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miR-221 and miR-222 Expression Affects the Proliferation Potential of Human Prostate Carcinoma Cell Lines by Targeting $p27^{Kip1}$*

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MicroRNAs are short regulatory RNAs that negatively modulate protein expression at a post-transcriptional level and are deeply involved in the pathogenesis of several types of cancers. Here we show that miR-221 and miR-222, encoded in tandem on chromosome X, are overexpressed in the PC3 cellular model of aggressive prostate carcinoma, as compared with LNCaP and 22R*v*1 cell line models of slowly growing carcinomas. In all cell lines tested, we show an inverse relationship between the expression of miR-221 and miR-222 and the cell cycle inhibitor $p27^{Kip1}$. We recognize two target sites for the microRNAs in the 3′ untranslated region of p27 mRNA, and we show that miR-221/222 ectopic overexpression directly results in p27 down-regulation in LNCaP cells. In those cells, we demonstrate that the ectopic overexpression of miR-221/222 strongly affects their growth potential by inducing a G_1 to S shift in the cell cycle and is sufficient to induce a powerful enhancement of their colony-forming potential in soft agar. Consistently, miR-221 and miR-222 knock-down through antisense LNA oligonucleotides increases $p27^{Kip1}$ in PC3 cells and strongly reduces their clonogenicity *in vitro*. Our results suggest that miR-221/222 can be regarded as a new family of oncogenes, directly targeting the tumor suppressor $p27^{Kip1}$, and that their overexpression might be one of the factors contributing to the oncogenesis and progression of prostate carcinoma through $p27^{Kip1}$ down-regulation.

MicroRNAs (miRNAs)[5] are a wide class of small, noncoding RNAs that negatively regulate protein expression at the post-transcriptional level. Through the specific targeting of the 3′ UTRs of multicellular eukaryotic mRNAs, miRNAs down-regulate gene expression by either inducing degradation of target mRNAs or impairing their translation (1, 2). The expression of many microRNAs was shown to be temporally and spatially regulated, whereas the disruption of their physiological expression patterns was associated with several examples of human tumorigenesis, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes (3). In fact, single or small sets of microRNAs were demonstrated to be dysregulated in diverse cancer subtypes including Burkitt lymphoma (4), colorectal cancer (5), lung cancer (6), breast cancer (7), papillary thyroid carcinoma (8), hepatocellular carcinoma (9), and glioblastoma (10, 11). The general rule stemming from these studies is that the non-physiological modulation of micro-RNA expression frequently characterizes cancer, thus making the comprehension of microRNA expression an important goal for diagnostic and prognostic applications, especially when this knowledge is further strengthened by the discovery of the molecular targets specifically modulated by microRNAs.

Prostate cancer, the most common malignant disease in the Western world, causes about 80,000 deaths a year in Europe (12). Despite considerable efforts made in recent years to understand prostate tumorigenesis, the molecular mechanisms involved in its initiation and progression remain largely unknown. Among factors whose misregulation was tightly linked to prostate cancer (PCa) progression, the cyclin-dependent kinase inhibitor $p27^{Kip1}$ is a well established marker of poor prognosis as it was shown that absent or decreased $p27^{Kip1}$ expression is associated with high tumor grade and poor prognosis of PCa and of several other human cancers (13–18). It is also well known that $p27^{Kip1}$ regulation, both in physiological and pathological conditions, is exerted mostly at a post-transcriptional level (19).

In this work we describe the differential expression of two microRNAs, miR-221 and miR-222, encoded in tandem from a gene cluster located on chromosome X, in three human prostate carcinoma cell lines, the androgen-independent, strongly aggressive PC3 cell line, the androgen-responsive 22R*v*1, and the androgen dependent, slowly growing LNCaP, which represent models of distinct stages of prostate carcinoma progression. Consistently with the proposed role of microRNAs as regulators of key components of cell cycle progression, here we

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[5] The abbreviations used are: miRNA, microRNA; UTR, untranslated region; siRNA, small interfering RNA; PCa, prostate cancer; MTS, 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2h-tetrazolium, inner salt.

identify $p27^{Kip1}$ as a target for miR-221/miR-222. We show that $p27^{Kip1}$ expression in the three PCa cell lines inversely correlates with that of miR-221/miR-222, and that the ectopic overexpression of miR-221 or both microRNAs in LNCaP, where they are normally almost undetectable, has deep consequences on the proliferation rate and the cell cycle phase distribution. We propose that $p27^{Kip1}$ is an important functional target for miR-221/222 in prostate carcinoma, and that the modulation of these microRNAs might be used as a molecular marker to characterize the progression of this tumor.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfections—All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM L-glutamine, 100 units/ml of penicillin G sodium, and 100 μg/ml streptomycin sulfate in a humidified atmosphere containing 5% CO_2 at 37 °C.

Transfections were performed by Lipofectamine 2000 reagent (Invitrogen) using 8 μg of plasmid DNA in Opti-MEM I (Invitrogen), as recommended by the manufacturer. For transient transfections, 7 μg of pCDNA(+)3.1-based plasmids were co-transfected with a 1:7 relative amount of a reporter plasmid, pEGFP-C3 (Clontech), to monitor transfection efficiency. Cells were analyzed by fluorescence microscopy 48 h after transfection to calculate the transfection efficiency. For each well, the cell number in four random microscopic fields was counted and transfection efficiency for each sample was estimated as the mean value of green fluorescent protein expressing cells over 100 cells per field. When establishing stable transfectants, the transfected cells were selected by adding 0.4 mg/ml G418 to the culture medium.

