through G1. The G1-slowdown was found to depend on dacapo, as knockdown of dacapo restored germcell growth. In addition, p27^{Kip1} is a tumor suppressor, as studies in mice have shown that loss of p27^{Kip1} increases tumor incidence and tumor growth rate in either specific genetic backgrounds or when mice are challenged with carcinogens (Fero et al., 1998). In human cancers, p27^{Kip1} protein levels are often lowered, however complete loss of p27^{Kip1} is rare. Increased proteolysis is a common mechanism involved in lowering p27^{Kip1} levels in tumors. However, several studies have indicated that the genes controlling the stability of p27^{Kip1} protein might not always account for its lower expression in cancer. p27^{Kip1} can also be controlled at the translational level (Chilosì et al., 2000; Hengst and Reed, 1996; Millard et al., 1997), leaving a role for regulation of p27^{Kip1} by miRNAs.

In order to screen for miRNAs that regulate p27^{Kip1}, we constructed a retroviral sensor vector expressing the 3'UTR of p27^{Kip1} downstream of the GFP reporter gene. We transduced a monoclonal GFP-p27-3'UTR expressing HeLa cell line with the miRNA library, and FACS-sorted the low GFP-expressing cells from the total population. Comparing the relative abundance of each of the miR-Vec inserts between these populations on the microarray revealed enrichment of miR-221 in the low GFP-expressing cell population.

Subsequent validating experiments confirmed miR-221, but also miR-222, a miRNA that shares seed sequence with miR-221, as the miRNAs in control of p27^{Kip1}.

Different reports that describe the differential expression of miRNAs in primary glioblastomas, papillary thyroid carcinomas and pancreas tumors, have indicated the upregulation of miR-221, as part of a miRNA cancer signature (Ciafre et al., 2005; He et al., 2005; Lee et al., 2007; Pallante et al., 2006). Because p27^{Kip1} is a target of these miRNAs, we hypothesized that in cancer the balance between miR-221 and miR-222 (miR-221&222) and p27^{Kip1} expression is altered in favor of higher miR-221&222 expression. To examine this, we designed miR-221&222 antagomiRs, sequences antisense to the miRNAs, to block miRNA function in U87 glioblastoma and MDA-MB-231 breast cancer cells, two cell lines that endogenously express miR-221&222. After 48 hours treatment with antagomiRs we observed a block in cellular proliferation in both cell lines, whereas no effect was observed in miR-221&222 negative cell lines. Further examination of this effect revealed an increase in p27^{Kip1} protein in U87 and MDA-MB-231 cells after antagomiR treatment. As a consequence of increased p27^{Kip1}, cells went into a G1 arrest, which is consistent with the role of p27^{Kip1} in controlling progression through this phase of the cell-cycle. To demonstrate that p27^{Kip1} is essential for the proliferation arrest in U87 and MDA-MB-231 cells, we showed that the antagomiR-induced G1 arrest could be overcome by knocking down p27^{Kip1}.

Finally, we extended the correlation between miR-221&222 and p27^{Kip1} to human malignancy. By comparing the levels of miRNA and p27^{Kip1} expression in glioblastoma tumors from 5 patients in relation to healthy tissue controls, our study revealed high miR-221&222 expression in tumor cells, but low miR-221&222 expression in surrounding healthy cells. In contrast, p27^{Kip1} protein levels were low in the tumors compared to surrounding tissue. Based on these results, we believe that a correlation exists between low p27^{Kip1} protein and high miR-221&222 levels in the tumor, contrasting high p27^{Kip1} protein and low miR-221&222 levels in healthy tissue.

Our results presented in this chapter illustrate the use of the miR-Lib and miR-Array tools in the search for miRNA function in a growth-independent screen setup. The direct target approach directly ties a gene of interest to regulation by specific miRNAs, which led to the identification of miR-221&222 as miRNAs acting on the 3'UTR of p27^{Kip1}. Furthermore, we showed that regula-

tion of p27^{Kip1} by miR-221&222 is important for the proliferation of certain tumor cell lines, and possibly glioblastoma tumors.

Our studies raise several unresolved issues that require further investigation. An important point to address for example is whether a change in the expression of miR-221&222 is causal to, and/or a consequence of tumorigenesis. In other words, is altered miR-221&222 expression essential solely for tumor growth, after the tumor has established, or is it sufficient to initiate tumor formation as well? Correspondingly, do human tumors, such as glioblastomas, require continuous miR-221&222 expression to maintain tumor growth? Currently, we are testing the miRNAs influence on tumor growth *in vivo*, by intracranially injecting mice with reporter-expressing glioblastoma cells. After tumor establishment, the mice are then injected with miR-221&222 antagomiRs, near the tumor location, and screened for tumor growth, as visualized by reporter gene intensity. In this way, the impact of antagomiRs on the growth rate of the tumor can be monitored *in vivo*.

Does altered expression of miR-221&222 suffice for tumor onset? Mouse p27^{Kip1} knockout experiments have demonstrated that loss of p27^{Kip1} expression promotes cell proliferation, but not tumor formation. However, tumor incidence and growth rate were significantly enhanced in mice that had lost tumor suppressor genes, such as PTEN or Rb, in addition to p27^{Kip1}. This would suggest that alteration in the expression levels of miR-221&222 or elimination of miR-221&222 sites in p27-3'UTR, could cooperate with specific mutating events in the onset of tumor growth. On the other hand, besides p27^{Kip1}, miR-221&222 may target other mRNAs, that could be involved in tumorigenesis, which would eliminate the need for other mutating events during tumorigenesis. Finding these targets, and showing that altered miR-221&222 expression can induce tumor formation, are topics that need further investigation.

Another interesting issue involves the mechanism that drives miR-221&222 expression. miR-221&222 are expressed in several tissues in the human body, among which are brain, kidney, pancreatic islets, and fibroblasts (Landgraf et al., 2007). It would be important to know what causes the increase in their expression as there is no evidence which suggests that amplification is involved. One possibility is a change in tran-

scription rate, which could be due to a higher availability of the transcription factor(s) that regulates miR-221&222 expression. Several studies have shown that particular miRNAs are regulated by transcription activators such as p53 (Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007) and Myc (O'Donnell et al., 2005). p53 and Myc are examples of genes that are often mutated or dysregulated in cancer, resulting in a change in miRNA expression. A similar event might explain miR-221&222 dysregulation.

In conclusion, I think it is important to address these issues which will help to demonstrate that a change in miR-221&222 expression could play an important role in the control of cellular behaviour and tumorigenesis.

