UNIVERSITY OF NAPLES
"FEDERICO II"

Doctorate School in Molecular Medicine

Doctorate Program in
Genetics and Molecular Medicine

Coordinator: Prof. Lucio Nitsch
XXIII Cycle - 2007/2010

"miR-191 downregulation plays a role in thyroid follicular tumors through CDK6 targeting"

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“miR-191 downregulation plays a role in thyroid follicular tumors through CDK6 targeting”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

Marianna Colamaio, Eleonora Borbone, Lucia Russo, Angelo Ferraro, Daniela Califano, Gennaro Chiappetta, Pierlorenzo Pallante, Giancarlo Troncone, Sabrina Battista, Alfredo Fusco. mir-191 Downregulation Plays a Role in Thyroid Follicular Tumors through CDK6 Targeting.(*submitted for publication).*

Sabrina Battista, **Marianna Colamaio**, Angelo Ferraro, Lucia Russo, Daniela Califano, Gennaro Chiappetta Giancarlo Troncone, Alfredo Fusco.Let-7a Microrna Affects Adhesion and Migration Ability of Thyroid Cell Lines by Targeting Dysadherin. *(in preparation).*

ABSTRACT

miR-191 expression is frequently altered in several neoplasias, being upregulated in some, such as pancreatic, colon, lung and prostate carinomas, and downregulated in others, such as severe medulloblastomas and melanomas. I have investigated the expression of miR-191 in thyroid neoplasias.
In my thesis I report that miR-191 is downregulated in follicular adenomas and carcinomas, with a reduction that is almost 2-fold higher in follicular carcinomas with respect to adenomas. Conversely it is upregulated in thyroid carcinomas of the papillary histotype (PTCs), in comparison with the normal thyroid tissue. Consistent with a putative tumour suppressor role of miR-191 in the development of thyroid neoplasias of the follicular histotype, functional studies showed that restoration of miR-191 expression in a follicular thyroid cancer cell line reduces cell growth and migration rate. Furthermore, I found that miR-191 negatively regulates the expression of CDK6 protein, involved in cell cycle control, without changing the CDK6 mRNA level and decreases the activity of a luciferase reporter construct containing the CDK6-3'untranslated region. These results demonstrate that miR-191 regulates CDK6 at the post-transcriptional level, resulting in inhibition of cell proliferation and migration of the follicular thyroid carcinoma cell line; I will show that restoration of miR-191 expression reduces cell proliferation leading to an increased number of the cells in the G1 phase of the cell cycle. Finally, in follicular adenomas and carcinomas, we immunohistochemically detected CDK6 over-expression, suggesting that miR-191 may have a pathogenic role in thyroid cancer by controlling CDK6 expression.

1. BACKGROUND

Carcinoma of the thyroid gland is an uncommon cancer, but one of the most frequent malignancies of the endocrine system. Most thyroid cancers are derived from the follicular cells; follicular carcinoma is considered more malignant than papillary thyroid carcinoma (PTC) and anaplastic thyroid cancer (ATC) is one of the most lethal human cancers.
Some years ago, several studies were undertaken to analyze the expression of microRNAs (miRNAs or miRs) in thyroid carcinoma to evaluate a possible role of their deregulation in the process of carcinogenesis. These studies showed an aberrant microRNA expression profile that distinguishes unequivocally among tumoral and normal thyroid tissue.

1.1 Thyroid gland

The thyroid gland, which is the largest endocrine organ in humans, regulates systemic metabolism through thyroid hormones. It is composed of two distinct hormone-producing cell types that have been designated follicular cells and parafollicular C cells (reviewed in Kondo T. et al. 2006). Follicular cells comprise most of the epithelium and are responsible for iodine uptake and thyroid hormones (triidothyronine and thyroxine) synthesis, which play an important role in development, differentiation, and metabolism (Zhang and Lazar 2000). The synthesis, storage and secretion of thyroid hormones L-triiodothyronine (T3) and L- thyroxine (T4) is under the control of the hypothalamic–pituitary axis with negative feedback by the thyroid hormones (Figure 1). Thyrotropin releasing hormone (TRH), which is secreted from the hypothalamus, stimulates the release of thyroid-stimulating hormone (TSH) from the anterior pituitary gland and TSH stimulates the follicular cells to synthesize and secrete thyroid hormones (Martin 1996).
C cells are scattered intrafollicular or parafollicular cells that are dedicated to the production of the calcium-regulating hormone calcitonin (reviewed Kondo T. et al. 2006).

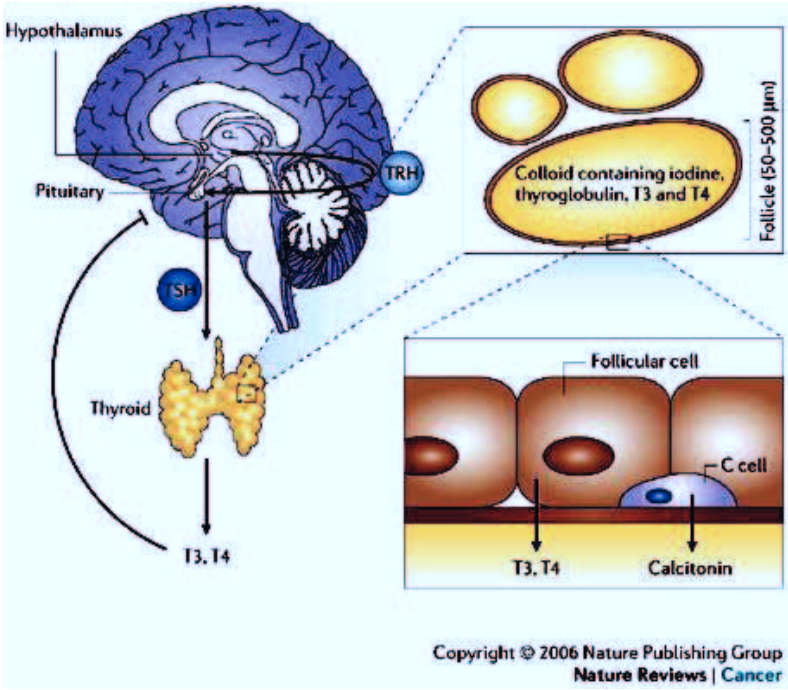


Figure 1 Schematic representation of thyroid gland structure and function. This butterfly-shaped organ that weighs 15– 25 g is located on the anterior surface of the trachea at the base of the neck. It is comprised of aggregates or lobules of spherical follicles that are filled with colloid. Thyroid follicular cell synthesize, store and secrete thyroid hormones T3 and T4 under the control of the hypothalamic-piyuitary, which axis with negative feedbach by the thyroid hormones.

1.1.1 Thyroid cancer

Cancer of the thyroid is the most common endocrine malignancy and incidence rates have steadily increased over recent decades (Kondo T. et al. 2006). Thyroid nodules can be hyperplastic benign adenomas or malignant lesions, and can be derived from thyroid follicular epithelial cells or C cells. Cancers that arise in the thyroid are the most common malignancy of endocrine organs (Parkin, D. M. et al. 2005). Age-adjusted global incidence rates vary from 0.5 to 10 cases per 100,000 population (DeLellis, R. A. et al. 2004). More than 95% of thyroid carcinomas are derived from follicular cells (Table 1); a minority of tumours (3%), referred to as medullary thyroid carcinoma, are of C-cell origin. Most carcinomas that are derived from follicular epithelial cells are indolent tumours that can be effectively managed by surgical resection. However, a subset of these tumours can behave aggressively and there is currently no effective form of treatment. Radiation exposure, reduced iodine intake, lymphocytic thyroiditis, hormonal factors and family history are putative risk factors for thyroid carcinoma. Iodine is required for thyroid hormone organification and a dietary iodine deficiency results in thyroid proliferation, known as goitre, as a compensatory mechanism.

Tumour type	Prevalence	Sex ratio female:male	Age (years)	Lymph-node metastasis	Distant metastasis	Survival rate (5 year)	References
Papillary thyroid carcinoma	85–90%	2:1–4: 1	20–50	<50%	5–7%	>90%	Hundahl et al. 1998, DeLellis et al. 2004, LiVolsi et al. 1994.
Follicular thyroid carcinoma	<10%	2:1–3: 1	40–60	<5%	20%	>90%	Hundahl et al. 1998, DeLellis et al. 2004, LiVolsi et al. 1994.
Poorly differentiated thyroid carcinoma	rare–7%	0,4:1–2,1: 1	50–60	30–80%	30–80%	50%	Hundahl et al. 1998, DeLellis et al. 2004, Carcangiu et al. 1984, Rodriguez et al. 1998.
Undifferentiated thyroid carcinoma	2%	1,5: 1	60–80	40%	20–50%	1–17%	Hundahl et al. 1998, DeLellis et al. 2004, Kebebew et al. 2005.
Medullary thyroid carcinoma	3%	1:1–1,2: 1	30–60	50%	15%	80%	Hundahl et al. 1998, DeLellis et al. 2004.
Mixed medullary and follicular-cell carcinoma	rare						DeLellis et al. 2004,

Table 1 Clinico-pathological features of thyroid cancer (copyroght © 2006 Nature Publishing Group).

Follicular-cell-derived carcinomas are broadly divided into well-differentiated thyroid carcinoma (WDTC), poorly differentiated thyroid carcinoma (PDTC) and undifferentiated types on the basis of histological and clinical parameters (Table 1). WDTCs include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) types. Although initially defined by architectural criteria, the histological diagnosis of papillary carcinoma rests on a number of nuclear features that predict the propensity for metastasis to local lymph nodes (DeLellis et al. 2004). The diagnosis of this most frequent type of thyroid malignancy (85–90% of thyroid malignancies) has been increasing, possibly owing to the changing recognition of nuclear morphological criteria (ground-glass nuclei). Radiation exposure as a consequence of nuclear fallout is associated with papillary carcinoma, as evidenced by the sequelae of the atomic bombs of Hiroshima and Nagasaki (1945), nuclear testing in the Marshall Islands (1954) and Nevada (1951–1962), and the more recent nuclear accident in Chernobyl (1986) (DeLellis et al. 2004, Kazakov et al. 1992).

On the other hand, follicular thyroid carcinoma is characterized by haematogenous spread, and the frequency of its diagnosis has been decreasing (LiVolsi and Asa 1994). FTC is well differentiated, usually unifocal,

encapsulated, with a tendency to metastasize via the vascular system to the bones and lungs. It is likely that FTCs can develop from pre-existing benign follicular adenomas (FTAs) or by directly bypassing the stage of adenoma. The differential diagnosis between the FTCs and the far more frequent FTAs is difficult. The incidence of follicular carcinoma in areas of iodine deficiency is higher than in areas of an iodine-rich diet (DeLellis et al. 2004, Harach et al. 2002). Interestingly, in animal models, iodine supplementation causes experimental thyroid cancers to change from follicular to papillary morphology, indicating that one of the roles of iodine in thyroid carcinogenesis is modulating tumour morphology, rather than cancer initiation (Gasbarri *et al.* 2004).

Most WDTCs behave in an indolent manner and have an excellent prognosis; they manifest in patients who are 20–50 years of age, and the disease is 2–4 times more frequent in females than in males (Hundahl et al. 1998, DeLellis et al. 2004). By contrast, undifferentiated or anaplastic thyroid carcinoma (UTC or ATC) is a highly aggressive and lethal tumour (DeLellis et al. 2004, Kebebew et al. 2005) and insensitive to conventional radiotherapy and chemotherapy. The presentation is dramatic, with a rapidly enlarging neck mass that invades adjacent tissues. There is currently no effective treatment and death usually occurs within 1 year of diagnosis. PDTCs are morphologically and behaviourally intermediate between well-differentiated and undifferentiated thyroid carcinomas (Table 1) (DeLellis et al. 2004; Carcangiu et al. 1984; Rodriguez *et al.* 1998).