Assay of Luciferase Activity—The 3′ UTR of the human $p27^{kip1}$ gene was PCR amplified using primers 5′-cagctcgaattaagaatatg-3′ and 5′-gtgtaacaataattggcatc-3′, and cloned downstream of the *Renilla* luciferase stop codon in pRLTK vector (Rl-luc, Promega), giving rise to the p3′UTR-p27 plasmid. This construct was used to generate, by inverse PCR, the p3′UTRmut-p27 plasmid (primers: reverse mut1269, 5′-aaccacccaacgcttttagaggcagatc-3′, forward 1270, 5′-cattatgcaattaggtttcc-3′, reverse 1331, 5′-ggtaaaactatatacacagg-3′, forward mut1336, 5′-ggaggttcacataaactttggggaagg-3′). LNCaP cells were transfected by Lipofectamine 2000 (Invitrogen) with p3′UTR-p27 or p3′UTRmut-p27 plasmid plus pGL3 control vector (Pp-luc, Promega). Cells were harvested 48 h post-transfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

Plasmid Constructs—The miR-221, miR-222, and miR-222/221 cluster sequences were amplified by PCR from genomic human DNA using the following primers: miR-221 sense, 5′-cgagatctgtgagaattacttgcaagctg-3′; miR-221 antisense, 5′-ccgctcgagcattggtgagacagccaatg-3′; miR-222 sense, 5′-cgcagatcttttcttccacagagcccctcc-3′; miR-222 antisense, 5′-ggggatcctctcaggacactgaagcaga-3′; miR-222/221 sense, 5′-cgcagatcttttcttccacagagcccctcc-3′; miR-222/221 antisense, 5′-gctcgaggcggtcctttctctgcactct-3′.

The correct sequences of amplified products were verified by sequencing and cloned into the BamHI site of pCDNA(+)3.1 vector (Invitrogen) for miR-222, and BamHI-XhoI sites for miR-221 and miR-222/221. Their expression was detected by Northern blot analysis after 48 h from transfection in LNCaP and 22R*v*-1 cells lines.

miRNA and p27 Knockdown—Fluorescein isothiocyanate-labeled LNA oligonucleotides against miR-221 and miR-222 were obtained from Exiqon. Knockdown oligos were transfected by Lipofectamine 2000 (Invitrogen) into PC3 cells at a final concentration of 40 nM. After 24 h, the cells were collected and miRNAs and p27 protein levels analyzed. Before loading onto 7 M urea polyacrylamide gels, RNA samples were denatured for 5 min at 65 °C in 3.5 M urea. SMARTpool siRNA against p27 were obtained from Dharmacon and transfected by Lipofectamine 2000 into LNCaP cells at a final concentration of 100 nM.

Cell Viability Assay—Cell viability was measured by using Promega's Cell Titer Aqueous assay with MTS tetrazolium, in which viable cells convert MTS tetrazolium into a formazan-colored product ($OD_{490\ nm}$). 2,500 cells/well were seeded in a 96-well plate and incubated for 24 h before treatment to let them attach to the bottom of the well. The cell growth was then measured at 48 and 96 h after the cellular adhesion. Following the manufacturer's instructions, 20 μl of MTS solution were added to 100 μl of culture media and incubated for 1 h at 37 °C and the optical density was measured at 490 nm. Five independent experiments were performed in quadruplicate.

Transfection of miR-222 into Anti-p27 siRNA-treated LNCaP Cells and Cell Viability Assay—1×10^4 cells/well were seeded in a 96-well plate, incubated for 24 h to let them attach to the bottom of the well, and then transfected with 80 nM anti-p27 SMARTpool siRNAs or control siRNAs (Dharmacon) using Oligofectamine (Invitrogen). 24 h after this first transfection, cells were transfected again with the appropriate combination of anti-p27 siRNA or control siRNA, mixed with 100 nM synthetic miR-222 (Exiqon) or a control microRNA. Thus, in this experimental setting, reported in Fig. 4*C*, time 0 corresponds to the second transfection with siRNA + miRNA, and cell growth was measured by MTS from that time on, at 24, 48, and 72 h.

Soft Agar Colony Assay—Anchorage-independent growth was determined by soft agar analysis as follows: 2.5×10^3 LNCaP or PC3 cells per 35-mm dish were seeded in 0.35% agar on top of a base layer containing 0.5% agar. In the assays involving anti-p27 siRNAs or anti-miR-221 or anti-miR-222 LNA oligos, cells were plated 24 h after transfection. Plates were incubated at 37 °C at 5% CO_2 in a humidified incubator for 2 weeks and stained with 0.005% crystal violet for 1 h. Colonies >0.1 mm (for LNCaP cells) or >0.15 mm (for PC3 cells) in diameter were counted under a microscopic field at ×10 magnifications. Each assay was performed in triplicate on two independent occasions.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from PC-3, LNCaP, and 22R*v*-1 cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For Northern blot analysis of miRNAs, 15 μg of total RNA were separated on 10% denaturing polyacrylamide gels and electrotransferred to Immobilon Nylon$^+$ membrane (Millipore Corporation). The specific probes, end-labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP, were: miR-221, 5′-gaaacccagcagacaatgtagc-3′; miR-222, 5′-gagacccagtagccagat-3′; and U6, 5′-cacgaatttgcgtgtcatccttgcgcaggggcc-3′. Bands

were quantified with ImageJ 1.34s or OptiQuant 3.1 Packard Instrument software.

Bromodeoxyuridine Incorporation/FACS Analysis—5×10^5 transfected cells were plated in 60-mm dishes and incubated at 37 °C for 48 h. Cells were then pulsed with 10 μM 5-bromodeoxyuridine (Sigma) for 30 min, harvested with trypsin, washed, and incubated for 30 min at room temperature with 2 M HCl to denature DNA into single-stranded molecules. After washing in borax buffer (0.1 M sodium tetraborate, pH 8.5), cells were incubated with 0.65 μg/ml anti-bromodeoxyuridine antibody (BD Biosciences) for 1 h at room temperature. Then cells were resuspended in 200 μl of washing buffer and labeled with 5 μl of anti-mouse fluorescein isothiocyanate-conjugated antibody (Calbiochem) for 30 min in the dark. Labeled cells were washed and resuspended in phosphate-buffered saline containing 10 μg/ml propidium iodide for 25 min before flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences) using "Cell Quest" software. For anti-p27 assay, 5×10^5 cells were transfected and incubated at 37 °C. After 72 h, cells were harvested and fixed in ice-cold 70% ethanol, washed in phosphate-buffered saline, and then treated with 10 μg/ml propidium iodide and 50 μg/ml RNase A for 25 min at 37 °C. All assays were performed 3 times.