Cell-phenotype screening

A third approach we established to screen for miRNA function is to measure a gain or loss of cellular phenotypic traits induced by individual miRNAs. This growth-independent screen type can assess differences in cellular characteristics, such as cell size, shape, and movement, in a single-well based format, and therefore establishes a link between miRNA expression and cellular phenotype. This type of screening was used to identify miRNAs that can stimulate cell migration, invasion and metastasis (Huang, submitted).

miRNAs and cancer

A hallmark in the development of cancer is the deregulated expression of genes, both at the genetic and epigenetic level. On one hand, there are genes whose gene upregulation drives tumorigenicity. These oncogenes arise as a result of mutations, rendering them more active, or are uncoupled from their controlled regulation. Alternatively, oncogenes appear through gene amplifications, producing an overload of the oncoprotein.

Opposing the oncogenes are tumor suppressor genes. These genes act as safe-guards by controlling cellular activities, and are involved in cell-cycle checkpoint responses, controlling mitogenic signalling, and are crucial for the DNA damage response. In cancers, these fail-safe mechanisms must be deactivated, and as such, tumor suppressors are frequently mutated, deleted or silenced (e.g. by means of methylation or miRNA).

Interestingly, accumulating evidence implicates miRNA genes in human cancer. At present, both oncogenic and tumor suppressive miRNAs have

been catalogued, demonstrating the devastating effects these tiny ncRNAs can have at the cellular level when abnormally expressed.

miRNAs and their targets may show differential regulatory modes of interaction, resulting in distinct regulatory outcomes (summarized in Figure 1). One group of miRNAs has switch-like functions, where the expression of the miRNA is either fired up or shut down, whereas another group of miRNAs has fine-tuning abilities. Switch-like miRNAs are crucial in the process of development and differentiation. Their expression enables cells to clear mRNAs for a certain period of time, after which the miRNA is switched off to allow the production of protein. The small temporal RNAs lin-4 and let-7 are 'classical' examples. Their expression patterns must be accurate in order to provide the correct cues for the developing *C. elegans* larvae. Having a miRNA expressed at times when it should not, or silencing its expression when presence is required, could lead to abnormal phenotypes.

One such example includes the prolonged or restored expression of miR-372&373 involved in the tumorigenesis of TGCTs. Aberrant expression of these miRNAs makes cells insensitive to respond to the fail-safe mechanism which is activated upon continuous oncogenic signaling.

Fine-tuning miRNAs on the other hand, may control the amount of target mRNA allowed to be translated into protein. These modulating miRNAs do not completely silence their targets, but rather function to dampen fluctuations in protein output by restricting available mRNAs. This delicate setup may easily be imbalanced by either miRNA- or target-discrepancies. A change in miRNA expression results in a change in target availability, whereas altering target availability affects miRNA efficiency. miR-221&222 are examples of fine-tuning miRNAs.

Other ways of disturbing the miRNA-target interaction, involve mutation, deletion or translocation of the miRNA recognition site(s) within the 3'UTR of the target. This behaviour of the target to avoid regulation by miRNAs has been reported to occur. The High Mobility Group A (HMGA) family of proteins regulate numerous cellular processes, including cell-cycle, and differentiation. HMGA proteins are undetectable in differentiated cells, yet present in proliferating cells, and tumor cells. Earlier this year, HMGA2 expression was found to be inversely correlated with let-7 expression, suggesting that HMGA2 is a target of this miRNA (Lee and Dutta, 2007; Shell et al., 2007). However, in tumors, this correlation is disrupted.

HMGA2 is known to translocate, whereby it usually loses part of the coding region, but more importantly, it loses its 3'UTR. Without the 3'UTR, HMGA2 is no longer sensitive to let-7 regulation. Furthermore, *in vitro* studies have shown that interference of the let-7 mediated HMGA2 regulation results in increased proliferation rates of cell lines, and even transformation of NIH-3T3 cells, further suggesting a role for this disruption between miRNA and target in the contribution to tumorigenesis. Still the *in vivo* relevance of HMGA2 avoiding let-7 regulation in the process of tumorigenesis has to be proven.

Finally, gene modifications can result in the creation of a miRNA binding site. The modified mRNA is suddenly regulated by a miRNA. This is known to occur, as exemplified by myostatin, a growth factor that limits muscle tissue growth. A natural occurring mutation in myostatin was observed in Texel sheep (Clop et al., 2006). There, a SNP (single nucleotide polymorphism) within the 3'UTR of myostatin, creates a binding site for miR-1 and miR-206, two miRNAs expressed abundantly in skeletal muscle. Suppressing myostatin expression creates a strong phenotypic alteration, which is predominantly associated with muscle hypertrophy.

Disturbing each of these regulatory modes, either by changing the miRNA input or by altering target sensitivity towards a particular miRNA, results in an imbalance. This imbalance might be a cause or consequence of malignancies, such as tumorigenesis.

miRNAs and therapy

miRNAs are involved in diseases such as cancer. Expression profiling studies (microarray, Q-RT-PCR) in cancer cell lines and tumors have shown that the expression of most miRNAs is down-regulated, while the expression of other miRNAs is upregulated. This suggests roles for miRNAs as oncogenes or tumor suppressor genes. Still, a change in the expression of a particular miRNA might be a consequence of, rather than causative to cells becoming tumorigenic. Because miRNAs are complementary to their targets, they may serve as ideal candidates for therapy. For the miRNAs that have been shown to possess either tumor suppressive or oncogenic traits, different antitumor strategies can be designed.

Ideally, the miRNA expression level should be restored to its original level. In case of increased or unwanted/sudden miRNA expression, strategies to lower the expression can be deployed. Currently, different formats of anti-miRNA oligonucleotides (AMOs), such as LNAs or morpho-

nos are available to that purpose. These oligos are complementary to the miRNA and as such their function relies on sequestration of the target miRNA, thereby alleviating the suppression of the targets of the miRNA.

In case of decreased miRNA expression, due to mutation, deletion, translocation or

, miRNA expression can be restored by means of viral delivery. Or, when methylation is involved in lowering the expression of miRNAs, drugs like methylation inhibitors (5-aza-2'-cytidine) could be used.

In the rare event that a miRNA is mutated (Landgraf et al., 2007), but still expressed at normal levels, this may cause loss of target recognition of, for example, oncogenes. Less tight control of oncogene expression could subsequently lead to complications. Alternatively, miRNA mutation might induce recognition of new targets, to which it has become sufficiently complementary to. When the new target is a tumor suppressor, this also could lead to complications. A solution to the first problem would be to express the wild-type miRNA, which would restore the miRNAs normal functions, and regulation of its targets. To prevent unwanted targeting of new/gained targets on the other hand, an AMO specifically designed to target the mutated miRNA could be used in combination with expression of the wildtype miRNA, that should restore target regulation.

When the target is mutated, it might avoid regulation by a particular miRNA. In cancer, this could be the case for an oncogene. A counteracting strategy involves expressing a custom designed miRNA that targets only the changed target, which therefore would restore the regulation of this target.