Two models of thyroid cancerogenesis have been proposed: 1) The theory of sequential progression of well-differentiated thyroid carcinoma through the spectrum of poorly differentiated to undifferentiated thyroid carcinoma (Figure 2), supported by the presence of pre- or co-existing well-differentiated thyroid carcinoma with less differentiated types, and the common core of genetic loci with identical allelic imbalances in co-existing well differentiated components (Van der Laan et al. 1993; Hunt *et al.* 2003); 2) The thyroid cancer stem cell origin that holds thyroid cancer stem cells originate either from normal stem cells, progenitor cells, or more mature cells that have dedifferentiated. Although any of these origins is possible, most researchers believe that stem cells or progenitor cells are the most likely culprits. Zhang *et al.* (2006) presented a model for the origin of the four types of thyroid carcinomas based on their differentiation levels. Anaplastic cancer could form directly from a stem cell because it is poorly differentiated. Follicular and papillary carcinomas, which are well-differentiated, could arise from bipotential stem cells. Meduallary carcinoma, another well-differentiated cancer, could originate from progenitor C cells (Zhang *et al.* 2006). Although the discovery of stem cell markers in the thyroid gland indicates the potential for the existence of thyroid cancer stem cells, the identification of these cells could prove to be quite difficult due to the extremely low lifetime turnover in the thyroid gland.

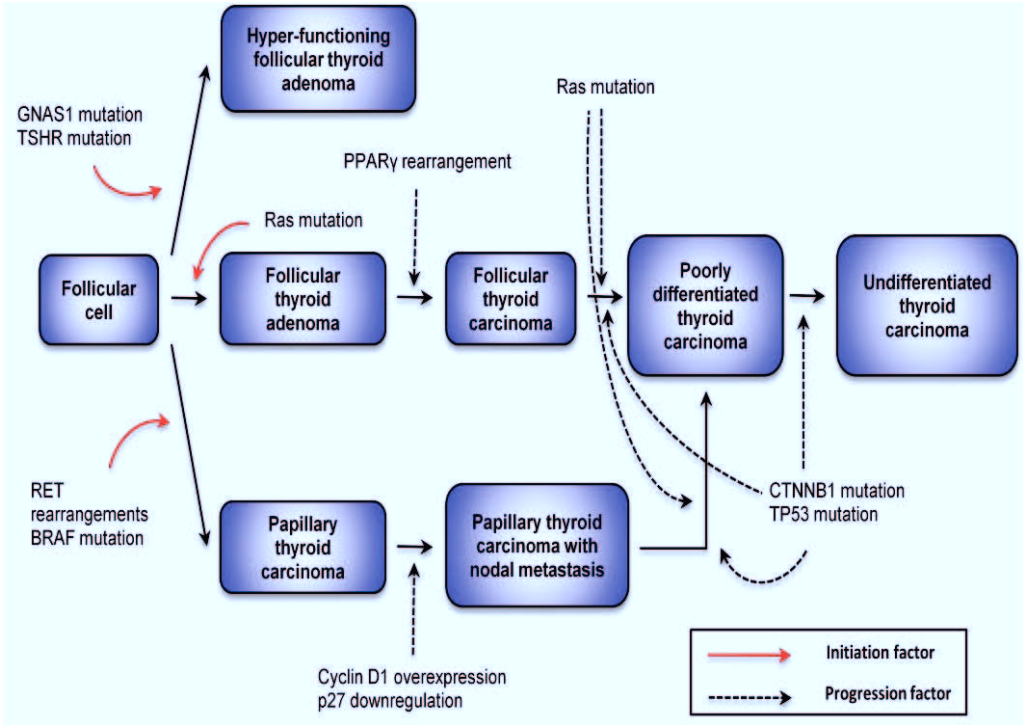


Figure 2 Model of multi-step carcinogenesis of thyroid neoplasms. The proposed model of thyroid carcinogenesis is based on general concepts and specific pathways. On the basis of clinical, histological and molecular observations, three distinct pathways are proposed for neoplastic proliferation of thyroid follicular cells, including hyper-functioning follicular thyroid adenoma (tumours that are almost always benign lesions without a propensity for progression), follicular thyroid carcinoma and papillary thyroid carcinoma. Genetic defects that result in activation of RET or BRAF represent early, frequent initiating events that can be associated with radiation exposure. Underexpression of the cyclin-dependent-kinase inhibitor p27KIP1 and overexpression of cyclin D1 are strong predictors of lymph-node metastases in papillary thyroid carcinomas. Most poorly differentiated and undifferentiated thyroid carcinomas are considered to derive from pre-existing well-differentiated thyroid carcinoma through additional genetic events, including β-catenin (which is encoded by CTNNB1) nuclear accumulation and p53 inactivation, but de novo occurrence might also occur. GNAS1, guanine nucleotide-binding α-subunit 1; PPARγ, peroxisome proliferation-activated receptor-γ; TSHR, thyroid-stimulating-hormone receptor (reviewed on Kondo et al. 2006).

Genomic alternations, such as RET/PTC and PAX8-PPARγ1 rearrangements and a mutation in the BRAF gene, play an oncogenic role by preventing thyroid fetal cells from differentiating (Figure 3). This hypothesis well explains the clinical and biological features and recent molecular evidence of thyroid cancer. Further, it underscores the importance of identifying stem cells and clarifying the molecular mechanism of organ development. Such data will lead to better understanding of thyroid carcinogenesis and the establishment of more accurate diagnostic methods and more effective therapies (Takano 2007).

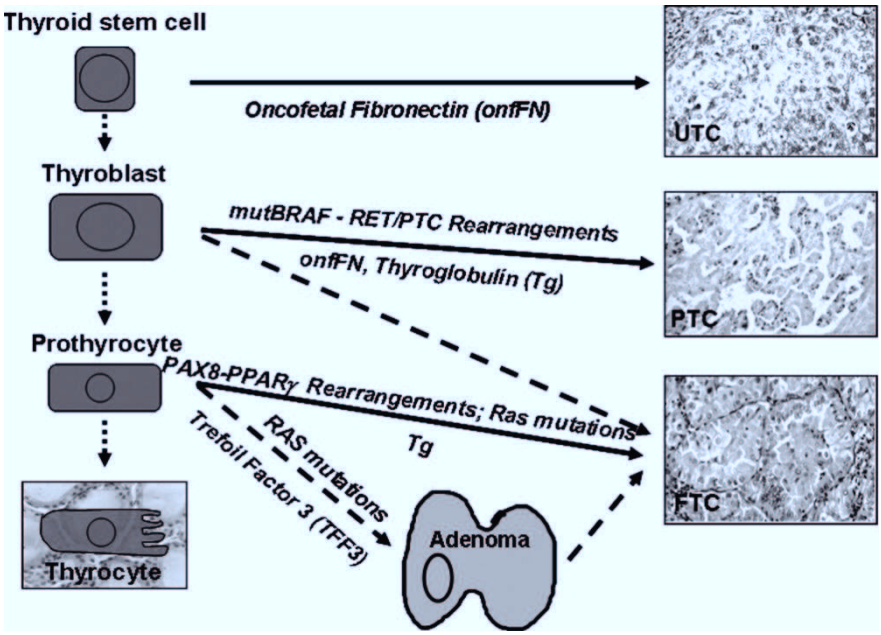


Figure 3 Schematic representation showing a developmental hierarchy of fetal thyroid stem/progenitor cells as a potential cause of specific follicular thyroid cancer types (Klonisch 2009, Takano 2007). Examples of mutations associated with and factors expressed in certain thyroid cancer types are depicted. Trefoil factor 3 (TFF3) may be used to distinguish foci of thyroid $TTF3^{+}$ adenoma from $TTF3^{weak/-}$ FTC. FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; UTC, undifferentiated anaplastic thyroid carcinoma.

1.1.2 Genetic alterations in signalling pathways

Genetic factors other than the environmental ones may as well play a role in thyroid carcinogenesis. There is also a genetic component to thyroid follicular cell-derived carcinoma, as a family history increases risk 3.2- and 6.2-fold when a parent and a sibling, respectively, have had thyroid cancer (Hemminki et al. 2005). Familial thyroid cancers associated with tumour syndromes caused by known germline mutations include familial polyposis coli (associated with mutations in the adenomatosis polyposis coli gene (*APC*)), Cowden disease (associated with mutations in the phosphatase with tensin homology gene (*PTEN*)) and Werner syndrome (associated with mutations in the *WRN* Gene) (Lindor and Greene 1998). It has recently been shown that an important role to the onset of familial PTCs and FTCs can be attributed to the common variants of thyroid transcription factor 2 (TTF2) or factor 1 (TTF1) genes; these variants account for an increased risk of both PTCs and FTCs (Gudmundsson et al. 2009).

Several oncogenes have been involved in thyroid carcinoma development (Table 2). In PTCs, nonoverlapping mutations of genes involved in the activation pathway of mitogen-activated protein kinase (MAPK), such as RET, TRK, RAS, and BRAF, have been found in about 70% of the cases (Kimura et al. 2003; Soares et al. 2003; Frattini et al. 2004), while a fraction of about 30% of PTCs presents rearrangement of RET proto-oncogene (Santoro et al. 2004). The RET/PTC rearrangements consist in the fusion of tyrosine kinase (TK) domain of RET with other genes that provide to the chimeric gene the promoter and the 5' coding region. RET/PTC1 and RET/PTC3 are the most frequent isoforms found in PTCs.

In most PTC cases, a specific point mutation of BRAF is found (T1799A, resulting in a valine>glutamate substitution at residue 600 (V600E). The proto-oncogene form of *BRAF* encodes a serine/threonine kinase that transduces regulatory signals through the Ras–Raf–MEK–ERK cascade. Gain-of-function *BRAF* mutation provides an alternative route for the aberrant activation of ERK signalling that is implicated in the tumorigenesis of several human cancers — for example, melanoma and colon carcinoma (Davies *et al.* 2002). In PTCs, BRAF mutation and RET/PTC rearrangements are mutually exclusive and cannot be found simultaneously in the same patients, yet they are not completely equivalent, since it has been shown that PTCs positive for BRAF are more aggressive than those positive for RET/PTC (Kimura et al. 2003; Soares et al. 2003). It has recently been demonstrated that some types of PTCs developed after the Chernobyl nuclear accident present a different BRAF alteration since they harbored a rearrangement similar to the RET/PTC rearrangements, which results in the fusion between BRAF kinase domain and the AKAP9 gene and is caused by a paracentric inversion of the long arm of chromosome 7 (Ciampi et al. 2005).

RAS gene mutations represent early molecular lesions since they are also frequently found in FTAs which are considered FTC precursors. Three Ras

proto-oncogenes — *HRAS, KRAS*, and *NRAS* — are implicated in human tumorigenesis (Downward 2003). Ras mutations are more common in iodine-deficient than iodine-sufficient areas and in lesions with follicular architecture (including follicular carcinoma and follicular variant papillary thyroid carcinoma) than in typical papillary thyroid carcinoma (Shi *et al.* 1991, Zhu et al. 2003) and highly specific analysis indicates that Ras mutations are more common in poorly differentiated and undifferentiated thyroid carcinoma, implicating this phenomenon in tumour progression (Ezzat *et al.* 1996, Garcia-Rostan *et al.* 2003). Another FTC group presents the fusion of two nuclear proteins with transcriptional activity: PAX8 and peroxisome proliferator-activated receptor γ (PPARγ; Kroll et al. 2000; Table 2).

PDTCs and ATCs can originate from WDTCs and, in particular, from PTCs, therefore, mutations that occur in the early stages of WDTCs are also observed in PDTCs and ATCs.

Alterations of PIK3CA and PTEN (effectors of phosphoinositide 3-kinase) have also been found in thyroid carcinomas. These alterations are generally observed in the later stages of thyroid carcinogenesis and are more frequent in ATCs than in WDTCs or FTCs (Paes & Ringel 2008; Table 2). This is in contrast with mutations of the MAPK effectors that are preferentially associated with the early stages of thyroid carcinogenesis.

More recently, an AKT1 oncogene mutation in PDTCs has also been observed (Ricarte-Filho et al. 2009) and this mutation result commonly associated with BRAF mutations (Ricarte-Filho et al. 2009).

Impairment of the p53 tumor suppressor gene function represents a usual feature of ATCs with p53 mutations being common both in PDTCs and in ATCs, but rare or absent in WDTCs (Ito et al. 1992, Donghi et al. 1993, Fagin et al. 1993; Table 2).

Moreover, mutations of CTNNB1 (b-catenin) have been found in PDTCs and ATCs (Garcia-Rostan et al. 2001, Miyake et al. 2001;Table 2).