Immunoblot Analysis—For each of three independent experiments, 20–40 μg of total protein extract was separated on 12% SDS-PAGE gels and transferred to nitrocellulose membrane. The levels of p27 expression were evaluated by using the rabbit polyclonal anti-p27 antibody (AB3003; Chemicon) or the monoclonal anti-p27 antibody (610241; BD Biosciences). As a loading control, β-actin expression levels were measured by rabbit polyclonal anti-actin antibody (A2066 Sigma). The secondary horseradish peroxidase-conjugated antibody (AP132P or AP160P; Chemicon) was detected using ECL Plus Western blotting detection reagents (Amersham Biosciences). Bands were quantified with ImageJ 1.34 or OptiQuant 3.1 Packard Instrument software.

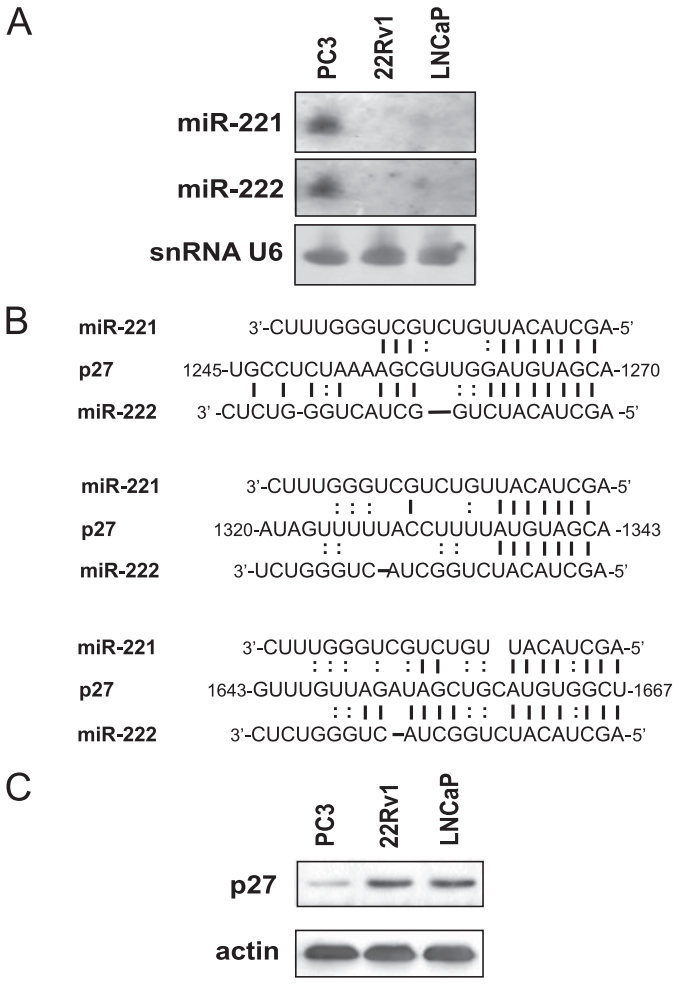


FIGURE 1. **Expression profile of miR-221, miR-222, and p27 proteins in prostate carcinoma cell lines.** *A*, Northern blot of total RNA from PC3, 22Rv1, and LNCaP cells was probed for miR-221 and miR-222. Small nuclear RNA (*snRNA*) U6 is included as a loading control. *B*, p27 mRNA 3′ UTR putative sites targeted by miR-221 and miR-222. *C*, total proteins, from the same sample set of cells utilized for the Northern blot, were analyzed by Western blot with anti-p27 antibodies. Western blot analysis of β-actin was performed as a loading control.

RESULTS

MicroRNAs miR-221 and miR-222 Are Expressed in PC3 Cells but Are Absent in LNCaP and 22Rv1 and Their Expression Is Inversely Linked to That of p27^{Kip}—Our experimental goal was to search for microRNAs differentially expressed in prostate carcinoma. To this aim, we studied three human PCa cell lines, PC3, LNCaP, and 22R*v*1, virtually representing three stages of prostate carcinoma progression. This study, performed by Northern blot analysis, revealed that the microRNAs miR-221 and miR-222 show a differential expression: they are easily detectable in PC3 cells, deriving from a distal metastasis of an androgen-independent, highly aggressive tumor, whereas they are almost absent in LNCaP, derived from a local lymph node metastasis of an androgen-dependent, slowly growing carcinoma (Fig. 1*A*). The expression in the 22R*v*1 cell line, an androgen-responsive primary cell line, was nearly undetectable, like in LNCaP (Fig. 1*A*).

We performed a bioinformatic search (20) for putative target mRNAs of both miRNAs, and we found that the 3′ UTR of human p27^{kip1} harbors two sites likely recognized by the miR-221 and miR-222 (Fig. 1*B*). In fact, the 5′ "seed" regions of these two miRNAs are identical, and theoretically target the same sites, located at nucleotides 1262–1269 and 1336–1342 of the p27^{kip1} mRNA (NM_004064.0). Moreover, another putative site matching miR-222 and miR-221 is recognizable at nucleotides 1660–1667 in the 3′ UTR of p27 mRNA, where it is also associated with additional flanking matches (Fig. 1*B*).

To experimentally validate the possible relationship inversely linking p27^{Kip1} and miR-221 and miR-222, we started by studying p27 expression levels in the same three prostate carcinoma cell lines: Western blot analysis showed that p27 is clearly detectable in LNCaP and 22R*v*1, whereas it is strongly reduced in PC3 cells (Fig. 1*C*). The whole of these results gave us a first hint that the expression of miR-221 and miR-222 might be one of the mechanisms acting to negatively regulate p27^{Kip1} in prostate carcinoma cells.