Another issue is systemic delivery of miRNAs. Important studies have shown the difficulties of bio-availability with systemic delivery of miRNAs in mice. Most of the molecules remain in the liver, thereby preventing an equal systemic distribution. Additionally, increased administration of miRNA drugs has proven to be toxic, as shRNA/miRNAs compete with one another within the RNAi pathway. The important limiting factor was shown to be exportin-5, important for the maturation of both shRNA and miRNA species.

Because miRNA drugs are much more specific due to their sequence, they can inhibit targets that normally are out of reach for conventional drugs. They are easy to make, and simple to test. A miRNA most probably regulates multiple targets, and its misexpression therefore might

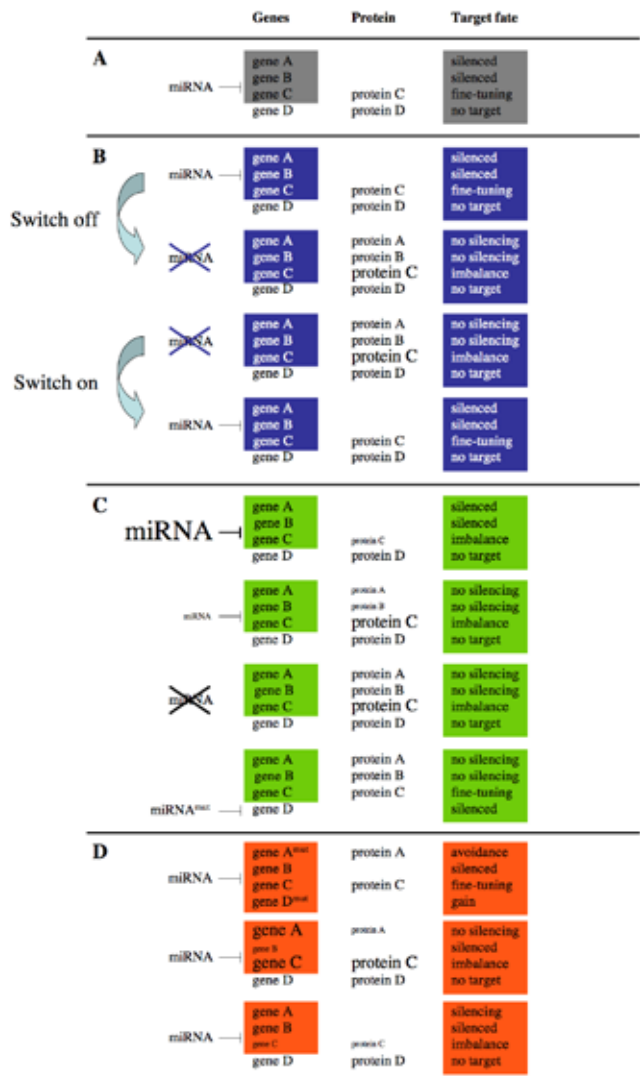


Figure 1. Schematic drawing of miRNA-mRNA relationships and implications following miRNA or mRNA alterations. (A) The normal regulation of target genes by a miRNA. Genes A and B are silenced, while gene C is fine-tuned. Gene D is not a target. (B) Target gene regulation by switch-like miRNAs. (C) A change in miRNA expression level or miRNA sequence. *Upper panel:* an increase in miRNA expression (e.g. by amplification) causes an imbalance in the fine-tuning of gene C, leading to less protein C. *Second panel:* a decrease in miRNA expression (e.g. by deletion or methylation) causes silenced genes A and B to become resistant enough to produce proteins A and B. Fine-tuning of gene C is also deregulated, leading to more protein C. *Third panel:* miRNA silencing (e.g. by deletion or methylation) causes full recovery of silenced genes A and B. Gene C is not fine-tuned anymore. Overall, more protein A, B and C. *Lower panel:* the expression of a mutated miRNA may cease regulation of the expression of genes A, B and C. Additionally, gene D might bear recognition sites for the mutated miRNA, and may become a target. (D) A change in mRNA expression level or mRNA sequence. *Upper panel:* mutation in gene A may avoid regulation by the miRNA. This causes appearance of protein A. A mutation in the miRNA unregulated gene D could make it a target. *Middle panel:* an increase in the expression of gene C, overwhelms miRNA regulation, and may lead to the production of protein A. Increased expression of gene C, which is fine-tuned by the miRNA, may cause an imbalance leading to increased production of protein C. *Lower panel:* lower expression of gene C might cause an imbalance in which the same number of miRNAs regulate less numbers of C mRNA, leading to a lowering in protein C.

interfere with the regulation of the different pathways that depend on correct expression of these targets. It is possible that different pathways can be repaired by targeting (expressing/inhibiting) just one miRNA.

In order to perfect miRNA-based drugs for clinical use in the treatment of pathologic conditions, improvements in bio-availability, stability, cellular uptake, as well as reducing off-target effects are important. A challenge indeed...

Conclusions

Manipulation of -and regulation by- noncoding RNAs, such as miRNAs, constitute another level of complexity within higher eukaryotes. How important miRNAs are, is illustrated by the fact that misexpression of miRNAs, or any participant involved in the pathways required for producing and processing miRNAs, or mandatory for executing miRNA-dependent actions, results in diseases, like cancer. We have shown that our recently developed miRNA tools allow us to perform functional genetic screens in human cells. And as such we were able to uncover the oncogenic potential of the miR-372&373 and miR-221&222 families. Ultimately, these and future discoveries can be used for the development of new therapeutic approaches for treatment of cancer.