Tumor histotype	Cell type	Etiological factors	Molecular lesion	Prevalence (%)	References
Papillary thyroid carcinoma	Follicular cell	Ionizing radiation	RET rearrangement	30	Santoro et al. (2004)
			NTRK1 rearrangement	10	Pierotti et al. (1995)
			BRAF mutation (V600E)	40	Kimura et al. (2003) and Soares et al. (2003)
			RAS mutation (codons 12, 13, and 61)	10–20	Namba et al. (1990), Ezzat et al. (1996) and Vasko et al. (2004)
Follicular thyroid Adenoma	Follicular cell	Iodine deficiency	RAS mutation (codons 12, 13, and 61)	20–40	Suarez et al. (1990), Esapa et al. (1999), Basolo et al. (2000) and Motoi et al. (2000)
			PAX8/PPARg rearrangement	2–10	Dwight et al. (2003), French et al. (2003) and Nikiforova et al. (2003)
Follicular thyroid carcinoma	Follicular cell	Iodine deficiency	RAS mutation (codons 12, 13, and 61)	40–50	Suarez et al. (1990), Esapa et al. (1999), Basolo et al. (2000) and Motoi et al. (2000)
			PAX8/PPARg rearrangement	35	Dwight et al. (2003), French et al. (2003) and Nikiforova et al. (2003)
			PIK3CA mutation	8	Paes & Ringel (2008)
			PTEN mutation	7	Paes & Ringel (2008)
Poorly differentiated thyroid carcinoma	Follicular cell	Tumor progression	RET rearrangement	13	Santoro et al. (2002)
			BRAF mutation (V600E)	12–17	Garcia-Rostan et al. (2005), Hou et al. (2007) and Santarpia et al. (2008)
			RAS mutation (codons 12, 13, and 61)	46–55	
			TP53 mutation	17–38	Ito et al. (1992), Donghi et al. (1993) and Fagin et al. (1993)
			CTNNB1 mutation	25	Garcia-Rostan et al. (2001) and Miyake et al. (2001)
Anaplastic thyroid carcinoma	Follicular cell	Tumor progression	BRAF mutation (V600E)	25–29	Garcia-Rostan et al. (2005), Hou et al. (2007) and Santarpia et al. (2008)
			RAS mutation (codons 12, 13, and 61)	6–52	
			TP53 mutation	67–88	Ito et al. (1992), Donghi et al. (1993) and Fagin et al. (1993)
			CTNNB1 mutation	66	Garcia-Rostan et al. (2001) and Miyake et al. (2001)
			PIK3CA mutation	16	Paes & Ringel (2008)
			PTEN mutation	14	Paes & Ringel (2008)

Table 2 Genetic alterations of human thyroid carcinomas of follicular cell origin.

1.1.3 Cell-cycle regulation in thyroid

The growth activity of well-differentiated thyroid carcinoma is low compared with poorly differentiated and undifferentiated thyroid carcinomas and altered cell-cycle regulators govern these differences in growth activity. Progression factors (for example, cyclin D1, cyclin E1, cyclin-dependent kinases (CDKs) and E2Fs) and competitor factors (for example, retinoblastoma protein (RB), p16INK4A, p21CIP1, p27KIP1 and p53) regulate the transition from G1 to S phase (Figure 4). The cyclin-dependent kinases (Cdks)[1] are a family of serine/threonine protein kinases (~34–40 kDa) that require the binding of a regulatory cyclin subunit for activity (in most cases, full activation also requires phosphorylation of a threonine residue near the kinase active site). Cyclin–CDK complexes promote cell-cycle progression through inactivation of RB. Although nuclear immunoreactivity for cyclin D1 and cyclin E1 are not detectable in normal thyroid follicular cells, expression of cyclin D1 and cyclin E1 is observed in approximately 30% and 76% of papillary thyroid carcinomas, respectively (Basolo F. *et al.* 2000; Brzezinski J. et al. 2004). Furthermore, cyclin D1 overexpression correlates with metastatic spread in papillary thyroid carcinomas, and significant overexpression of cyclin D1 is observed in undifferentiated thyroid carcinoma (Khoo, Beasley et al. 2002, Khoo, Ezzat et al. 2002, Wang *et al.* 2000). CDK inhibitors are commonly downregulated in thyroid neoplasia. There is progressive loss of p21CIP1 with advancing tumour stage of papillary thyroid carcinomas (Brzezinski et al. 2005). Normal and hyperplastic follicular cells show strong immunoreactivity for p27KIP1, whereas p27KIP1 expression is significantly reduced in papillary thyroid carcinoma, mainly metastatic forms and undifferentiated thyroid carcinoma (reviewed in Kondo et al. 2006). The E2F transcription-factor family — the main targets of RB — consists of six members: E2F1–E2F6. E2F1, but not other members of this family, is upregulated in WDTCs, PDTCs and ATCs (Volante et al. 2002, Onda *et al.* 2004).
The tumour-suppressor p53 seems to be a crucial gatekeeper of progression from indolent to lethal thyroid cancers; activation of wild-type p53 can lead to G1 cell-cycle arrest through p21CIP1, and apoptotic cell death, preventing replication of cells with damaged DNA, while loss of- function mutation of *p53* induces genomic instability, owing to weakened DNA repair systems, and subsequent cancer progression (Hosal *et al.* 1997).

[1] Cyclin-dependent kinases (CDK) belong to a group of protein kinases originally discovered as being involved in the regulation of the cell cycle, but they are also involved in the regulation of transcription and mRNA processing.

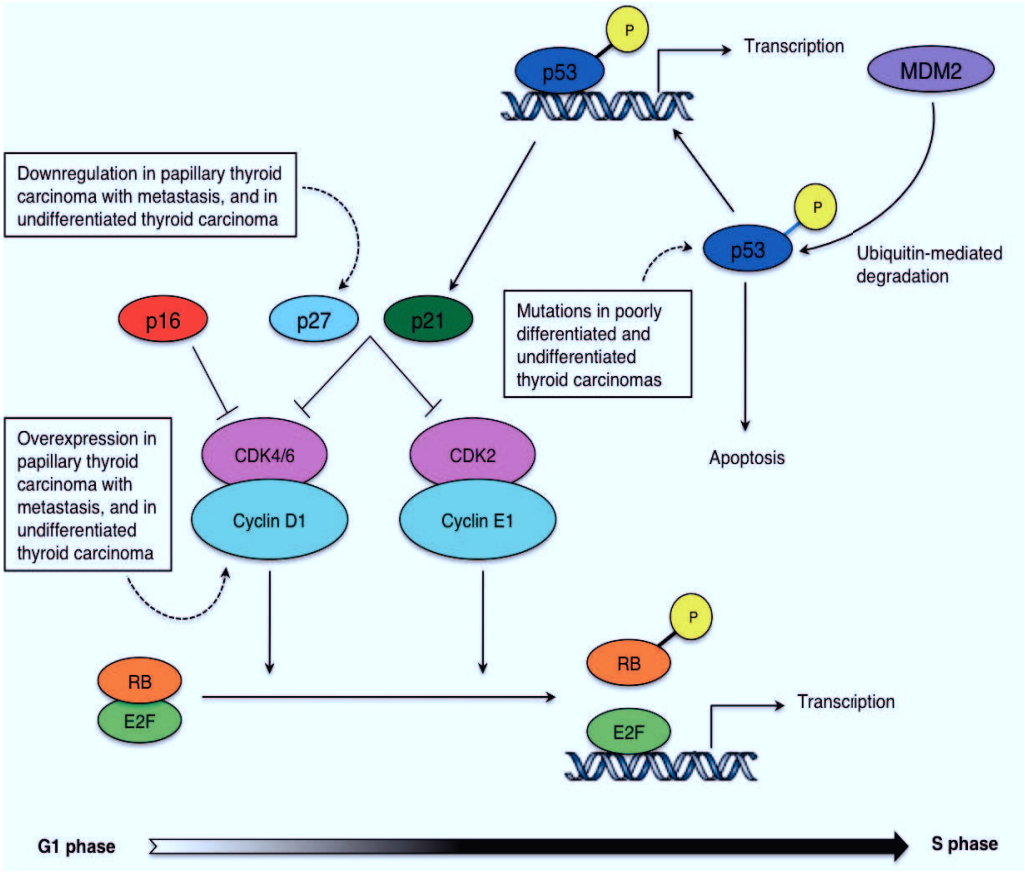


Figure 4 Cell-cycle regulation in follicular cells. Cyclin D1 and cyclin E1 cooperate to control the G1 to S phase transition through interactions with retinoblastoma protein (RB). Cyclin D1 and cyclin E1 heterodimerize with cyclin-dependent kinases (CDKs) 4 or 6 and 2, respectively, to inactivate the tumour suppressor RB by phosphorylation. Active RB functions as a repressor of E2F transcription factors, whereas inactivation (phosphorylation) of RB allows E2F transcriptional activity. E2F activates the transcription of genes that are involved in the G1 to S phase transition, such as DNA polymerase and thymidine kinase. The CDK inhibitors p16INK4A, p21CIP1 and p27KIP1 impair the activity of cyclin–CDK complexes, thereby preventing phosphorylation of RB. The CDK inhibitors therefore function as tumour suppressors. The tumour suppressor p53 induces cell-cycle arrest by upregulating p21CIP1, which initiates apoptosis. The function of p53 is controlled by negative regulators, including MDM2. The MDM2 protein targets p53 for ubiquitinmediated degradation, constituting a feedback loop to maintain a low concentration of p53 in the cells (reviewed in Kondo et al.2006).

1.2 MicroRNA

MicroRNAs (miRNAs or miRs) are a class of endogenous noncoding small RNA molecules of 19–25 nucleotides (nt), that negatively regulates gene expression by degradation of mRNA or suppression of mRNA translation (Bartel 2004). MiRNA plays important roles in physiological processes such as cellular development, differentiation, proliferation, apoptosis, and stem cell self-renewal. The first glimpse into the new world of small RNAs came with seminal papers from Ambros, Ruvkun and colleagues (Lee et al. 1993, Wightman et al. 1993): they reported that *lin-4* and *let-7*, the first microRNA (miRNA) genes identified, control developmental timing in nematodes by modulating the expression of other genes at the post-transcriptional level. We now know that miRNAs are a universal and pervasive feature of animal and plant genomes and are conserved among distantly related organism. The most recent release of the miRBase sequence (http://www.mirbase.org/, 14.0 released on September 2009) lists 10,886 different miRNAs identified in all species, among them are 721 human miRNAs. It is predicted that the human genome encodes about 1,000 miRNAs (Bentwich et al. 2005) and that they could regulate ~30% of human genes (Berezikov et al. 2005). Deregulation of miRNA contributes to a variety of human diseases, such as diabetes, immuno- or neurodegenerative disorders, and cancer (Bartel 2004, Pasquinelli et al. 2005, Harfe 2005, Carleton et al. 2007, Garzon et al. 2006, Wang 2007, Boehm and Slack 2006, Poy et al. 2004, Lu et al. 2005). Half of the known miRNAs are located inside or close to fragile sites and in minimal regions of loss of heterozygosity, minimal regions of amplifications, and common breakpoints associated with cancer (Calin et al. 2004). For example, the miRNA cluster *17-92* is located at 13q31, a region commonly amplified in lymphomas (Ota et al. 2004); *miR-143* and *miR-145* are located at 5q33, which is frequently deleted in myelodysplastic syndromes (Calin et al. 2004); and a rearrangement of *miR-125b-1*, juxtaposed to the immunoglobulin heavy chain locus, was described in a patient with B cell acute lymphocytic leukemia (Sonoki et al. 2005). MiRNAs may function as oncogenes (OncomiRs) or tumor suppressors (TSmiRs) in tumors; mounting evidence shows that miRNAs are aberrantly expressed during cancer development (O'Donnell 2005), invasion and metastasis (Zhu et al. 2008), angiogenesis (Hua et al. 2006) and play crucial roles in cancer stem cell regulation (Yu et al. 2007).

Several groups, including ours, have systematically analyzed miRNA expression in cancer samples and their corresponding normal tissues (Visone et al. 2007, Pallante et al. 2006). Consequently, miRNA "signatures" were discovered that distinguish between tumoral and normal cells, and in some instances are associated with the prognosis and the progression of cancer (Calin et al. 2005).