The Overexpression of miR-221, miR-222, or miR-221/miR-222 Reduces p27^{Kip1} Expression in LNCaP and 22Rv1 Cells and the Knock-down of the Same MicroRNAs Increases p27^{Kip1} in PC3 Cells—To check if these microRNAs actually affect p27^{Kip1} expression in LNCaP cellular environment, we analyzed the

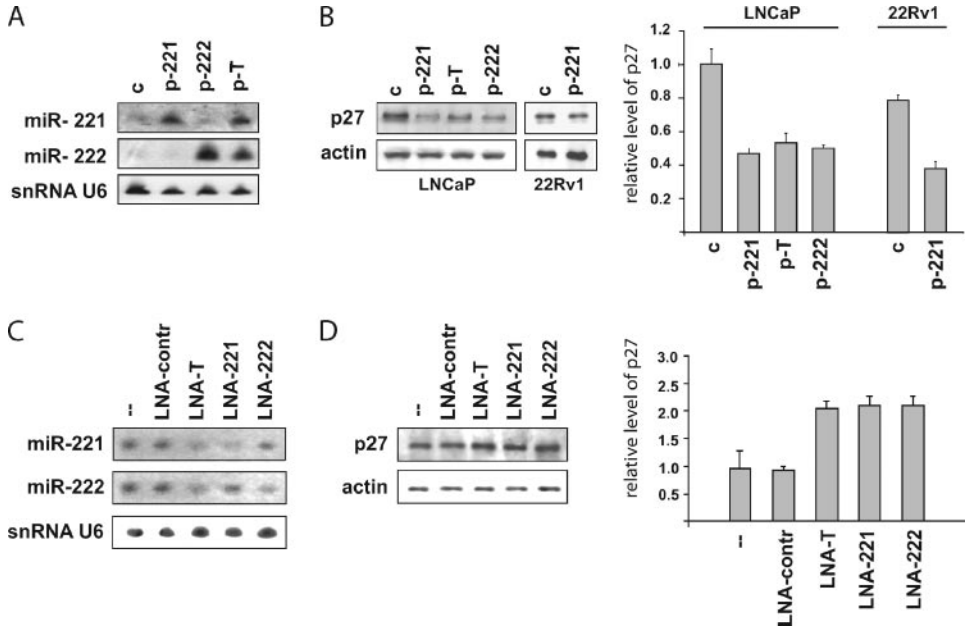


FIGURE 2. **Ectopic expression and knockdown of miR-221 and miR-222.** *A*, Northern blot analysis of total RNA extracted from LNCaP transfected with the empty vector (*c*), and plasmids p-221, p-222, or p-T. Hybridization to the U6 small nuclear RNA (*snRNA*) is shown as a loading control. *B*, Western blot analysis of p27. Total proteins were extracted from the same LNCaP cells utilized in *A* and from 22R*v*1 cells transfected with the empty vector (*c*) or p-221. Analysis of β-actin was performed as a loading control. *C*, Northern blot hybridizations of total RNA extracted from non-transfected PC3 cells (−), or PC3 cells transfected with LNA oligos against an *Arabidopsis thaliana* miRNA (*LNA-contr*), miR-221 (*LNA-221*), miR-222 (*LNA-222*), or both miR-221 and miR-222 (*LNA-T*). *D*, total proteins from the same sample set of cells utilized for *C* were analyzed by Western blot with anti-p27 antibodies. Relative signal intensities for p27 in the Western blots normalized to the β-actin loading control, are included for comparison of p27 levels. *Error bars* represent S.D. and were obtained from three independent experiments.

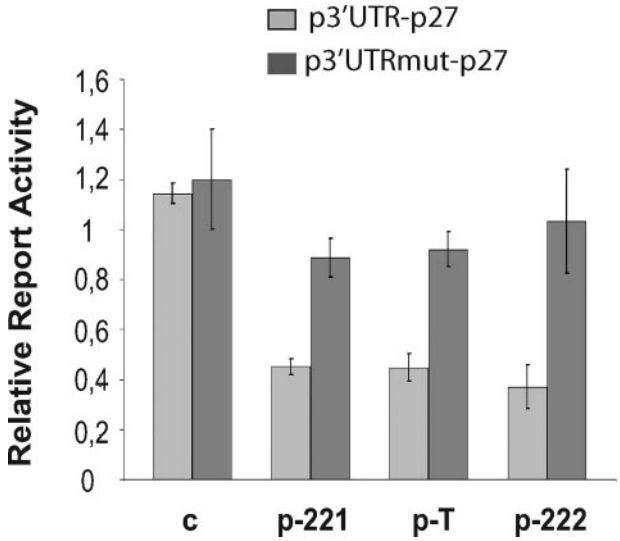


FIGURE 3. **p27 3′ UTR is a target of miR-221 and miR-222.** p3′UTR-p27 or p3′UTRmut-p27 luciferase constructs containing a wild-type (*light gray columns*) or a mutated (*dark gray columns*) p27 3′ UTR were transfected into LNCaP cells stably transduced with empty vector (*c*), or plasmids p-221, p-T, or p-222. Luciferase activity was determined 48 h after transfection. The ratio of normalized sensor to control luciferase activity is shown. *Error bars* represent S.D. and were obtained from three independent experiments.

consequences of the ectopic expression of miR-221 and miR-222. We made three constructs, p-221, p-222, and p-T, containing, respectively, miR-221, miR-222, or the whole genomic region with miR-221 and miR-222 encoded in tandem, under the control of cytomegalovirus promoter. Fig. 2*A* shows that LNCaP cells transduced with p-221, p-222, or p-T clearly expressed high levels of the expected mature microRNAs, whereas very little expression was detected in control-transduced cells. Similar results were obtained with the 22R*v*1 cell line (data not shown). Notably, no pre-miRNA accumulation was detected in all cases, indicating efficient processing of the ectopically expressed miRNAs in the cells. A Western blot performed on the same cells (Fig. 2*B*) shows that the $p27^{Kip1}$ protein was clearly reduced (50%) in both LNCaP and 22R*v*1 cells expressing the single miRNAs or a combination of both, as compared with cells transfected with the empty vector. These data strongly support the hypothesis that miR-221 and miR-222 post-transcriptionally regulate $p27^{Kip1}$ expression in LNCaP and 22R*v*1 cells.

To study the biological significance of these miRNAs in PC3 cells, where they are normally expressed, we knocked down miR-221 or miR-222 expression. To do this, we transfected PC3 cells with LNA antisense oligonucleotides targeting either miR-221 or miR-222, and we analyzed the effects on p27 production. PC3 cells transfected with anti-221 and anti-222 LNA showed a 2-fold decrease in miR-221 and miR-222 levels when compared with control cells transfected with LNA against a microRNA not expressed in these cells (Fig. 2*C*). As expected, the reduction of miR-221 or miR-222 was accompanied by an increase of p27 protein of about 2-fold (Fig. 2*D*). The results that we collected by both the overexpression and the knock down of miR-221 and/or miR-222 converge toward the identification of p27 as an actual target of miR-221/miR-222 in LNCaP, 22Rv1, and PC3 PCa cell lines.