References

- Bartel, D.P. (2004) microRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116, 281-297.
- Chang, T.C., Wentzel, E.A., Kent, O.A., Ramachandran, K., Mullendore, M., Lee, K.H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J., Arking, D.E., Beer, M.A., Maitra, A. and Mendell, J.T. (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell*, 26, 745-752.
- Chilosi, M., Chiarle, R., Lestani, M., Menestrina, F., Montagna, L., Ambrosetti, A., Prolla, G., Pizzolo, G., Doglioni, C., Piva, R., Pagano, M. and Inghirami, G. (2000) Low expression of p27 and low proliferation index do not correlate in hairy cell leukaemia. *Br J Haematol*, 111, 263-271.
- Ciafre, S.A., Galardi, S., Mangiola, A., Ferracin, M., Liu, C.G., Sabatino, G., Negrini, M., Maira, G., Croce, C.M. and Farace, M.G. (2005) Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem Biophys Res Commun*, 334, 1351-1358.
- Clop, A., Marcq, F., Takeda, H., Pirottin, D., Tor-doir, X., Bibe, B., Bouix, J., Caiment, F., Elsen, J.M., Eychenne, F., Larzul, C., Laville, E., Meish, F., Milenkovic, D., Tobin, J., Charlier, C. and Georges, M. (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet*, 38, 813-818.
- Fero, M.L., Randel, E., Gurley, K.E., Roberts, J.M. and Kemp, C.J. (1998) The murine gene p27^{Kip1} is haplo-insufficient for tumor suppression. *Nature*, 396, 177-180.
- Hatfield, S.D., Shcherbata, H.R., Fischer, K.A., Nakahara, K., Carthew, R.W. and Ruohola-Baker, H. (2005) Stem cell division is regulated by the microRNA pathway. *Nature*, 435, 974-978.
- He, H., Jazdzewski, K., Li, W., Liyanarachchi, S., Nagy, R., Volinia, S., Calin, G.A., Liu, C.G., Franssila, K., Suster, S., Kloos, R.T., Croce, C.M. and de la Chapelle, A. (2005) The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*, 102, 19075-19080.
- He, L., He, X., Lim, L.P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A.L., Linsley, P.S., Chen, C., Lowe, S.W., Cleary, M.A. and Hannon, G.J. (2007) A microRNA component of the p53 tumor suppressor network. *Nature*, 447, 1130-1134.
- Hengst, L. and Reed, S.I. (1996) Translational control of p27^{Kip1} accumulation during the cell cycle. *Science*, 271, 1861-1864.
- Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. *Science*, 294, 853-858.
- Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A.O., Landthaler, M., Lin, C., Socci, N.D., Hermida, L., Fulci, V., Chiaretti, S., Foa, R., Schliwka, J., Fuchs, U., Novosel, A., Muller, R.U., Schermer, B., Bissels, U., Inman, J., Phan, Q., Chien, M., Weir, D.B., Choksi, R., De Vita, G., Frezzetti, D., Trompeter, H.I., Hornung, V., Teng, G., Hartmann, G., Palkovits, M., Di Lauro, R., Wernet, P., Macino, G., Rogler, C.E., Nagle, J.W., Ju, J., Papavasiliou, F.N., Benzing, T., Lichter, P., Tam, W., Brownstein, M.J., Bosio, A., Borkhardt, A., Russo, J.J., Sander, C., Zavolan, M. and Tuschl, T. (2007) A mammalian microRNA expression atlas based on small RNA library se-

quencing. *Cell*, 129, 1401-1414.

Lau, N.C., Lim, L.P., Weinstein, E.G. and Bartel, D.P. (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science*, 294, 858-862.

Lee, E.J., Gusev, Y., Jiang, J., Nuovo, G.J., Lerner, M.R., Frankel, W.L., Morgan, D.L., Postier, R.G., Brackett, D.J. and Schmittgen, T.D. (2007) Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*, 120, 1046-1054.

Lee, R.C. and Ambros, V. (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. *Science*, 294, 862-864.

Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, 75, 843-854.

Lee, Y.S. and Dutta, A. (2007) The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev*, 21, 1025-1030.

Li, Y., Pei, J., Xia, H., Ke, H., Wang, H. and Tao, W. (2003) Lats2, a putative tumor suppressor, inhibits G1/S transition. *Oncogene*, 22, 4398-4405.

Lujambio, A., Ropero, S., Ballestar, E., Fraga, M.F., Cerrato, C., Setien, F., Casado, S., Suarez-Gauthier, A., Sanchez-Cespedes, M., Git, A., Spiteri, I., Das, P.P., Caldas, C., Miska, E. and Esteller, M. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res*, 67, 1424-1429.

McPherson, J.P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak-Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J., Kassam, F., Squire, J., Bruneau, B.G., Hande, M.P. and Hakem, R. (2004) Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. *Embo J*, 23, 3677-3688.

Millard, S.S., Yan, J.S., Nguyen, H., Pagano, M., Kiyokawa, H. and Koff, A. (1997) Enhanced ribosomal association of p27^{Kip1} mRNA is a mechanism contributing to accumulation during growth arrest. *J Biol Chem*, 272, 7093-7098.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V. and Mendell, J.T. (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*, 435, 839-843.

Pallante, P., Visone, R., Ferracin, M., Ferraro, A., Berlingieri, M.T., Troncone, G., Chiappetta, G., Liu, C.G., Santoro, M., Negrini, M., Croce, C.M. and Fusco, A. (2006) microRNA deregulation in human thyroid papillary carcinomas. *Endocr Relat Cancer*, 13, 497-508.

Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z. and Oren, M. (2007) Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell*, 26, 731-743.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R. and Ruvkun, G. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, 403, 901-906.

Shell, S., Park, S.M., Radjabi, A.R., Schickel, R., Kistner, E.O., Jewell, D.A., Feig, C., Lengyel, E. and Peter, M.E. (2007) Let-7 expression defines two differentiation stages of cancer. *Proc Natl Acad Sci U S A*, 104, 11400-11405.

Tarasov, V., Jung, P., Verdoodt, B., Lodygin, D., Epanchintsev, A., Menssen, A., Meister, G. and Hermeking, H. (2007) Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1 arrest. *Cell Cycle*, 6.

Nederlandse Samenvatting

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Het centrale dogma in moleculaire biologie, zoals bedacht door Francis Crick in 1958, beschrijft het doorgeven van genetische informatie vanaf DNA naar de aanmaak van eiwitten. Omdat DNA beperkt is tot de celkern en eiwitten in het cytoplasma worden aangemaakt, wordt gebruik gemaakt van messenger RNA, zogenaamde 'boodschappers', en dragers van genetische informatie, die worden gekopieerd van het DNA. Elke boodschapper fungeert als loopjongen en verbindt nucleaire transcriptie (boodschapper aanmaak) met cytoplasmatische vertaling (eiwit aanmaak). Verschillende boodschappers worden gekopieerd van verschillende stukken DNA, en zullen worden vertaald tot verschillende eiwitten.

Echter heeft RNA niet alleen de belangrijke taak genetische informatie door te geven naar de eiwit machinerie in de vorm van messenger RNAs (mRNAs), het heeft ook andere functies. Eén van deze taken is verbonden met de eiwit aanmaak op een directe manier. tRNAs, of transfer RNAs zijn onderdeel van de niet-coderende RNA familie. Zij zijn betrokken bij het leveren van de juiste aminozuren die passen bij de genetische code van de messenger RNAs tijdens het proces van eiwit aanmaak.

In de afgelopen decennia is de familie van niet-coderende RNAs sterk gegroeid. We moeten hierbij denken aan RNAs die, met de hulp van eiwitten, als enzymen kunnen fungeren. Zo kunnen deze zogenaamde RNA-eiwit complexen andere RNA moleculen modificeren, of zelfs afbreken.