Therefore, miRNAs are integral elements in the post-transcriptional control of gene expression and the challenge is now to understand their specific biological function and, hopefully exploit them in cancer diagnosis and

therapy.
I will briefly describe miRNA biogenesis and discuss how miRNAs can act as oncogenes and tumor suppressors. I will also address the issue of miRNA deregulation in the diagnosis, prognosis, and treatment of cancer.

1.2.1 miRNA biogenesis

miRNA genes are evolutionarily conserved and may be located either within the introns or exons of protein-coding genes (70%) or in intergenic areas (30%) (Rodriguez et al. 2004). Most of the intronic or exonic miRNAs are oriented in sense with their host gene, suggesting that they are transcribed in parallel with their host transcript. The second group of miRNAs is transcribed from intergenic regions or gene deserts comprising independent transcription units (Rodriguez et al. 2004). MiRNAs are preferentially transcribed by polymerase II (Pol II) into long primary transcripts, up to several kilobases (pri-miRNA) that are subsequently processed in the nucleus by the enzyme Drosha to become 70-nt-long precursor strands (pre-miRNA) (Figure 5) (Lee Y et al. 2004, Lee Y et al. 2003). This precursor is exported by exportin 5 to the cytoplasm (Bohnsack et al. 2004), where it is bound to the RNase Dicer and to the RNA-induced silencing complex (RISC). RISC is composed of the transactivation-responsive RNA-binding protein (TRBP) and Argonaute 2 (Ago2) (Hammond et al. 2000, Thimmaiah et al. 2005). Recent studies suggest that first Ago2 cleaves the pre-miRNA 12 nt from its 3' end (forming Ago2-cleaved precursor miRNA) and then the Dicer cleaves the Ago2-cleaved precursor miRNA into a mature 22-nt miRNA duplex (Diederichs and Haber 2007). While the active or mature strand is retained in RISC, the passenger strand is removed and degraded. miRNAs modulate gene expression through canonical base pairing between the seed sequence of the mature miRNA (nucleotides 2–8 at its 5′ end) and its complementary seed match sequence which is present in the 3' untranslated region (3′ UTR) of target mRNAs (Bartel 2009); in this way mRNA guides the miRNA-RISC complex to repress gene expression by inhibiting translation of target (Figure 5) (Bartel 2004, Hammond et al. 2000, Thimmaiah et al. 2005, Diederichs and Haber 2007). The RISC can inhibit the expression of the target mRNA through two main mechanisms that have several variations: removal of the polyA tail (deadenylation) by fostering the activity of deadenylases (such as CCR4–NOT), followed by mRNA degradation; and blockade of translation at the initiation step or at the elongation step. For example, by inhibiting eukaryotic initiation factor 4E (EIF4E) or causing ribosome stalling, RISC-bound mRNA can be localized to sub-cytoplasmatic compartments, known as P-bodies, where they are reversibly stored or degraded (Masafumi et al. 2010).
During miRNA biogenesis, miRNAs are subject to intense transcriptional and post-transcriptional regulation and the elucidations of these mechanisms and of alterations at the expense of these mechanism has improved our understanding

of miRNA deregulation in disease. A cluster of papers in *Nature* and *Molecular Cell* revealed that a family of miRNAs, *miR-34a*, *-b*, and *-c*, are induced directly by *TP53* and suggested that some of *TP53*'s effects could be mediated through transcriptional activation of miRNAs. Using different models, the authors compared miRNA expression in cells with high or low *TP53* expression and found that *miR-34* expression is increased in cells with high *TP53* levels. Chromatin immunoprecipitation experiments revealed that *TP53* binds to the *miR-34*s promoters. Restoring *miR-34* levels in both primary and tumor cell lines induced cell cycle arrest by targeting a gene program involved in cell cycle progression (He et al. 2007, Raver-Shapira et al. 2007, Chang et al. 2007). Our group has shown that HMGA protein activates miR-181 expression by binding to the microRNA promoter region (Mansueto et al. 2010).

An important epigenetic mechanism of miRNA regulation is miRNA expression silencing by promoter DNA hypermethylation. Saito et al. first reported that *miR-127* is silenced by promoter hypermethylation in bladder cancer cell lines and patients, and its expression could be restored by using hypomethylating agents (Saito Y et al. 2006).

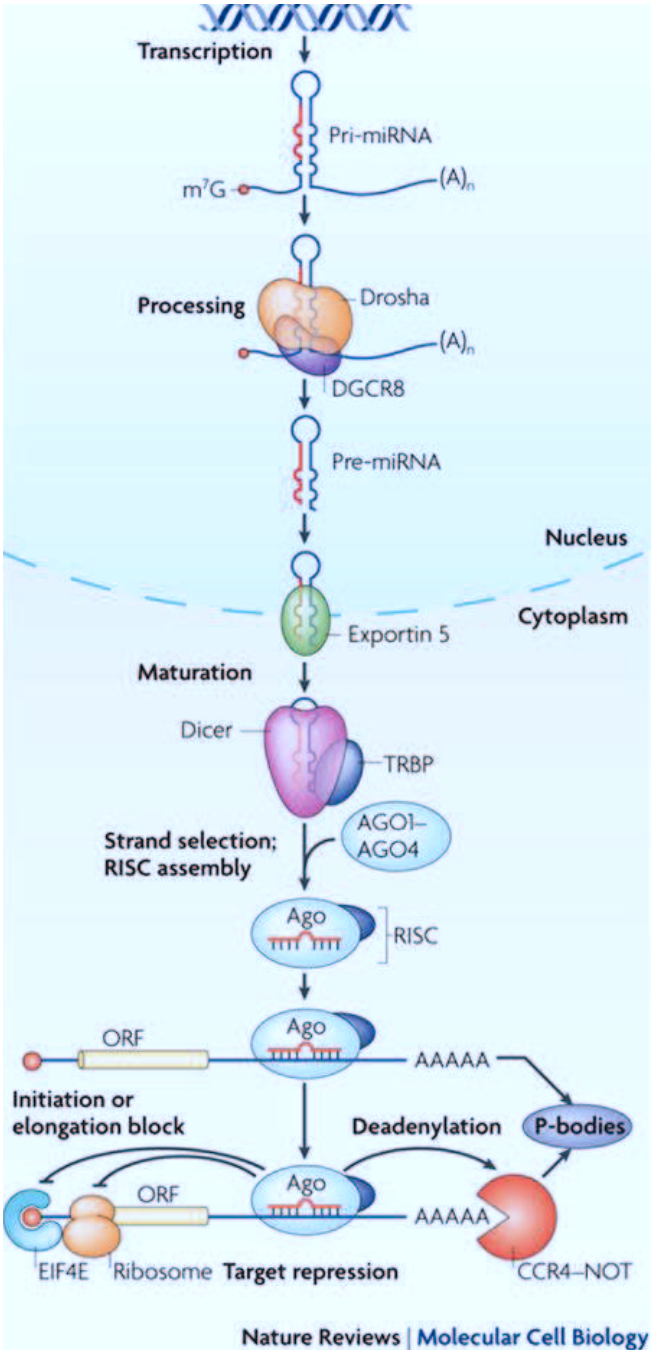


Figure 5 The biogenesis of microRNAs. MicroRNAs (miRNAs) are transcribed by RNA polymerase II (Pol II) as primary transcripts (pri-miRNAs) in the nucleus, which are capped (7MGpppG) and polyadenylated (AAAAA). Each pri-miRNA contains one or more hairpin structures that are recognized and processed by the microprocessor complex, which consists of the RNase III type endonuclease Drosha and its partner, DGCR8 (also known as PASHA) (see the figure). The microprocessor complex generates a 70-nucleotide stem loop known as the precursor miRNA (pre-miRNA), which is actively exported to the cytoplasm by exportin 5. In the cytoplasm, the pre-miRNA is recognized by Dicer, another RNase III type endonuclease, and TAR RNA-binding protein (TRBP; also known as TARBP2). Dicer cleaves this precursor, generating a 20-nucleotide mature miRNA duplex. Generally, only one strand is selected as the biologically active mature miRNA and the other strand is degraded. miRNA duplex is loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC), which includes the Argonaute (Ago) proteins, and the mature single-stranded miRNA is preferentially retained in this complex. The mature miRNA allows the RISC to recognize target mRNAs to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation. However, recent evidence indicates that miRNAs that bind to mRNA targets with imperfect complementarity might also affect on mRNA stability. In particular, perfect base pairing between the seed sequence of the miRNA (from the second to the eighth nucleotide) and the seed match sequences in the mRNA 3′ UTR are crucial. The RISC can inhibit the expression of the target mRNA through two main mechanisms that have several variations: removal of the polyA tail (deadenylation) by fostering the activity of deadenylases (such as CCR4–NOT), followed by mRNA degradation; and blockade of translation at the initiation step or at the elongation step; for example, by inhibiting eukaryotic initiation factor 4E (EIF4E) or causing ribosome stalling RISC-bound mRNA can be localized to sub-cytoplasmatic compartments, known as P-bodies, where they are reversibly stored or degraded (Masafumi et al. 2010).

1.2.2 miRNAs as tumor suppressors (TSmiRs)

Like a protein-coding gene, a miRNA can act as a tumor suppressor (TSmiRs) when its function loss can initiate or contribute to the malignant transformation of a normal cell. The loss of function of a miRNA could be due to several mechanisms, including genomic deletion, mutation, epigenetic silencing, and/or miRNA processing alterations (Calin et al. 2002, Calin et al. 2005, Saito et al. 2006, Nakamura 2007). For example, the miR-15b and miR-16 clusters at 13q14 were identified as the first TSmiRs because they are deleted and/or downregulated in about 68% of B-cell chronic lymphocytic leukemia (CLL) samples (Bullrich et al. 2001, Doehner et al. 2000). MiR-15b and miR-16 have been shown to control the expression of VEGF, a key proangiogenic factor involving in tumor angiogenesis (Hua et al. 2006). In addition, miR-15b and miR-16 also induce apoptosis of leukemic cells by affecting the antiapoptotic protein Bcl-2 (Calin et al. 2005, Cimmino et al. 2006) (Tabella 3). Furthermore, ectopic expression of *miR-16* negatively regulates cell growth and cell cycle progression and induces apoptosis in several human cancer cell lines and in a leukemic xenograft model (Linsley et al. 2007, Calin et al. 2008).

Reduced expression of another group of tumor suppressive miRNAs, the let-7 family members, is a common genetic event in non-small cell lung cancer and is associated with a poor prognosis. The let-7 family is able to negatively regulate let-60/RAS, providing a possible mechanism for let-7 deficiency in cancers (Johnson et al. 2005, Akao et al. 2006). In addition, let-7 regulates late embryonic development by suppressing the expression of a number of genes, such as c-Myc and RAS, and the embryonic gene, high-mobility group A2 (HMGA2) (Lee and Dutta 2007). Let-7 family induces cancer stem cell differentiation in breast cancer (Yu et al. 2007).

MiRNAs not only are regulated by DNA methylation but also modulate DNA methylation in cancer by interfering with the DNA methylation machinery (Garzon R et al. 2008). For example, it has been reported that the *miR-29* family, which includes targets *DNMT3A* and *-3B*, induces global DNA hypomethylation and tumor suppressor gene re-expression in lung cancer (Garzon R et al. 2008). Interestingly, *miR-29*s are downregulated in lung cancer patients, and an inverse correlation was found between *miR-29b* and *DNMT3B* expression, suggesting that the downregulation of this miRNA may contribute to increased DNMT3 levels, as well as hypermethylation and silencing of tumor suppressor genes in lung cancer (Yanaihara et al. 2006, Fabbri M et al. 2007).

Taken together, reduction of TSmiRs expression in cancers activates the oncogenic genes and promotes the tumor initiation and progression. Many other miRNAs are believed to act as tumor suppressors, although the evidence supporting those claims is merely correlative. Substantial experimental data are lacking, and miRNA knockout mice that develop or are predisposed to cancer have not been yet reported. It is noteworthy that most of the miRNAs with a

clear tumor suppressor role (e.g., *miR-15b and 16*, *miR-29*s, and *let-7*) have more than one genomic location, and although they are transcribed from different precursors, the mature miRNA is identical. The presence of more than one genomic copy of the miRNA could be an evolutionarily conserved mechanism to preserve function of an important miRNA if one allele is deleted or silenced.