The Insertion of the 3′ UTR of p27 mRNA Downstream of a Luciferase Reporter Gene Confers Responsiveness to miR-221 and miR-222—To show that the 3′ UTR of p27 mRNA actually contains matching sites for the interaction with miR-221 and miR-222 and is likely responsible for the effect of these miRNAs on p27 expression, we cloned the whole 3′ UTR region downstream of the luciferase open reading frame, and we used this reporter construct to transfect LNCaP cells. The same luciferase-3′UTR reporter construct was transfected into LNCaP cells stably transfected with plasmids p-221, p-222, or p-T. Fig. 3 shows that the presence of miR-221, miR-222, or miR-221 and miR-222 in tandem strongly affected luciferase expression (>50%), measured as relative luciferase activity. The reduction of luciferase activity was totally comparable in all transfected cell lines, either expressing miR-221 or miR-222, or a combina-

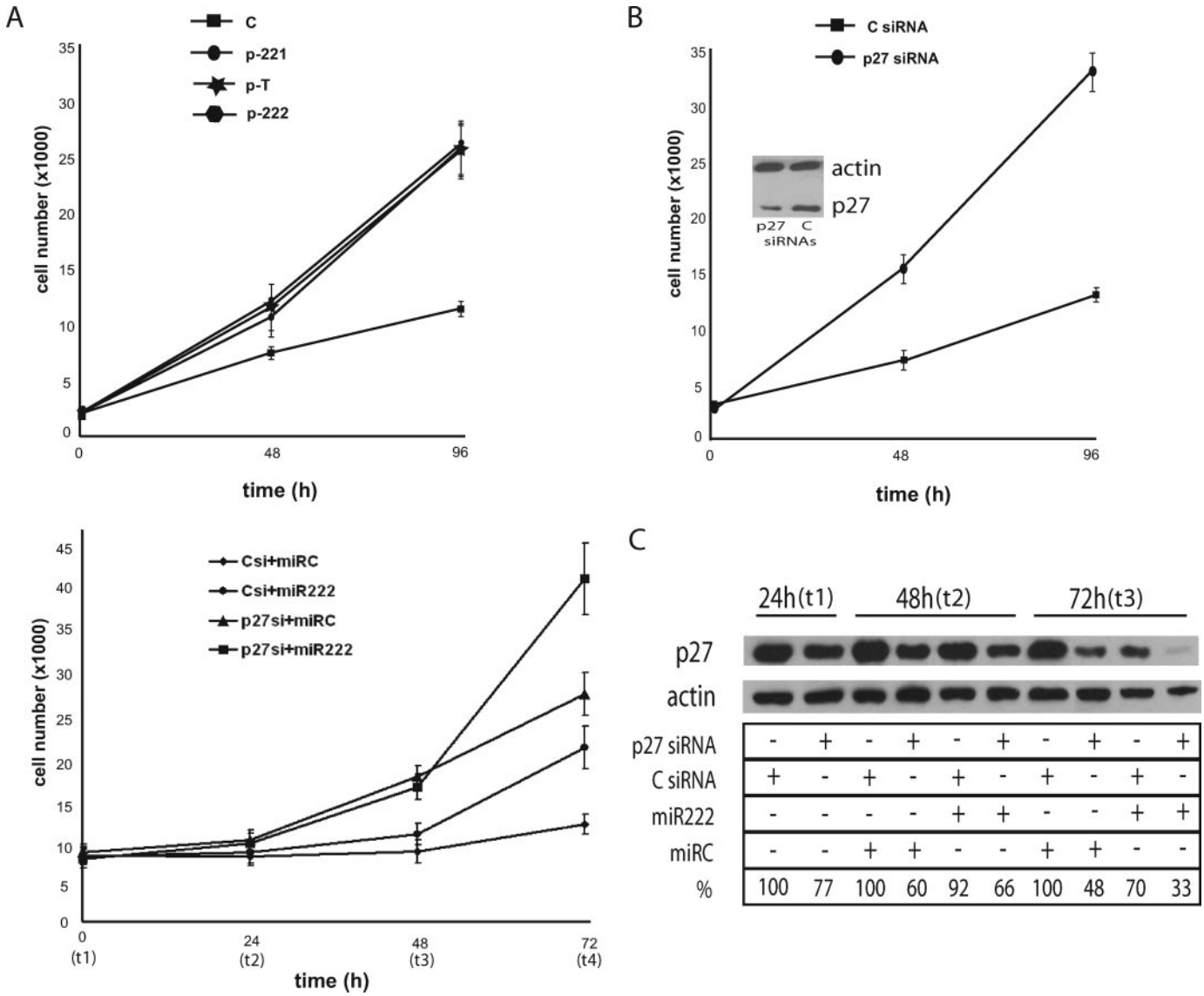
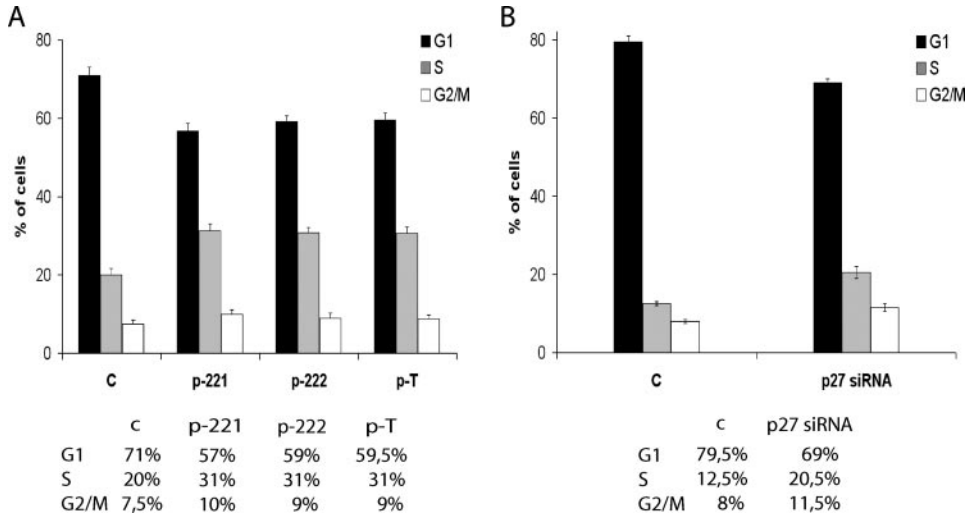