Een belangrijk deel van de niet-coderende RNA familie wordt ingenomen door de microRNAs. Samen met gespecialiseerde eiwitten pakken zij messenger RNAs aan. Dit gebeurt nog voordat de messenger RNA de genetische boodschap door heeft gegeven aan de eiwit productie machinerie, waardoor uiteindelijk minder tot veel minder eiwit gemaakt wordt.

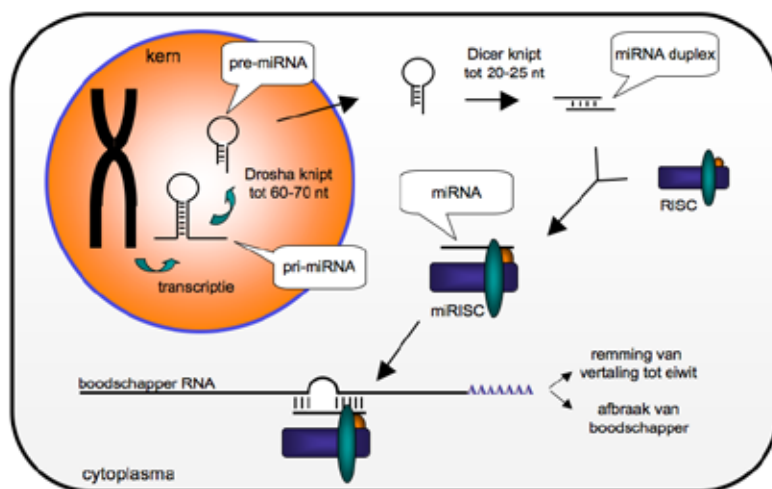
Hoe wordt een microRNA gemaakt en wat is het doel van microRNAs?

Een microRNA wordt doorgaans geproduceerd alsof het een messenger RNA is (Figuur 1). Daarmee houdt de vergelijking ook direct op. microRNAs hebben een dusdanige RNA sequentie waardoor ze op zichzelf terugvouden, waardoor een haarspeld structuur (de primaire microRNA) wordt gevormd. Deze structuur wordt in de kern

van de cel herkend door een enzym, Drosha, die de haarspeld verkort. Eenmaal korter wordt de microRNA-haarspeld (nu microRNA precursor geheten) de kern uitgeloodst naar het cytoplasma voor een verdere verkortings sessie. Ditmaal door het Drosha familielid, Dicer. Dicer knipt de lus van de microRNA haarspeld weg, en het grootste gedeelte van de haarspeld poten. Wat overblijft is een klein stukje dubbelstrengs RNA, de gerijpte microRNA. Wat begon als een RNA keten van enkele honderden schakeltjes (nucleotiden), eindigt als een 22 nucleotiden korte dubbelstrengs RNA molecuul. Uiteindelijk wordt één van deze twee ketens gekozen en in een speciaal complex genaamd RISC ingebouwd. RISC staat voor RNA Induced Silencing Complex. Dit complex is gespecialiseerd in het stoppen van de boodschappers in hun taak de genetische informatie door te geven aan eiwitten. Zoals de naam verder weergeeft is dit proces afhankelijk van RNA, in dit geval de microRNA. RNA van tegenovergestelde sequentie (complementaire sequentie) kan aan elkaar binden en ditzelfde gegeven wordt gebruikt bij de binding van microRNAs aan hun doelwit messenger RNAs. Opvallend genoeg vindt de binding van een microRNA aan een messenger RNA plaats op telkens hetzelfde stukje van de messenger, de 3'UTR (UnTranslated Region). De 3'UTR is een stuk RNA achter het eiwit-coderende deel van de messenger RNA en blijkt de plek te zijn waar microRNAs hun functionaliteit ontplooien. De 3'UTRs van messenger RNAs variëren van enkele 100-en tot 1000-en nucleotiden. De microRNAs zijn ieder zo'n 22 nucleotiden lang, dus is het niet moeilijk om in te zien dat één microRNA meerdere doelwitten kan hebben. Tevens kunnen meerdere verschillende microRNAs aan één en dezelfde messenger binden.

Uit onderzoek naar de associatie tussen microRNA en messenger RNA is gebleken dat de twee niet perfect met elkaar binden. Verder is aangetoond dat hoe perfecter deze binding is, des te groter de kans dat het messenger RNA wordt afgebroken. Echter, hoe incompleter de binding, des te groter is de kans op remming van vertaling. Beide mechanismen leiden tot hetzelfde effect: minder eiwit aanmaak.

Daarmee is een nieuw regulatie mechanisme geboren waarbij eiwit kwantiteit gemanipuleerd kan worden nog voordat er eiwit gemaakt is. De



Figuur 1. De aanmaak en werking van microRNAs.

door de microRNA veroorzaakte afbraak of remming van de messenger kan van een dusdanige aard zijn dat de messenger geen kans meer ziet de genetische code door te geven aan de eiwit productie machinerie. In dat geval spreken we van een switch microRNA. Expressie van de microRNA legt de productie van een eiwit stil, en omgekeerd kan hetzelfde eiwit alleen gemaakt kan worden als de microRNA expressie stopt. In een ander geval komt de microRNA tot expressie, maar legt de productie van een eiwit niet stil, het moduleert het. Het eiwit wordt nog steeds gemaakt, maar de aanmaak ervan staat onder controle. Hier spreken we van fine-tuning. Op beide voorbeelden komen we later terug.

microRNAs en kanker

microRNAs hebben een belangrijke rol in de regulatie van eiwit expressie. Ondanks dat we nog lang niet weten welke messenger RNAs door welke microRNAs worden gecontroleerd, is wel duidelijk geworden dat veranderingen in de expressie van microRNAs tot problemen kunnen leiden, zoals kanker. Voor bepaalde microRNAs is zelfs gevonden dat ze het gedrag vertonen van tumor onderdrukkende (tumor suppressor) of juist tumor stimulerende (oncogen) genen. In tumoren worden tumor suppressors vaak geïnactiveerd, terwijl oncogenen worden geactiveerd. Hetzelfde geldt voor microRNAs en onderzoek heeft dan ook aangetoond dat er microRNAs zijn

met tumor onderdrukkende of oncogene activiteiten.

microRNAs binden niet volledig aan hun doelwit messenger RNAs en hierdoor is het erg moeilijk te bepalen welke messenger door welke microRNA wordt gereguleerd. Vandaar dat de ontdekkingen van nieuwe miRNAs ver voor liggen op het aantal beschreven doelwitten voor diezelfde microRNAs. Er bestaan inmiddels echter speciale computer algoritmen, die gebruik maken van de informatie verkregen uit reeds bekende microRNA-messenger RNA koppels. Deze algoritmen voorspellen welke messengers het doelwit kunnen zijn van een bepaalde microRNA en dat aantal kan oplopen tot over de 100. De algoritmen zijn gebaseerd op een aantal dezelfde maar ook een paar verschillende aanname's, waardoor ze deels overlappende maar ook deels andere output genereren. Worden alle voorspelde messengers gereguleerd door de microRNAs, zijn dat er slechts enkele, of zelfs geen? Om deze vragen te kunnen beantwoorden, en om zelf microRNAs te kunnen koppelen aan messenger RNAs, hebben we een eigen methode opgezet, waar in de volgende sectie dieper op wordt ingegaan.