1.2.3 miRNAs as oncogenes (Oncomir)

The list of miRNAs that function as oncogenes is short, but the evidence for their role in cancer is very strong (Tabella 3). *MiR-155* was one of the first described (Metzler et al. 2004, Kluiver et al. 2005). It is embedded in a host noncoding RNA named the B cell integration cluster (*BIC*) and is located in chromosome 21q23. A previous study showed the ability of *BIC* to cooperate with *c-myc* in oncogenesis. The coexpression of *c-myc* and *BIC*, either singly or pairwise, in cultured chicken embryo fibroblasts using replication-competent retrovirus vectors caused growth enhancement of cells (Tam et al. 2002). Several groups have shown that *miR-155* is highly expressed in pediatric Burkitt lymphoma (Metzler et al. 2004.), Hodgkin disease (Kluiver et al. 2005), CLL (Calin et al. 2005), AML (Garzon et al. 2008), lung cancer (Yanaihara et al. 2006), and breast cancer (Iorio et al. 2005). Little is known, however, about the regulation of *miR-155/BIC* and the mechanism of its overexpression in cancer. A role for this miRNA in early leukemogenesis was proven in a transgenic mouse model with a B cell–targeted overexpression of *miR-155*, which underwent a polyclonal preleukemic pre–B cell proliferation followed by full-blown B cell malignancy (Tabella 3) (Costinean et al. 2006).
The *miR-17-92* cluster, which comprises six miRNAs (*miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1*, and *miR-92-1*), is located within 800 base pairs in the noncoding gene C13orf25 at 13q31.3. This region is frequently amplified in follicular lymphoma and diffuse large B cell lymphoma (Ota A et al. 2004). Members of the *miR-17-92* cluster are highly expressed in a variety of solid tumors and hematological malignancies, including cancers of the breast, colon, lung, pancreas, prostate, and stomach as well as lymphomas **(**Tabella 3) (Mendell 2008). These miRNAs promote proliferation, inhibit apoptosis, induce tumor angiogenesis, and cooperate with *c-myc* to cause lymphoma in mice (Mendell 2008). Interestingly, the *miR-17-92* cluster is transactivated by *c-myc*, an oncogene that is frequently activated in cancer (O'Donnell KA et al. 2005). The effects of this cluster's expression on cell cycle and proliferation are partly due to its regulation of E2F transcription factors, which are critical regulators of the cell cycle (O'Donnell KA et al. 2005). Genes in the E2F family, particularly *E2F1*, *E2F2*, and *E2F3,* activate multiple genes resulting in cell cycle progression from G1 to S phase. Conversely, both *E2F1* and *E2F3* can activate the *miR-17-92* cluster, establishing a regulatory loop. Critical also to the antiapoptotic effects of this cluster is the down-modulation of two

validated targets: the antiapoptotic protein Bim and the tumor suppressors *PTEN* and *p21* (Ventura et al. 2008, Petrocca et al. 2008). The current theory is that like *miR-155*, this cluster induces lymphoid proliferation that predisposes to secondary genetic abnormalities that will ultimately become a full-blown malignancy. Deletion of the *miR-17-92* genomic locus has been described in 16.5% of ovarian cancers, 21.9% of breast cancers, and 20% of melanomas (Mendell 2008, , Hossain et al. 2006). These observations raise the question whether there is a fine post-transcriptional mechanism that regulates the expression of individual miRNAs in this cluster. In addition, these data support a tumor suppressor role for *miR-17-5p*, which seems to contradict extensive data consistently showing upregulation of this cluster in cancer (Volinia et al. 2006). This dual role, oncogene and tumor suppressor, has also been described in protein-coding genes involved in the pathogenesis of cancer, such as *TP53* (Lane and Benchimol 1990). It is possible that a miRNA can act either as an oncogene or as a tumor suppressor depending on the tissue and its transcriptome, including the miRNA targets expressed in that particular tissue.

MicroRNA	Expression in patients	Confirmed targets	Experimental data	Function
miR-15a miR-16-1	downregulated in CLLa	Bcl-2, Wt-1	induce apoptosis and decrease tumorigenicity	TSa
let-7 (a,-b,-c,-d)	downregulated in lung and breast cancer	RAS, c-myc, HMGA2	induce apoptosis	TS
miR-29 (a,-b,-c)	downregulated in CLL, AMLa (11q23), lung and breast cancers, and cholangiocarcinoma	TCL-1, MCL1, DNMT3s	induce apoptosis and decrease tumorigenicity	TS
miR-34 (a-b-c)	downregulated in pancreatic,colon and breast cancers	CDK4, CDK6, cyclinE2, E2F3	induce apoptosis	TS
miR-155	upregulated in CLL, DLBCL,a FLT3-ITDa AML, BL,a and lung and breast cancers	c-maf	induces lymphoproliferation, pre-B lymphoma/leukemia in mice	OGa
miR-17-92 cluster	upregulated in lymphomas and in breast, lung, colon, stomach, and pancreas cancers	E2F1, Bim, PTEN	cooperates with c-myc to induce lymphoma in mice, transgenic miR-17-92 develop lymphoproliferative disorder	OG
miR-21	upregulated in breast, colon,pancreas, lung, prostate, liver, and stomach cancer; AML(11q23); CLL; and glioblastoma	PTEN, PDCD4, TPM1	induces apoptosis and decreases tumorigenicity	OG
miR-72/ miR-373	upregulated in testicular tumors	LATS2	promote tumorigenesis in cooperation with RAS	OG

Tabella 3 MiRNAs with experimental data supporting a tumor suppressor or oncogene function in cancer

1.2.4 miRNAs in tumor invasion and metastasis

Very few studies have addressed the role of miRNAs in tumor invasion and metastasis. For example it has been found that *miR-10b* was upregulated in metastatic breast cancer cells with respect to the primary tumors (Ma et al. 2007). It has been reported that enforced expression of *miR-10b* in nonmetastatic breast tumor cells positively regulated cell migration and invasion and that the level of *miR-10b* expression in primary breast cancer tissues correlated with clinical progression. Using a different approach, Huang et al. analyzed a nonmetastatic breast cancer cell line migration after transduction with a miRNA expression library (Huang et al. 2008). The authors identified two miRNAs, *miR-373* and *miR-520c*, that stimulated cancer cell migration and invasion in vitro and in vivo by blocking the adhesion molecule CD44. A significant upregulation of *miR-373* and negative correlation with CD44 expression was found in breast cancer patients with metastasis.

1.2.5 miRNA profiling to improve cancer diagnosis and therapy

It has been shown that miRNAs can differentiate between tissue types with high accuracy (Lu et al. 2005). Therefore, MiRNA profiling could represent an invaluable tool to classify tumors that represent diagnostic challenges. The discovery of distinctive miRNA signatures will likely improve the molecular classification of cancer. Several studies have shown that miRNA expression is predictive of outcome in solid tumors and hematological malignancies (Calin et al. 2005, Garzon et al. 2008, Takamizawa et al. 2004, Schetter et al. 2008). However, further studies will be needed to test whether miRNAs could be used to better stratify patients for treatment. Because aberrant miRNA expression is now recognized as a common feature of cancers, specific miRNAs are being considered as potential new biomarkers for cancer diagnosis and prognosis (Yu et al. 2008).
On the other hand, since loss of function or gain of function of miRNAs contributes to the cancer initiation and progression, correction of these miRNAs and their regulated gene network could change the behavior of cancer cells, thereby making them a promising target for new drug development. There are several reasons to pursue a miRNA based therapeutic approach. First, a single miRNA can have many targets that are involved in different oncogenic pathways. For example, *miR-29b* targets MCL-1 (apoptosis) and DNMT3A and -3B (methylation); *miR-181* targets BCL-2 (apoptosis), TCL-1 (AKT pathway), and CD69 (adhesion); and *miR-17-92* targets the E2F family (cell cycle), Bim (apoptosis), and angiogenesis. Therefore, modulating the level of a single miRNA could eventually affect many pathways at the same time. Second, since a small group of miRNAs, including *miR-155*, *let-7a*, *miR-21*, and the *miR- 17-92* cluster, are consistently deregulated in a wide variety of hematological malignancies and solid tumors, developing strategies to silence

or re-express these miRNAs will likely affect several groups of patients. Third, as a proof of principle, preliminary data indicate that using cholesterol-modified antisense oligonucleotides to the mature miRNAs (named antagomirs[2]) is an effective approach to silence miRNA expression in mice (Krutzfeldt et al. 2005). This could be a valuable approach to silence miRNAs upregulated in cancer, such as *miR-155* or *miR-21*. However, the use of cholesterol-based oligonucleotides could be too toxic for humans. Further research is needed to determine the best formulation. In addition, precise delivery to the cancer cell is needed to avoid unwanted miRNA effects that could result from targeting important genes in other healthy tissues. Promising miRNA formulations should be further evaluated by detailed pharmacokinetics and pharmacodynamics studies in animal models.

Experimental evidence demonstrates that the modulation of specific miRNA alterations in cancer cells using miRNA replacement[3] or anti-miRNA technologies can restore miRNA activities and repair the gene regulatory network and signaling pathways, in turn, reverse the phenotype of cancerous cells (Wu 2011). Numerous animal studies for miRNA-based therapy offer the hope of targeting miRNAs as alternative cancer treatment. Developing the small molecules to interfere with miRNAs could be of great pharmaceutical interest in the future. This approach has been used, for example, to increase Let-7 expression, which is significantly reduced in lung cancer cell lines and lung cancer tissues (Takamizawa et al. 2004).

Interestingly, specific miRNA is capable of reprogramming the cancer cells into a pluripotent embryonic stem cell-like state (mirPS), which could be induced into tissue-specific mature cell types (Wu 2011).

[2]Antagomir is a member of a novel class of chemically engineered oligonucleotides, which are efficient and specific silencers of endogenous miRNAs. They are synthesized from a hydroxyprolinol linked cholesterol solid support and 2'-*O*Me phosphoramidites.

[3]miRNA replacement therapy is a strategy that consist of adding the miRNAs missing, (for example miRNA-mimic oligos or vector-based miRNA expression) in the cancer cells to restore their "normal" functions.

2. AIM OF THE STUDY

Thyroid carcinomas are one of the most common malignancies of the endocrine system. Well-differentiated thyroid carcinomas include papillary (PTC) and follicular (FTC) carcinomas and the latter is usually a more aggressive form of cancer than the more common papillary type. Unlike PTC, it can be difficult to diagnose FTC without performing surgery because there are no characteristic changes in the way the thyroid cells look. Therefore, the only way to tell if a follicular neoplasm is a cancer, is to look at the entire capsule around the nodule and see if there is any sign of invasion. This difficulty in diagnosis is one of the most frustrating areas for physicians who study thyroid disease, because its surgery is often the only way of definitively diagnosing a thyroid nodule. The follicular variant of papillary thyroid carcinoma (FVPTC) is believed to behave in a clinical manner similar to classical papillary cancer and to follow a similar indolent course.

The setting up of molecular assays to analyze clinical tissues in the diagnosis and management of thyroid cancer would allow far more accurate characterization of individual tumors and maybe the early diagnosis of recurrence.

MicroRNAs have emerged as an important class of short endogenous RNAs that act as post-transcriptional regulators of genes. The analysis of microRNA expression profile in several types of cancer has shown an altered microRNA expression in cancer compared to the respective normal tissues, and several studies have delineated their role in predicting cancer progression and prognosis. Our lab has lately been looking for aberrant miRNA expression profile that distinguishes unequivocally among PTCs, ATCs and normal thyroid tissue (Pallante et al. 2006, Visone et al. 2007, Pallante et al. 2010). Recent results show that miR-191 expression is up-regulated in breast, colon and lung cancers, (Volinia et al. 2006, Xi et al. 2006), whereas it appears downregulated in ovarian cancer (Wynendaele et al. 2010), severe medulloblastoma (Ferretti et al. 2008), in AML with normal karyotype (Garzon e al. 2008) and in melanomas where the low miR-191 expression correlates with a poor prognosis (Caramuta et al., 2010). However, no targets for miR-191 have been so far identified.