FIGURE 4. **Ectopic expression of miR-221 and miR-222 affects the growth of LNCaP cells by targeting p27.** *A*, growth curves of LNCaP cells transfected with the empty vector (*c*), and plasmids p221, p-T, or p-222, and analyzed after 48 and 96 h. Data represent the mean ± S.D. from five independent experiments. *B*, growth curves of LNCaP cells transfected with siRNAs against p27 or a siRNA negative control (*c siRNA*), and analyzed after 48 and 96 h. Data represent the mean ± S.D. from five independent experiments. The *inset* shows a representative Western blot analysis of p27 protein levels after anti-p27 (*p27*) or control (*c*) siRNA transfection. *C*, *left panel*, growth curves of LNCaP cells transfected with anti-p27 siRNAs, incubated 24 h in the presence of anti-p27 siRNA, and then re-transfected with one of the described combinations of microRNA and siRNA. Time 0 corresponds to the time of the second transfection with microRNAs + siRNAs and, for this reason, is also indicated as *t1*. Data represent the mean ± S.D. from three independent experiments. *Right panel*, Western blot of p27 in LNCaP cells at 24, 48, and 72 h after the first anti-p27 siRNA transfection, corresponding, respectively, to time 0, 24, and 48 h from the transfection with microRNAs + siRNAs, as depicted in the *left panel*. In both *left* and *right panels*, *t1*, *t2*, and *t3* identify the matching time points of the procedures. The *lower table* schematically resumes the type of combined molecules used to transfect cells, and the relative amount of p27 compared with control-treated LNCaP cells (*C siRNA* + *miR C*), set as 100%. Western blot analysis of β-actin is shown as a loading control. Abbreviations used are: *p27 si*, anti-p27 siRNA; *Csi*, control siRNA; *miRC*, control miRNA.

tion of miR-221 and miR-222. On the contrary, when we used, as a reporter construct, a plasmid harboring the 3′ UTR of p27 mRNA where two core binding sites for miR-221 and miR-222 were inactivated by site-directed mutagenesis, we observed only a very slight effect on luciferase activity, compared with LNCaP cells that did not express any microRNA. These results support the bioinformatic prediction indicating the 3′ UTR of p27 mRNA as a target for miR-221 and miR-222, and denote that the two matching sites thus identified strongly contribute to the miRNA-mRNA interaction mediating the post-transcriptional inhibition of the expression.

The Ectopic Expression of miR-221/222 Enhances Growth of LNCaP Cells and Induces a Progression to the S Phase of Cell Cycle—p27^{Kip1} is known to play a key role as a regulator of cell cycle progression, strongly inhibiting G_1/S transition. We tested if the cell growth potential of stably transfected LNCaP cells expressing miR-221, miR-222, or both miR-221 and miR-222 was modified, as a consequence of the demonstrated p27 reduction. Fig. 4*A* shows the results of an MTS assay where cell viability of empty vector-transfected LNCaP cells is compared with that of the microRNA-expressing cells: the expression of the microRNAs induced a marked increase in growth rate



	c	p-221	p-222	p-T
G1	71%	57%	59%	59,5%
S	20%	31%	31%	31%
G2/M	7,5%	10%	9%	9%

	c	p27 siRNA
G1	79,5%	69%
S	12,5%	20,5%
G2/M	8%	11,5%

FIGURE 5. **Ectopic expression of miR-221 and miR-222 affects the cell cycle distribution of LNCaP cells.** Flow cytometric distributions of LNCaP cells transfected with empty vector (*c*), and p-221, p-222, or p-T (*A*), or transfected with the anti-p27 siRNAs (*B*). Data represent the mean ± S.D. from three independent experiments.

(more than 2-fold at 96 h after the start of the experiment), both in the presence of miR-221 or miR-222 alone, or the two microRNAs in tandem. These results found a parallel confirmation in a similar experiment we performed to show that a reduction of p27 dosage by means different from microRNA expression leads to analogous outcomes: when we transfected LNCaP cells with anti-p27 siRNAs that were able to reduce p27 of about 60% (Fig. 4*B*, *inset*), we observed a sharp increase in cell growth, as compared with control siRNA-transfected cells (Fig. 4*B*). Thus, reducing p27 levels in LNCaP cells, either by miR-221/222 expression or by anti-p27 siRNA transduction, is sufficient to induce a comparable cell growth increase.

To strongly prove the p27 dependence of these effects of miR-221/222, we set up a different experiment, where we measured the proliferation variations induced by microRNAs in LNCaP cells previously transfected with anti-p27 siRNAs. The aim of this experiment was to study if and how the p27-depleted cellular environment responds to miR-221 or miR-222 addition. When one of the microRNAs, miR-222, was transfected into LNCaP cells previously treated with anti-p27 siRNAs, we observed that, after 48 h from miR-222 transfection, anti-p27 siRNA and miR-222 seemed to co-operate to the reduction of p27 protein (Fig. 4*C*, *right panel*), and consistently to additively enhance the growth rate. This latter effect is clearly observed at 72 h, when the functional consequences of p27 depletion are expected (Fig. 4*C*, *left panel*).

We then investigated LNCaP cell cycle phase distribution through flow cytometric analysis. This analysis revealed that LNCaP cells expressing miR-221, miR-222, or the miR-221/222 cluster had a significant increase in S phase population, as compared with empty vector-transfected cells, with a concomitant decrease of the G_1 portion (Fig. 5*A*). As for cell growth assays, cell cycle phase distribution was also consistently affected by anti-p27 siRNAs, clearly driving cells out from G_1 toward S phase (Fig. 5*B*). These results demonstrate the ability of miR-221 and miR-222 to overcome a p27-mediated cell cycle arrest, and support the role of these two miRNAs as negative regulators of $p27^{Kip1}$.