Op zoek naar functionele microRNA-mRNA koppels

Eerst zijn we begonnen met het bouwen we een bibliotheek van al de tot dan toe bekende

microRNA genen. Ieder van de destijds 300 microRNA genen werd gecloneerd in een retrovirale vector, handig voor de aanmaak van microRNA dragend virus. Bovendien is deze vector uitermate geschikt voor de selectie van geïntegreerd virus na infectie van cellen middels een resistentie-gen. Zodra geïnfecteerde cellen resistent zijn, betekent dit dat ze kunstmatig de microRNA tot expressie brengen. Tegelijkertijd werden microRNA-microarrays gemaakt, glaasjes waarop ieder van de microRNAs uit de bibliotheek gefixeerd werden in kleine spotjes. De microRNA bibliotheek en microRNA-microarrays vormen twee handige tools voor het uitvoeren van zogenaamde genetische screens. Met het doen van microRNA genetische screens gaan we op zoek naar (het screenen) microRNAs (het genetische deel) die onder bepaalde gecontroleerde omstandigheden iets kunnen veroorzaken, of juist iets kunnen voorkomen. Als een microRNA tot zoiets in staat is, kunnen we dit zien, simpelweg omdat de microRNA afwijkend gedrag vertoont ten opzichte van alle andere microRNAs, die dit niet kunnen.

Hieronder volgt een voorbeeld van een screen setup (zie tevens hoofdstuk 2 Figuur 1, pagina 29).

De microRNA bibliotheek kan bijvoorbeeld tot expressie worden gebracht in een bepaalde cellijn die gevoelig is voor een specifiek medicijn. Door het medicijn stoppen de cellen met groeien. De vraag is dan of er een microRNA is die de cellen ongevoelig maakt voor dit medicijn, waardoor ze door kunnen blijven groeien. Stel voor het gemak dat iedere cel binnen de populatie geïnfecteerd is geraakt met één type microRNA en dat microRNA-X in staat is de cellen ongevoelig te maken voor de medicijn behandeling. Tijdens de behandeling zullen alle cellen die microRNA-X tot expressie brengen door blijven groeien, terwijl alle andere cellen stoppen met groeien. Vervolgens moet achterhaald worden dat microRNA-X degene is die resistentie geeft aan de cellen, en daar komt de microRNA-microarray van pas. De microRNAs van behandelde en niet-behandelde cellen worden uit de cellen geïsoleerd, en de relatieve aanwezigheid van ieder van de microRNAs wordt vergeleken op de microarray. Omdat tijdens de behandeling cellen met microRNA-X zijn blijven groeien, is er relatief meer van deze microRNA in de behandelde cellen ten opzichte van de onbehandelde cellen en dit komt naar voren met de microarray methode.

microRNA-372&373

In hoofdstuk 3 passen we de bibliotheek en mi-

croarray tools toe op het vinden van een microRNA die senescence kan voorkomen. Senescence is het verschijnsel waarbij cellen de capaciteit verliezen om te delen. Senescence kan verschillende oorzaken hebben en één daarvan is de continue aanwezigheid van een groei-stimulerend signaal (oncogene stress). Dit wordt door de cellen aangevoeld als iets abnormaals, waardoor een controle mechanisme (checkpoint) wordt geactiveerd. Dit checkpoint zorgt ervoor dat de cellen stoppen met groeien, ondanks blijvende groei-stimulatie. Dit proces, vanaf het registreren van een oncogeen signaal, tot en met de reactie daarop en uiteindelijke groeistop, wordt senescence genoemd. Senescence kan geïnduceerd worden in kweekcellen die functionele checkpoint pathways hebben, zoals primaire cellen. Als oncogeen werd RAS^{V12} gebruikt, een gemuteerde vorm van RAS, dat constitutief actief is. Introductie van RAS^{V12} in primaire cellen veroorzaakt senescence. Senescence kan doorbroken worden door onder andere inactivering van het checkpoint. Een belangrijk onderdeel van het checkpoint is de tumor suppressor p53 en zonder p53 gaan de cellen niet in senescence met RAS^{V12} expressie. Dit is belangrijk voor kanker, omdat cellen alleen door kunnen groeien als het checkpoint stil ligt. Daarom zijn we gaan screenen voor microRNAs die p53 kunnen vervangen en daardoor in de aanwezigheid van RAS^{V12} cellen kunnen laten doorgroeien. Primaire cellen werden geïnfecteerd met de microRNA bibliotheek, geselecteerd voor microRNA expressie en vervolgens werd één schaal cellen geïnfecteerd met RAS^{V12} voor de inductie van senescence en de andere schaal niet (controle cellen). Na een paar weken werd de aanwezigheid van ieder van de microRNAs, verkregen uit RAS^{V12} geïnfecteerde en controle cellen, vergeleken op de microarray. Zo werden miR-372&373 geïdentificeerd als microRNAs die verrijkt waren in de populatie van RAS^{V12} geïnfecteerde cellen. Ze kunnen alleen verrijkt zijn geraakt omdat ze de cellen waarin ze tot expressie kwamen lieten groeien.

Primaire cellen krijgen de kenmerken van een tumorcel na transformatie. Om cellen te laten transformeren moet aan een aantal eisen worden voldaan, zoals het onderdrukken van p53, retinoblastoma en pp2a expressie, alsmede constitutieve expressie van het telomerase enzym en RAS^{V12}. Als aan één van deze onderdelen niet wordt voldaan zullen primaire cellen niet transformeren en dus niet groeien in soft agar of uitgroeien tot tumoren in immuun onderdrukte muizen, zoals veel tumor cellijnen dat doen. Verdere analyse liet zien dat deze twee microRNAs het

onderdrukken van p53 overbodig maken in het transformatie model. Dit betekende dat er een mogelijk uniek scenario kon bestaan waarbij tumoren die miR-372&373 tot expressie brachten niet noodzakelijkerwijs gemuteerd of verminderd p53 hoefden te hebben, iets wat bij veel tumoren normaal het geval is. Tumoren die voldeden aan dit scenario waren de zogenaamde testiculaire kiemceltumoren die vallen op te splitsen in seminoma's en non-seminoma's, afhankelijk van het weefsel van oorsprong. De meeste seminoma's en een groot deel van de non-seminoma's brachten miR-372&373 tot expressie. Interessant was dat bijna al deze tumoren in het bezit waren van normaal functionerend p53, en dat non-seminoma's, die geen miR-372&373 expressie hadden, verminderde p53 expressie of gemuteerd p53 hadden. Cellen met normale p53 functie zouden moeten reageren op bestraling, wat DNA schade veroorzaakt. Dit komt overeen met de meeste testiculaire kiemceltumoren, en deze worden dan ook succesvol behandeld met bestraling.