Since miR-191 is deregulated in several tumors, the aim of our work has been to investigate the expression of this microRNA in thyroid cancer, in order to evaluate the potential use of mir-191 as an additional diagnostic marker. On the other hand, the aim has been to assess the role that miR-191 has in thyroid cancer cells proliferation and migration, *in vitro*. Moreover, we have tried to unravel the mechanism of action miR-191 by validating bioinformatically predicted targets. Finally, we have looked for an inverse correlation between miR-191 and its target expression in thyroid cancers, in order to validate its pathogenetic role.

3. MATERIALS AND METHODS

3.1 Thyroid tissue samples

Human neoplastic thyroid tissues and normal adjacent tissue or the controlateral normal thyroid lobe were obtained from surgical specimens and immediately frozen in liquid nitrogen. Thyroid tumors were collected at the Service d'Anatomo- Pathologie, Centre Hospitalier Lyon Sud, Pierre Benite, France. Tissue from patients with follicular adenoma (n = 25), follicular thyroid cancer (n = 24), papillary thyroid cancer (n = 15), anaplastic thyroid cancer (n = 8) and follicular variant of papillary thyroid cancer (n = 6) was studied. All thyroid tissue diagnoses were confirmed histologically.

3.2 RNA extraction and quantitative reverse transcription (qRT-PCR)

Total RNA isolation from human tissues and cells was performed with Trizol (Invitrogen) according to manufacturer's instructions. RNA was extracted from paraffin-embedded specimens after pulverizing the tumors by using dry-ice-chilled stainless steel mortar and pestles. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis. qRT-PCR analysis was performed on a panel of FTC samples of human thyroid origin and on cells by using miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). One μg of total RNA was reverse-transcribed. After a 5-fold dilution, 2 μl of the cDNA were amplifyed by using miScript SYBR® Green PCR Kit (Qiagen) following manufacturer's instructions. Primers (miScript Primer Assays; Qiagen) were: Hs_miR-191_1 (amplifying human miR-191) and Hs_RNU6B_2 (amplifying U6, used as loading control). For CDK6 and PTEN mRNA detection, we reverse transcribed total RNA from cell lines by using QuantiTect® Reverse Transcription Kit and the qRT-PCR was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Charleroi, Belgium) and the following primers: CDK6_for 417: 5'-CCGAGTAGTGCATCGCGATCTAA-3' and CDK6_rev 823: 5'-CTTTGCCTAGTTCATCGATATC-3', PTEN_for 1823: 5'-GGGGAAGTAAGGACCAGAGAC-3' and PTEN_rev 1917: 5'-TCCAGATGATTCTTTAACAGGTAGC -3' and G6PD_for: 5'-ACAGAGTGAGCCCTTCTTCAA-3' and G6PD_rev: 5'-ATAGGAGTTGCGGGCAAAG-3'. G6PD was used as an internal standard for data calibration. The $2^{-\Delta\Delta Ct}$ formula was used for the calculation of differential gene expression.

3.3 Cell cultures and stable transfections

The human thyroid carcinoma WRO cells were grown in Dulbecco's

modified Eagle's medium (Gibco Laboratories) containing 10% fetal bovine serum (GibcoLaboratories), 1% glutamine (Gibco Laboratories), and 1% ampicillin/streptomycin (Gibco Laboratories) in a 5% CO_2 atmosphere.
For stable transfections, $5x10^5$ cells were plated in 100 mm plates and 1.8 μg of the pre-miR-191 expressing vector or the empty vector were co-transfected with 0.2 μg CMV-neomicin construct, using FuGENE® HD Transfection Reagent (Roche Diagnostics GmgH, Mannheim, Germany), following manufacturer's instructions.

3.4 Plasmids

The Lentivector-based Micro-RNA Precursor construct expressing pre-miR-191 (PreMiR-191) and the corresponding empty vector (pCDH-CMV-MCS-EF1-copGFP) were purchased by System Biosciences (Mountain View, CA) and stably transfected in WRO cells.
For the CDK6-3'UTR luciferase reporter construct, the 1133 bp 3'-UTR region of CDK6 gene, including 2 the putative binding sites for miR-191, was amplified from WRO cells DNA by using the following primers:
FwCDK6: 5'-CCACCACTCCCAACTTGAC-3' and RevCDK6 5'-ATGCAATCTAGACTAGCACCCAGTAAGACATCC-3'. The amplified fragment was cut with XbaI and cloned into pGL3-Control firefly luciferase reporter vector (Promega, Madison, Wisconsin, USA) at the XbaI site (pGL3-CDK6-3'UTR). Deletions of 8 and 7 bp into each of the seed regions of the CDK6 3'UTR construct were introduced by site directed mutagenesis (Eurofins MWG/Operon, Ebersberg Deutschland), in order to generate the mutant constucts Mut1 and Mut2, respectively (Figure 12). A mutant construct, in which both sites were deleted (Mut1+2) was also generated. Renilla luciferase vector (pRL-CMV), for transient transfection efficiency, was from Promega.

3.5 Proliferation and cell cycle analysis

Proliferation was assayed by using the Cell Titer (Promega) colorimetric assay, following manufacturer's instructions. Briefly, 1X 103 cells were plated in each well of a 96-well. The following day, 20 ul of Cell Titer were added to the medium, and the 96-well has been incubated at 37°C for 1 hour and then read in a 96-well plate reader at 450 nm. The experiment was performed in triplicate and assayed for 10 days, every other day.
For Flow cytometric analysis, WROpCDH and WROmiR-191 stable clones were serum starved and analyzed by flow cytometry after 48 h. Cells were harvested in PBS containing 2 mM EDTA, washed once with PBS, and fixed for 2 h in cold ethanol (70%). Fixed cells were washed once in PBS and treated with 25 μg/ml RNase A in PBS for 30 minutes, then washed again in PBS and stained with 50 μg/ml propidium iodide (Roche, Basel Switzerland) and 0,1%

Nonidet®P 40 Substitute (Sigma-Aldrich, St. Louis, MO). Stained cells were analyzed with a fluorescence activated cell sorter (FACS) Calibur (Becton-Dickinson, Franklin Lakes, NJ, USA), and the data were analyzed using a mod-fit cell cycle analysis program. The measurements were performed in triplicate.

3.6 Protein extraction, western blotting and antibodies

Cells were scraped in ice-cold PBS, and then lysed in ice-cold NP40 lysis buffer (0.5% NP40, 50 mM HEPES (pH 7), 250 mM NaCl,5 mM EDTA, 50 mM NaF, 0.5 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, Complete inhibitor (Roche)). For Western blotting, 30 micrograms of protein from WROpCDH and WRO miR-191 were electrophoresed in 10% SDS–PAGE minigels and transferred onto Hybond C nitrocellulose membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). The nitrocellulose membranes were incubated with primary antibodies anti-CDK6 (sc-177, Santa Cruz Biotechnology Inc., dilution 1: 1000), anti-PTEN (ab32199, Abcam, dilution 1: 500), anti-actin (sc-1616, Santa Cruz Biotechnology Inc., Santa Cruz, CA, dilution 1: 3000) or anti GAPDH (sc-32233, Santa Cruz, dilution 1: 5000) at 4°C overnight, and then washed three times with Tris-Buffered Saline Tween-20 (TBS 0,1% Tween). Afterwards, the nitrocellulose membranes were incubated with horseradish peroxydase (HRP)-conjugated donkey anti-rabbit (GE Healthcare UK Ltd, Buckinghamshire, England) (dilution 1: 3000) or donkey anti-goat IgG (Santa Cruz Biotechnology Inc.) (dilution 1:6000), respectively, for 1 hr at room temperature. After washing with TBST (TBS 0,1% Tween), blots were visualized by using the Western blotting detection reagents (GE Healthcare UK Ltd).

3.7 Dual-luciferase reporter assay

For dual-luciferase reporter assay, 3x105 WRO cells were co-transfected in 6-well plates with the pGL3-CDK6-3'UTR luciferase vector (400 ng) or with the mutated 3'UTR of CDK6 (Mut1, Mut2 and Mut1+2), together with the Renilla luciferase plasmid (4 ng) and the Pre-MiR-191 expressing constructs or the corresponding empty vector (1.6 μg), by using FuGENE HD Transfection Reagent (Roche Diagnostic Corporation, Indianapolis, IN). The pRL-TK control vector expressing Renilla luciferase (Promega) was used for normalization of cell number and transfection efficiency. Firefly and Renilla luciferase activity was measured 48h after transfection using the Dual-Luciferase Reporter Assay System (Promega) by a Lumat LB 9507 apparatus (Berthold Technologies, Bad Wildbad, Germany).

3.8 Cell migration assays

A wound-induced migration assay (scratch assay) was performed as described previously (Angers-Loustau et al. 1999), with some modifications. Briefly, WROpCDH or WROmiR-191 cell clones were plated to form a monolayer, in presence of serum. The cells were then trypsinized, washed in PBS and allowed to attach on vitronectin-coated 6-well plates. Using a sterile yellow tip, a scratch was made in triplicate. Detached cells were removed, and the scratches were monitored for 24 hours under serum-free conditions. Migration rate was quantified by measuring the distance between the edges of wound and compared at t=0 to t=24h. In addition, a more quantitative haptotactic migration assay was performed essentially as described previously (Keely et al. 1995). Briefly, transwell inserts (8 µm pore size) in triplicates were coated on the underside with vitronectin (5 µg/ml). 2 x 104 cells were placed in the upper chamber in absence of serum (DMEM + 1%BSA) and allowed to migrate across inserts for 24 hours at 37°C. Unmigrated cells from the upper chamber were removed, and migrated cells in the lower side of the membrane were stained with crystal violet (0,5% in 25% methanol); after washing with PBS, cells were lysed in 1% SDS and the absorbance at 570 nm was measured. Each set of experiments was performed in triplicate. Statistical analysis of the data was performed using the paired Student's t-test.

3.9 Immunohistochemistry

Five µm-thick paraffin sections were cleared in xylene and alcohol-rehydrated. After microwave antigen retrieval with citrate buffer, slides were treated with a 0,3% solution of hydrogen peroxidase in methanol to block the endogenous peroxidase activity for 30 min and then washed in phosphate buffer solution before immunoperoxidase staining. The slides were then incubated overnight at 4°C in a humidified chamber with the primary antibody (diluted 1:50 in PBS). Tissue sections were covered with biotinylated anti-rabbit/anti-mouse immunoglobulins (Dako LSAB2 System) for 20 min, and then stained with streptavidin labeled with peroxidase; the signal was developed by using as substrate the diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS pH 7.6 for 5 min. After chromogen development, slides were washed, dehydrated with alcohol and xylene, and mounted with cover slips using a permanent mounting medium. Negative controls were performed by omitting the first antibody.
The proportion of malignant cells positively stained was scored from 0 to 3: 0, no positive cells; 1+, ≤10% of positive cells; 2+, 11–50% of positive cells; and 3+, >50% of positive cells.

4. RESULTS

4.1 MicroRNA-191 expression is downregulated in thyroid follicular adenomas and carcinomas

In order to investigate the role of miR-191 in thyroid cancer, we analyzed mir-191 expression in 6 normal thyroid tissues and in a panel of thyroid neoplastic samples, including 25 follicular adenomas, 24 follicular carcinomas, 15 PTCs, 8 ATCs and 6 follicular variant of PTCs (FVPTCs), by performing quantitative stem-loop RT-PCR.
As shown in Figure 6, miR-191 is significantly downregulated in all the follicular adenomas (Figure 6A) and carcinomas (Figure 6B) compared to normal thyroid. However, the miR-191 expression levels are significantly lower in the carcinoma samples with respect to the adenomas. Conversely, in most PTCs, miR-191 expression is upregulated (Figure 6C) and it is upregulated just in 50% ATCs (Figure 6D) in comparison with normal thyroid tissues; interestingly, miR-191 expression in FVPTCs is downregulated (Figure 6E), similarly to what observed for FTCs, even though at a lower extent. This result is suggestive of miR-191 downregulation as a signature for a follicular-type behaviour (such as metastasis and blood invasion).