The Ectopic Expression of miR-221 or miR-222 Increases the Clonogenic Potential of LNCaP Cell, and miR-221/222 Knock-down Strongly Reduces It in PC3 Cells—A hallmark of cellular transformation is the ability of tumor cells to grow in an anchorage-independent way in a semisolid medium. To analyze the possible effects of miR-221/miR-222 expression on this cell property, we tested the ability of transfected LNCaP cells to grow and to form colonies when seeded at low density in soft agar. As shown in Fig. 6, *A* and *C*, LNCaP cells stably expressing miR-221 or miR-222 formed many more colonies than the empty vector-transfected cells (118 ± 9.5 *versus* 37.5 ± 6.9 for miR-221, and 146 ± 15.6 for miR-222). These results highlight that miR-221 and miR-222 expression affects another typical feature of tumor cell growth.

In addition, we compared the effect of miR-221 and miR-222 expression with that of anti-p27 siRNAs on the clonogenic potential of LNCaP cells. The colonies formed by LNCaP cells transduced with anti-p27 siRNAs were far more abundant than those formed by LNCaP cells transduced with a control siRNA (Fig. 6, *B* and *D*). Thus, as for proliferation studies, we obtained the same kind of results in both cases, independently of which sort of small RNA molecule was used to target p27.

Conversely, we also showed that the inhibition of miR-221 and miR-222 through LNA antisense oligonucleotides in PC3 cells was able to strongly reduce (4.5-fold) the number of the colonies growing in soft agar (Fig. 6, *E* and *F*), in accordance with our observation about the increase of p27 levels after transfection with anti-miR-221/222 LNA oligonucleotides. This strengthens our hypothesis that directly connects miR-221 and miR-222 action to p27 modulation, and to the consequent changes in cell properties under the control of p27.

DISCUSSION

In the last few years, an ever growing number of articles have been published describing a link between several forms of human cancer and the expression of microRNAs (for a review, see Ref. 21). Despite the great deal of work that has been done to date, however, only in a minority of the cases were the targets of the microRNAs that were shown to be specifically modulated in tumors successfully identified. As it is evident that full comprehension of the functional role of microRNAs in oncogenesis will be achieved only by elucidating their mechanism of action in each type of tumor, in this work we focused on a pair of microRNAs, miR-221 and miR-222, overexpressed in the specific context of prostate carcinoma. The data we present here are based on our observation that this couple of miRNAs are differentially expressed in a PCa cell line derived from a strongly aggressive tumor (PC3) *versus* other cell lines derived from slowly growing neoplasms (LNCaP and 22R*v*1). As these

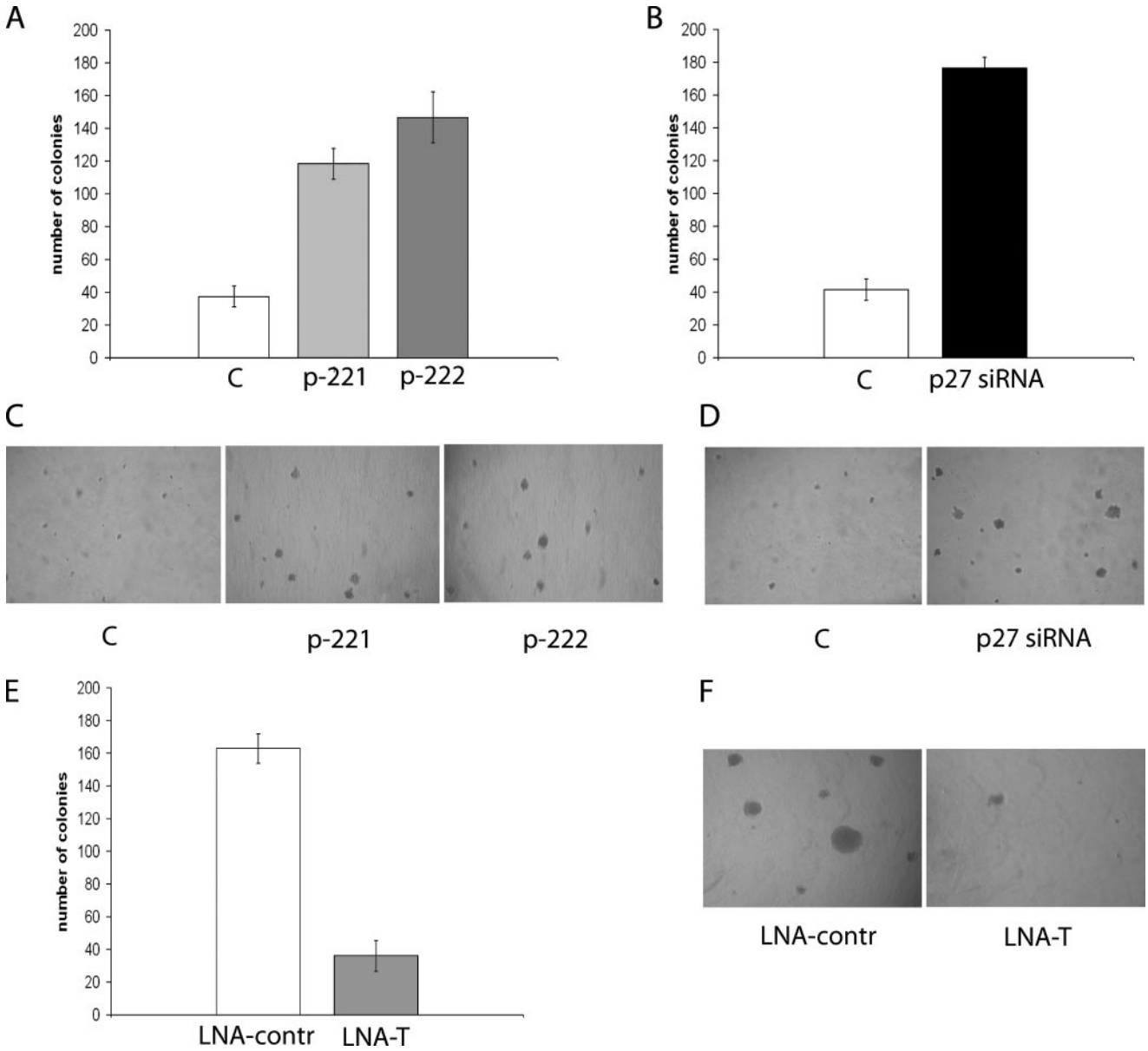


FIGURE 6. **Ectopic expression of miR-221 and miR-222 in LNCaP cells enhances colony formation in soft agar, and miR-221/222 knock-down strongly reduces it in PC3 cells.** *A*, colony formation of LNCaP cells stably transfected with empty vector (*c*), and plasmids p-221 or p-222. *B*, colony formation after transfection of LNCaP cells with anti-p27 siRNAs or with a control siRNA (*c*). *E*, colony formation after transfection of PC3 cells with LNA oligos against an *A. thaliana* miRNA (*LNA-contr*), or a mixture of anti-miR-221 and anti-miR-222 LNA oligos (*LNA-T*). Colonies >0.1 mm (for LNCaP cells) or >0.15 mm (for PC3 cells) in diameter were counted under a microscopic field at ×10 magnifications. Two independent experiments were performed in triplicate; the results shown are the average ± S.D. from one representative assay. In *C*, *D*, and *F*, one representative picture is shown for each cell type (×10 magnification).