Het enige wat overbleef was het doelwit van miR-372&373. Met behulp van een messenger RNA expressie array konden cellen die miR-372&373 tot expressie brachten worden vergeleken met controle cellen. microRNAs kunnen hun messenger RNA doelwitten afbreken of remmen in het doorgeven van de genetische informatie naar de eiwit machinerie. Alleen als microRNAs hun doelwit afbreken is dit te zien met een expressie array en dit gebeurde met het miR-372&373 doelwit LATS2, Large Tumor Suppressor 2. De 3'UTR van LATS2 heeft twee voorspelde bindingsplekken voor de microRNAs en door deze twee plekken te muteren was de 3'UTR resistent voor regulatie door de microRNAs. De afwezigheid van LATS2 kon tevens verklaren waarom de checkpoint functie van p53 niet voldoende werkte in cellen die miR-372&373 tot expressie brachten tijdens oncogene stimulatie met RAS^{V12}.

microRNA-221&222

In **hoofdstuk 4** hebben we, gebaseerd op de microRNA tools, een andere screenings methode bedacht. Hierbij begonnen we met een messenger RNA doelwit, dat als aas diende tijdens het screenen op microRNAs die dit doelwit kunnen reguleren. Om de regulatie te visualiseren, werd het doelwit gekoppeld aan GFP, een eiwit dat groen fluoresceert zodra het met een blauw licht wordt aangestraald. Cellen werden geïnfecteerd met een vector, waarin de 3'UTR van de cel cyclus remmer en tumor suppressor p27 was gecloneerd, gekoppeld aan GFP. Daarna

werden cellen geïnfecteerd met de microRNA bibliotheek. microRNAs die p27 reguleren zullen binden aan de 3'UTR van p27 en dit remmen zodat er minder p27 eiwit gemaakt wordt. GFP zit aan de 3'UTR van p27 vast, en fungeert als sensor. Als p27 door een microRNA omlaag gaat, kunnen we dat zien doordat het GFP signaal tegelijk omlaag gaat. Cellen met lage GFP expressie brengen de potentiële p27 microRNA tot expressie. Met behulp van de FACS konden cellen die lage niveau's van GFP tot expressie brachten gescheiden worden van de rest van de populatie cellen. Om de microRNA te identificeren die p27 kan remmen, werd de relatieve microRNA aanwezigheid van de laag-GFP cellen vergeleken met de totale populatie op de microarray. Hieruit bleek dat miR-221 verrijkt was in de populatie van cellen met laag GFP in vergelijking met de totale populatie. Daarna werd bevestigd dat miR-221, en familielid miR-222, beiden p27 kunnen reguleren en wel middels translationele repressie, wat wil zeggen dat de hoeveelheid p27 messenger RNA niet veranderde door de microRNAs, maar wel de hoeveelheid p27 eiwit. Door de door programma's voorspelde 2 microRNA-221 bindingssites in de 3'UTR van p27 te muteren kon worden aangetoond dat de microRNAs direct inspelen op p27 en afhankelijk zijn van deze 2 sequenties in het doelwit.

Vervolgens werd gezocht naar de functionele relevantie tussen p27 en miR-221&222, en of er een connectie bestond met kanker. Andere studies hadden al laten zien dat miR-221&222 hoger tot expressie komen in een aantal tumoren in vergelijking met omringend normaal weefsel. Eén van deze tumoren is de glioblastoma, een hersentumor. Glioblastoma cellijnen werden positief bevonden voor miR-221&222 expressie en de vraag was wat er zou gebeuren als de microRNA expressie zou worden geremd. p27 komt dan hoger tot expressie en als celcyclus remmer zou p27 een groeistop kunnen veroorzaken. De microRNA expressie werd geremd met kleine stukken RNA (zoals de microRNA) met een sequentie tegenovergesteld (complementair) aan de microRNA. Deze zogenaamde antagomiRs (zoals de term zegt, werkzaam tegen miRNAs) konden de microRNAs weghouden van wat ze normaal moeten doen, het remmen van p27. Inderdaad, p27 eiwit nam toe na het toedienen van deze microRNA-221&222 antagomiRs, wat leidde tot een celcyclus stop. Dat dit effect van microRNA remming puur afhankelijk was van p27 werd bewezen met een proef waarin cellen eerst werden onderdrukt in p27 eiwit productie, en daarna met antagomiRs werden behandeld. De

antagomiRs veroorzaken verhoging in p27 eiwit, maar dit wordt tegengewerkt door de eerdere onderdrukking van p27 productie. Daardoor hadden de cellen netto geen last van de antagomiRs en konden rustig verder groeien.

Tenslotte werd een poging gedaan om te zien hoe de microRNAs en p27 zich in werkelijkheid verhouden ten opzichte van elkaar. In tumor samples van glioblastoma patiënten vonden we de inverse correlatie van microRNAs en p27 terug. In de tumoren was microRNA-221&222 expressie hoog en p27 eiwit laag tot afwezig, terwijl in naastgelegen gezond weefsel de microRNA expressie laag was, met hoge niveau's van p27 eiwit.

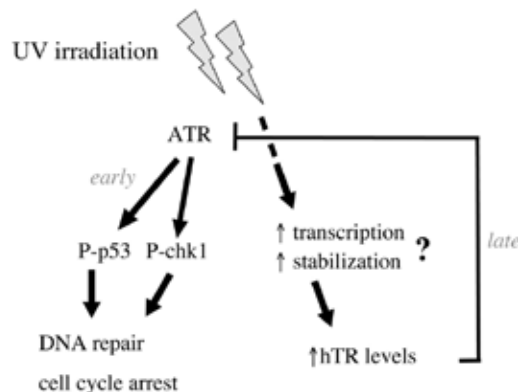
Interessant om te vermelden is het feit dat deze screening methode ook microRNA's heeft geïdentificeerd voor andere doelwitten, zoals de tumor suppressors APC (adenomatosis polyposis coli) en p16Ink4a. Op het moment wordt aan deze projecten gewerkt.

Concluderend laten we zien dat we met de microRNA bibliotheek en de microRNA microarray twee tools hebben vervaardigd voor het uitvoeren van genetische screens met als doel het vinden van een functie en een messenger RNA doelwit voor microRNAs.