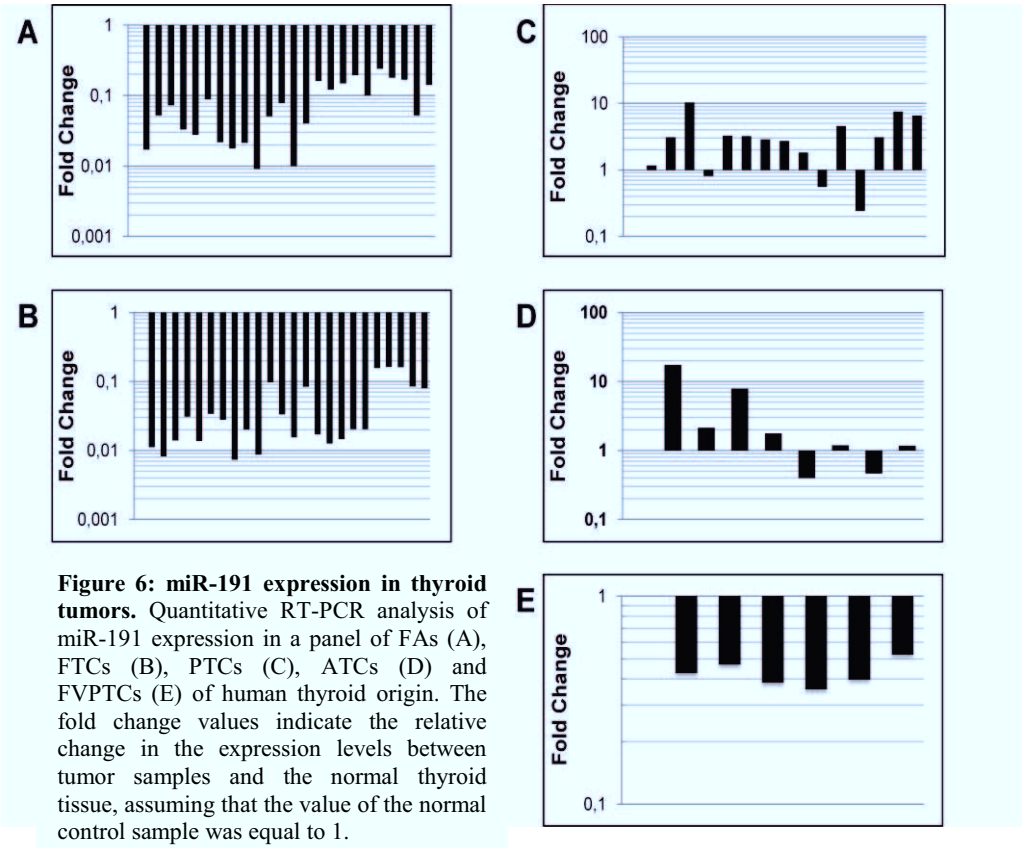


Figure 6: miR-191 expression in thyroid tumors. Quantitative RT-PCR analysis of miR-191 expression in a panel of FAs (A), FTCs (B), PTCs (C), ATCs (D) and FVPTCs (E) of human thyroid origin. The fold change values indicate the relative change in the expression levels between tumor samples and the normal thyroid tissue, assuming that the value of the normal control sample was equal to 1.

4.2 miR-191 is downregulated in human thyroid carcinoma and retrovirally transformed rat thyroid cell lines

Subsequently, we analysed miR-191 expression in a panel of thyroid carcinoma cell lines of different histotypes, including WRO (from follicular carcinoma), FB2 and BCPAP (from papillary thyroid carcinoma), FRO and FB1 (from anaplastic carcinoma), compared to normal thyroid. As shown in Figure 7A, apart from the BCPAP cell line, a drastic reduction in miR-191 expression with respect to normal thyroid tissue was observed in the thyroid carcinoma cell lines, independently from the histotype.
In order to verify whether miR-191 is a more general event in thyroid neoplastic cell transformation, we analysed miR-191 expression in panel of rat thyroid cells deriving from the transformation of normal differentiated thyroid cells, PC Cl 3 and FRTL5 (Figure 7B), with the following viral oncogenes: v-mos (PC 663), v-raf (PC raf), E1A plus v-raf (PC R15 raf), Ki-ras (PC kiki and FRTL5 Ki MSV), v-ras-Ha (PC Ha-ras) and Polyoma Middle T (PC Py), previously generated in our laboratory (Berlingieri et al., 1995). As shown in Figure 7B, all the transformed cell lines showed a drastic reduction in miR-191 expression in comparison to the normal thyroid cells PC Cl3 and FRTL5, suggesting that miR-191 downregulation may be a general event in thyroid cell transformation.

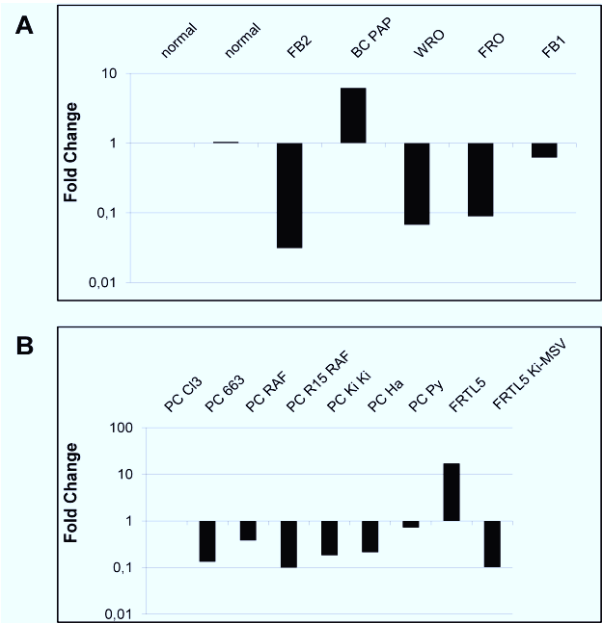


Figure 7: miR-191 expression in human and rat thyroid cell lines. Quantitative RT-PCR analysis of miR-191 expression in (A) human thyroid cell lines compared to normal thyroid and in (B) normal rat thyroid cell lines (PC Cl3 and FRTL5) and rat thyroid cell transformed by various oncogenes (PC 663, PC RAF, PC R15 RAF, PC KiKi, PC Ha, PC Py, FRTL5 Ki-MSV).

4.3 miR-191 expression affects cell shape, cell proliferation and migration of WRO cells

To define the role of miR-191 downregulation in the process of thyroid carcinogenesis we restored miR-191 expression levels in WRO cells by transfecting them with the PreMiR-191 Lentivector-based Micro-RNA Precursor construct, expressing preMiR-191, or with the corresponding empty vector (pCDH-CMV-MCS-EF1-copGFP), together with a plasmid confering neomycin-resistence. After selection, several GFP-positive clones were picked up, amplified and checked for miR-191 expression (Figure 8B), by quantitative RT-PCR. The selected WROmiR-191 cell clones (2 representative clones are showed in Figure 8A) showed an epithelial-like morphology, being more tightly packed, with respect to mock transfected WRO cells and to WRO cells transfected with the control vector (WRO-pCDH), which had a scattered distribution (Figure 8A). The acquisition of an epithelial phenotype is in agreement with a role of tumor suppressor, hypothesized for miR-191.

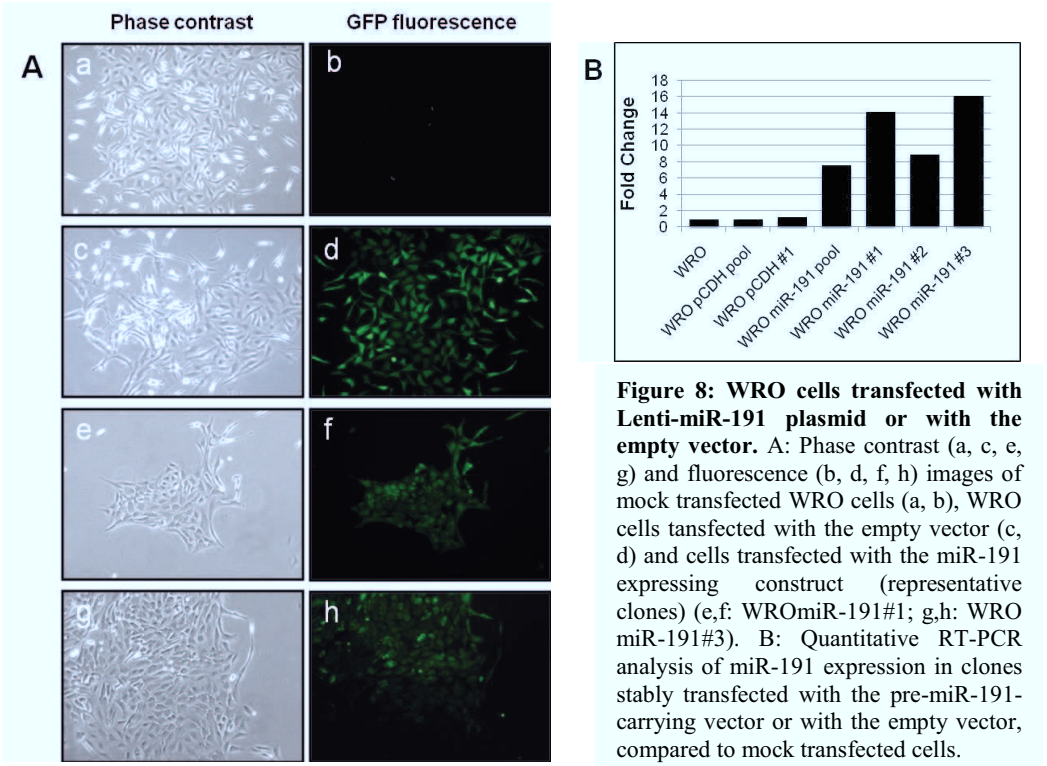


Figure 8: WRO cells transfected with Lenti-miR-191 plasmid or with the empty vector. A: Phase contrast (a, c, e, g) and fluorescence (b, d, f, h) images of mock transfected WRO cells (a, b), WRO cells tansfected with the empty vector (c, d) and cells transfected with the miR-191 expressing construct (representative clones) (e,f: WROmiR-191#1; g,h: WRO miR-191#3). B: Quantitative RT-PCR analysis of miR-191 expression in clones stably transfected with the pre-miR-191-carrying vector or with the empty vector, compared to mock transfected cells.

In order to demostrate the tumor suppressive activity of miR-191, we assessed the proliferation rate in miR-191 and empty vector transfected WRO cells. The analysis of cell growth revealed a lower proliferation rate of the WRO-miR-191 cell clones in comparison with the cells carrying the empy vector (Figure 9A) or with mock transfected cells (Figure 9A).
To better define the effect of miR-191 expression on cell cycle progression, we used flow cytometric analysis (FACS) to measure the DNA content of WRO miR-191 and WROpCDH cell clones. Transition from G1 to S phase was significantly lower in the WROmiR-191 cell clones than in the WROpCDH cells (Figure 9B). In fact, 19% of WROmiR-191 cells were in S phase and 52% in G0/G1 phase, whereas 24% of WROpCDH cells were in S phase and 45% in G0/G1 phase (Figure 9B).
These results indicate that miR-191 overexpression decreases cell proliferation rate of a thyroid carcinoma cell line, retaining the cells in the G1 phase.

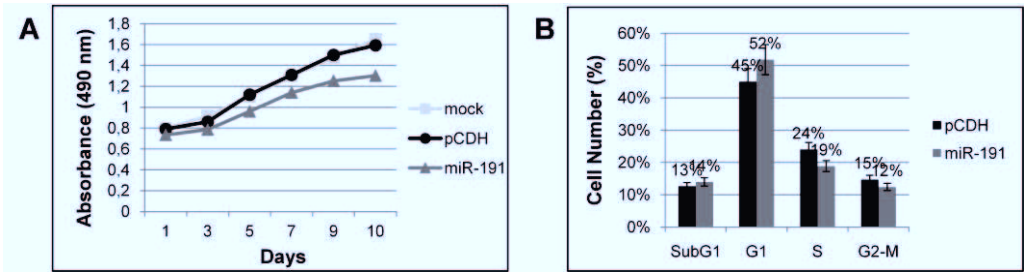


Figure 9: miR-191 reduces proliferation and slows down the G1/S transition in WRO cells. A: The effect of miR-191 on WRO cells proliferation was assayed by Cell Titer. The diagram showes a reduction in proliferation in miR-191 transfected cells compared to cells transfected with the empty vector and to mock transfected cells. B: The effect of miR-191 on cell cycle was assayed by Flow cytometric analysis of WRO cells stably transfected with the miR-191 or the control vector.