PCa cell lines are commonly recognized as models of different stages of PCa progression (22), we hypothesized that the expression of miR-221/222 in PC3 aggressive cells might be considered a marker of their increased tumorigenic potential. This hypothesis is strengthened by our finding that one target, recognized and modulated by miR-221/222 in PCa cell lines, is the cell cycle inhibitor p27^{Kip1}, properly placed in the family of tumor suppressors. Through the luciferase assay, we demonstrated that at least two effective binding sites are present in the p27^{kip1} 3′ UTR; consistently, we observed that miR-221 and miR-222 levels are inversely linked to those of p27 expression in PC3, 22R*v*1, and in LNCaP cell lines. Moreover, the ectopic expression of these miRNAs or their knock-down are able to induce the predictable opposite effects on p27 expression in LNCaP, 22R*v*1, and PC3 cells.

We provided functional evidence about the possible role of miR-221 and miR-222 in PCa, by showing that the forced expression of these microRNAs is able, *per se*, to induce an enhancement of LNCaP cell growth potential, which is comparable and additive to that induced by a specific pool of anti-p27 siRNAs. In turn, this increase in cell growth is tightly linked to the G_1 to S shift we observed in the same cells, which is in agreement with modulation of p27, a known regulator of the G_1/S cell cycle checkpoint. This property is accompanied by their increased ability to grow in an anchorage-independent way, a feature clearly connected to the tumorigenic nature of

PCa cells. The clonogenic potential was strongly enhanced when miR-221 or miR-222 were overexpressed in LNCaP cells, and was consistently reduced in PC3 cells where miR-221 and miR-222 were knocked down through LNA oligos, thus providing us with a further indication about the involvement of this microRNA in PCa development.

The inverse correlation between the cdk inhibitor p27 and prognosis in several human cancers, including prostate carcinoma, is well known (14, 23). Of note, p27 decrease is an early event in prostate carcinoma oncogenesis, as it is frequently observed in proliferative inflammatory atrophy, which is considered as a precursor lesion preceding carcinoma onset (24). The observation that p27 is a dose-dependent tumor suppressor is of great importance for the interpretation of our present data, as it would be in agreement with the typical regulatory mode of microRNAs acting by modulating the amount of their targets, rather than knocking them out. We believe that this fine-tuning regulatory action exerted by miR-221/222 on the levels of p27 protein present in the cell might be considered as another piece of the sophisticated puzzle made up of several post-transcriptional mechanisms ensuring a fast response of p27 amount to environmental and intracellular variations. Actually, at least in theory, one could draw tantalizing conclusions by reviewing numerous papers that describe an upward modulation of miR-221 and/or miR-222 in a variety of cancers, the majority of which is also known to be characterized by p27 loss or mutations. miR-221 and miR-222 are overexpressed in solid tumors of the colon, pancreas, and stomach (25, 26), and are strongly up-regulated (>10-fold) in papillary thyroid carcinoma (8). Furthermore, miR-222 is a marker of poorly differentiated hepatocellular carcinoma *versus* well differentiated cases of the same tumor, being overexpressed about 10-fold in the former tumors (9). miR-221 and miR-222 up-regulation was described also in a non-solid tumor, chronic lymphocytic leukemia, as associated with some important markers of poor prognosis (27). We previously demonstrated that miR-221 and miR-222 are the most significantly up-regulated microRNAs in glioblastoma multiforme (11), and we have also collected preliminary experimental evidence that suggest the existence of a link between miR-221/222 overexpression and p27 down-regulation in this totally different model of human tumor.[6] In only three cases was the modulation of miR-221 and miR-222 described in conjunction with the elucidation of their target, which was the tyrosine kinase receptor KIT. However, in one case, miR-221 and miR-222 appeared to be down-regulated in an erythroleukemic cell line with a consequent overexpression of KIT (28), whereas in the other the two miRNAs in tandem were overexpressed in papillary thyroid carcinoma, causing a down-regulation of KIT (8). In a third report, the overexpression of miR-221 and miR-222 in HUVEC reduced their response to the angiogenic activity of stem cell factor by targeting its receptor c-Kit (29), suggesting a role for miR-221 and miR-222 as modulators of the formation of new vessels in physiological and pathological conditions.

[6] S. Galardi, S. A. Ciafrè, and M. Giulia Farace, unpublished results.

We think that our results, which identify p27^{Kip1} as a target for miR-221 and miR-222 in the context of prostate carcinoma cell lines, perfectly fit within a dynamic view of the microRNA-mediated regulation of gene expression: it is well known and widely predicted that the relationship between microRNAs and target mRNAs is not a "one to one" connection, as the same mRNA can be regulated by more than one miRNA, and that the choice of how many and which miRNAs target one 3′ UTR is strongly determined by the specific cellular environment (30). An miRNA that regulates targets playing opposite roles in the control of cell proliferation may act as a tumor suppressor in some cancers and as an oncogene in others, depending on which targets are driving tumorigenesis in that specific cellular milieu.

In conclusion, our results suggest that overexpression of miR-221 and miR-222 may contribute to the growth and progression of prostate carcinoma, at least in part by blocking p27 mRNA translation. Additional functional studies are now needed for the comprehension of the molecular basis of the formation of this carcinoma, and give new clues to develop innovative therapies targeting specific tumor markers, such as the overexpressed microRNAs.

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miR-221 and miR-222 Expression Affects the Proliferation Potential of Human Prostate Carcinoma Cell Lines by Targeting p27 ^{Kip1}

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