In **hoofdstuk 5** ten slotte onderzoeken we een nieuwe functie voor het niet coderende RNA hTR

(human Telomerase RNA). hTR is de RNA component van het telomerase RNA-eiwit. Telomerase is belangrijk bij de aanmaak en bescherming van de chromosoom uiteinden, de telomeren. Bij iedere verdubbeling van het DNA zorgen DNA polymerasen voor de aanmaak van de nieuwe DNA strengen. Echter, zodra een DNA polymerase aan het uiteinde van een chromosoom begint, kan deze de laatste paar nucleotiden niet verdubbelen, simpelweg omdat de DNA polymerase zelf die ruimte inneemt. Het gevolg is dat een aantal nucleotiden daarmee verloren gaan, en dus de chromosoom uiteinden korter worden (erosie). Telomerase gaat dit tegen. De telomeren worden gevormd door een keten van herhaalde sequenties, die door telomerase worden herkend, en worden uitgebreid zodra verkorting optreedt. Dat telomerase deze sequenties kan herkennen dankt het aan hTR. Veel meer dan deze functie is niet bekend van hTR, behalve dat tumoren vaak verhoogde expressie van hTR laten zien. Hierbij komt hTR hoger tot expressie dan normaal, zonder dat het de enzymactiviteit van telomerase beïnvloedt.

In deze studie laten we de betrokkenheid van hTR in het moduleren van de respons op DNA schade zien, een nieuwe functie van hTR. Zodra hTR RNA expressie wordt geremd met behulp van RNAi technologie, stoppen cellen met groeien. Dit komt door een snelle activatie van de checkpoint eiwitten ATR, Chk1 en p53. De groeistop kan dan ook voorkomen worden



Figuur 2. Model van de negatieve terugkoppeling in de DNA schade respons.

door naast hTR verlaging, tevens Chk1 of p53 te verminderen middels RNAi. Dit suggereert dat zonder hTR, de telomeer beschermende functie van telomerase wegvalt, wat leidt tot extra snelle chromosoom erosie. De cel registreert dit en activeert een groei-remmende respons, de checkpoint respons.

Omgekeerd kan kunstmatige overexpressie van hTR de checkpoint activatie onderdrukken. Dit kan worden aangetoond door cellen te bestralen met UV, waar onder normale omstandigheden ATR wordt geactiveerd als respons op DNA schade. Vervolgens worden Chk1 en p53 geactiveerd en dit leidt tot een groeistop, totdat de cellen de schade hebben gerepareerd. Echter, hTR overexpressie kan ATR activatie voorkomen en dus een groeistop, die nodig is voor DNA herstel, verhinderen.

UV bestraling activeert de ATR/Chk1/p53 pathway binnen een aantal minuten, maar veroorzaakt tevens een verhoging in de hTR expressie. Echter gebeurt dit pas na ongeveer 5 uur na de bestraling. Samenvattend leidt dit tot een negatieve terugkoppeling waarbij na UV bestraling de cellen eerst stoppen met groeien, veroorzaakt door de ATR checkpoint pathway (vroeg respons), waarna hTR opkomt om diezelfde groeistop te onderdrukken (late respons; Figuur 2).

Curriculum Vitae

Carlos le Sage werd geboren op 16 juni 1979 te Sluiskil. Na het behalen van zijn VWO diploma in 1997 aan de Stedelijke Scholengemeenschap De Rede te Terneuzen, begon hij in datzelfde jaar de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. Tijdens deze studie liep hij zijn eerste stage bij de vakgroep Moleculaire Celbiologie en Immunologie onder begeleiding van Dr. H. Honing en Dr. T.K. van den Berg aan de Vrije Universiteit. Hier werd de negatieve invloed van de transmembrane glycoproteïne receptor SIRP α op receptor tyrosine kinase-gekoppelde pathways in bloedcellen onderzocht. De tweede stage werd gelopen bij de afdeling Tumor Biologie in het Nederlands Kanker Instituut. Onder begeleiding van Dr. I. Jordens en Prof. Dr. J.J. Neefjes werd bestudeerd of melanosoom localisatie/transport afhankelijk is van de opeenvolgende werking van twee GTPases, Rab7 en Rab27a, die

ieder een deel van de melanosoom maturatie en localisatie op zich nemen.

De studie Medische Biologie werd in januari 2002 afgerond, waarna hij als promovendus begon op de afdeling Tumor Biologie onder begeleiding van Dr. R. Agami. Hier onderzocht hij de functionele implicaties van het verwijderen van checkpoint eiwitten voor het induceren van de DNA damage respons. Daarnaast werd gekeken of het mechanisme waardoor de levensduur van het cycline D1 eiwit drastisch wordt verkort na ioniserende bestraling kon worden verklaard. Uiteindelijk werd de juiste koers bepaald met de aanmaak van een microRNA library, en voortvloeiend onderzoek gericht op het vinden van betrokkenheid van deze niet-coderende RNAs in kanker.

Na de promotie blijft hij voor als nog als post-doc in de groep van Dr. R. Agami verbonden aan het NKI-AvL.

List of Publications

Perspectives

Diverse ways to control p27^{Kip1} function: miRNAs come into play

C. le Sage*, R. Nagel* and R. Agami

Cell Cycle 6 (22): (November 2007)

Immense promises for tiny molecules: uncovering miRNA functions

C. le Sage and R. Agami

Cell Cycle 5 (13): 1415 - 1421 (July 2006)

Articles

Regulation of the p27^{Kip1} tumor suppressor by miRNA-221 and miRNA-222 promotes cancer cell proliferation

C. le Sage*, R. Nagel*, D. Egan, M. Schrier, E. Mesman, A. Mangiola, C. Anile, G. Maira, N. Mercatelli, S. Ciafre, M. Farace, R. Agami.

EMBO Journal 26 (15): 3699 - 3708 (July 2007)

Telomerase-independent regulation of ATR by human telomerase RNA

M. Kedde, **C. le Sage**, A. Duursma, E. Zlotorynski, B. van Leeuwen, W. Nijkamp, R. Beijersbergen, R. Agami

Journal of Biological Chemistry 281 (52): 40503 - 40514 (December 2006)

A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors

M. Voorhoeve*, **C. le Sage***, M. Schrier, A. Gillis, H. Stoop, R. Nagel, Y.

Liu, J. van Duijse, J. Drost, A. Griekspoor, E. Zlotorynski, N. Yabuta, G. de Vita, H. Nojima, L. Looijenga, R. Agami

Cell 124 (6): 1169 - 1181 (March 2006)

Homologous recombination and nonhomologous end-joining repair pathways regulate fragile site stability

M. Schwartz, E. Zlotorynski, M. Goldberg, E. Ozeri, A. Rahat, **C. le Sage**, B. Chen, D. Chen, R. Agami, and B. Kerem

Genes & Development 19 (22): 2715 - 2726 (November 2005)

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