Since WROmiR-191 cells appeared less scattered on the cell culture plate, we evaluated cell motility in WROmiR-191 and control cells, by performing "scratch" and transwell chamber assays. As shown in Figure **10**A, the migration (% wound closure) was comparable in WROmiR-191 and control cells, when assayed on plastic cell culture plates; conversely, when the scratch was performed on vitronectin treated plates, the motility of the WROmiR-191 cells was reduced (about 31%) compared with control cells (Figura 10A).
Furthermore, we measured the invasive ability of the WRO-mir191 and WRO-pCDH cells by performing a transwell migration assay and as shown in Figure 10B, the ability of WRO cells to invade and cross the polycarbonate Nucleopore filters was reduced by the restoration of the miR-191 expression in WRO cells. This effetct was more evident on vitronectin (VN) coating (Figure 10B). In this case the ability of WRO-mir191 cells to cross the membrane was

reduced by 30% compared to WROpCDH cells. This differential behaviour in presence or absence of VN suggest that at least one miR-191 target has to be implicated in the $\alpha_v\beta_3$–integrin-mediated inhibition.

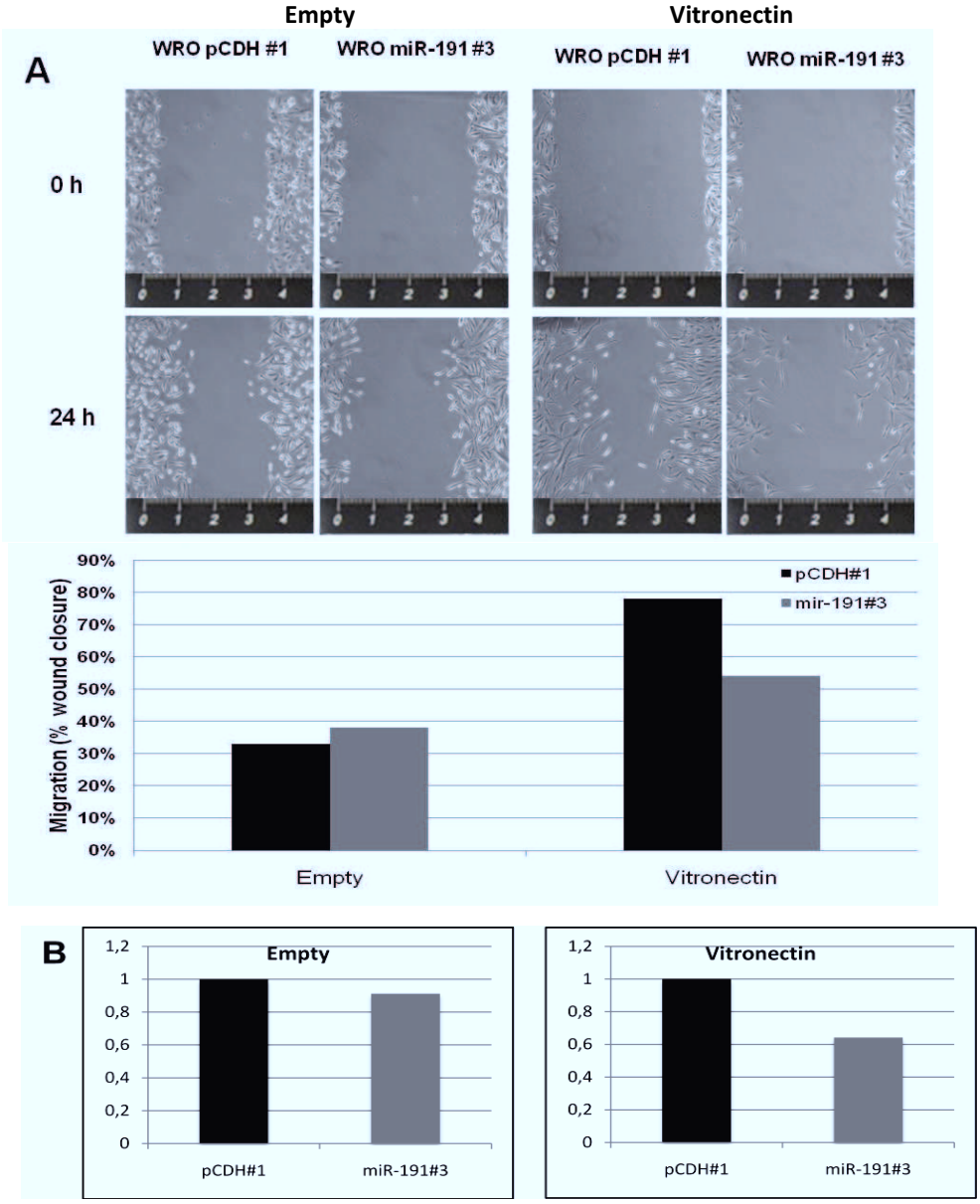


Figure 10: miR-191 expression reduces cell migration on Vitronectin. A: A wound-induced migration assay, in presence or absence of a vitronectin matrix coating, was performed using WROpCDH and WROmiR-191 cell clones in the absence of serum or growth factors. The extent of cell migration into the wounded area was photographed under phase-contrast microscopy at 0 hour and after 24 hours. Magnification, x100. Data shown are representative of three separate experiments and are diagrammed in the lower panel. Migration rate was quantified by measuring the distance between the edges of wound and compared at t=0 to t=24h. The migration rate is represented as percentages in graphics illustrating the reduction of wound closure in WROmiR-191 cell clones, plated on VN. B: Diagram of transwell assays, performed in presence (right panel) or absence (left panel) of VN coating. Migration is expressed as percent of adsorbance at 570 nm, compared to control cells.

4.4 miR-191 downregulates CDK6 expression

MicroRNAs act on many cellular processes by modulating the expression of mRNA targets. Therefore, to investigate the mechanism by which miR-191 may affect tumorigenesis, cell proliferation and migration, we used bioinformatic tools (mirGen, Target Scan, Pictar and miRanda), to search for potential mRNA targets. We identified several genes involved in DNA replication, cell cycle control and cell death regulation that were potentially targeted by miR-191. Among them, we selected the CDK6 gene, which encodes a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition (Serrano et al. 1993). We selected this gene also because the activity or amount of CDK6 is elevated in some cancer cells and lymphomas (Costello et al. 1997, Timmermann et al. 1997, Corcoran et al. 1999). Two sites matching the miR-191 seed sequences were predicted in the 3'UTR of the CDK6 gene (Figure 11A). To validate the influence of miR-191 on the CDK6 target, we evaluated the expression of CDK6 protein levels in WRO, WROpCDH and WROmiR-191 cell clones, by Western blot analysis (Figure 11B). The introduction of miR-191 significantly decreased by almost 50%) CDK6 protein level.
Interestingly, there were no significant changes in CDK6 mRNA levels in cells transfected with the miR-191 or with the empty vector (Figure 11C). These results are consistent with post-transcriptional regulation of the CDK6 protein by miR-191 and exclude its role in CDK6 mRNA degradation.

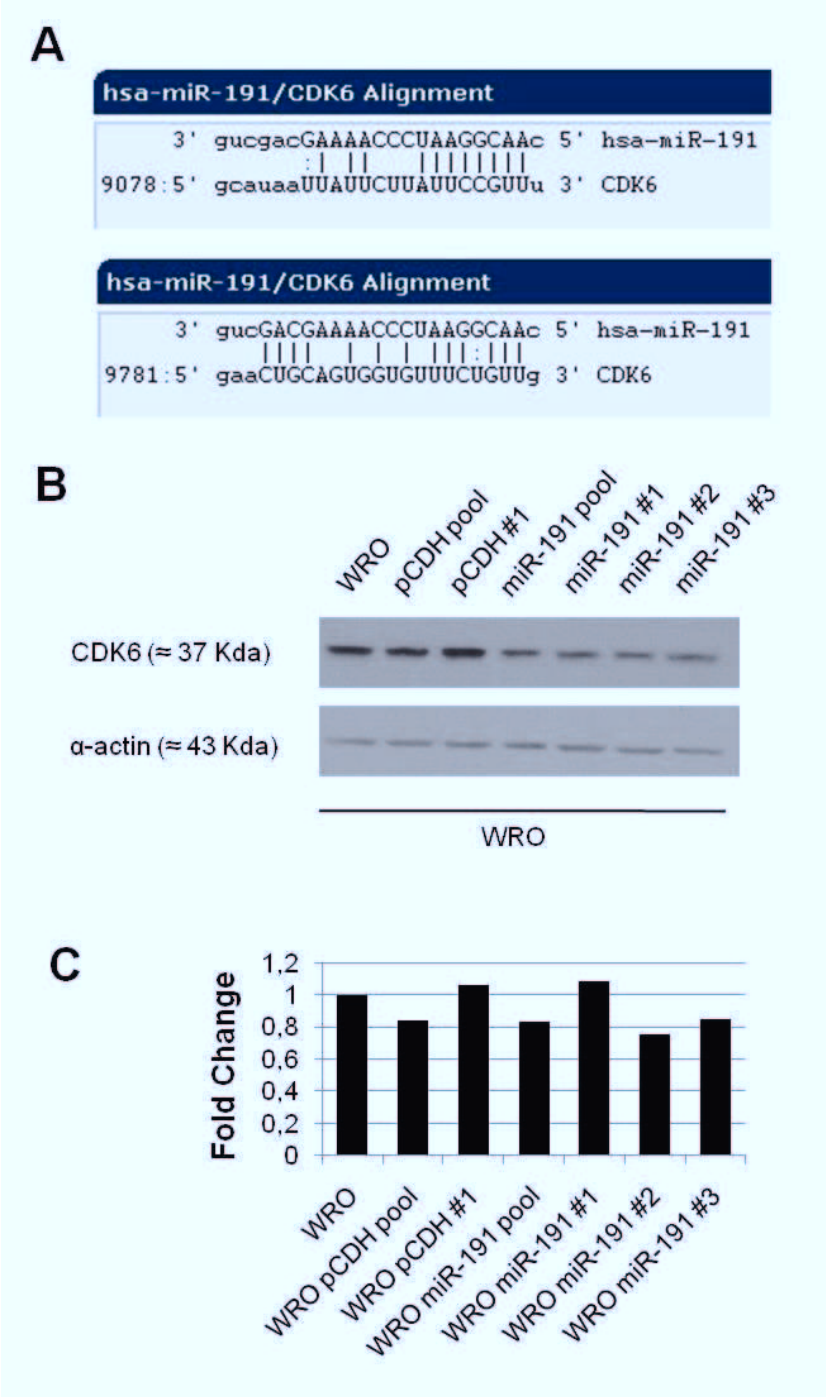


Figure 11: MiR-191 expression downregulates CDK6 protein, but not mRNA expression. A: Alignment of miR-191 with the putative seed sequences in the CDK6 3'-UTR. B: Immunoblots for CDK6 in WRO, WROpCDH and WROmiR-191 cell clones. Immunoblot for α-actin has been used as loading control. C: qRT-PCR analysis of CDK6 mRNA in samples as in B. The fold change values indicate the relative change in CDK6 mRNA expression levels between control and miR-expressing cells, normalized with G6PD.

To determine whether direct interaction between miR-191 and CDK6 mRNA caused the decrease in CDK6 protein expression, we performed luciferase assays. To this aim, we inserted a sequence corresponding to the CDK6 3'UTR downstream of the luciferase gene, by cloning into the pGL3-control vector. This reporter vector was co-transfected into WRO cells, together with the miR-191 expressing construct or the corresponding empty vector. Luciferase activity, measured 48 h later, resulted significantly reduced by miR-191 expression, in a dose-dependent manner, as compared with cells transfected with the control vector (Figure 12B). These results indicate that miR-191 interferes with CDK6 mRNA translation by directly interacting with the 3'UTR. This conclusion is supported by similar experiments in which we used a reporter construct carrying target sites modified by 8 nucleotides-deletion in one or both sites. MiR-191 ability of downregulating luciferase activity was lost when either or both sites were mutated, suggesting that miR-191 acts through interaction with both sites (Figure 12B).
These data show that miR-191 directly downregulate CDK6 protein expression, by interacting with two seed sequences in its 3'UTR.

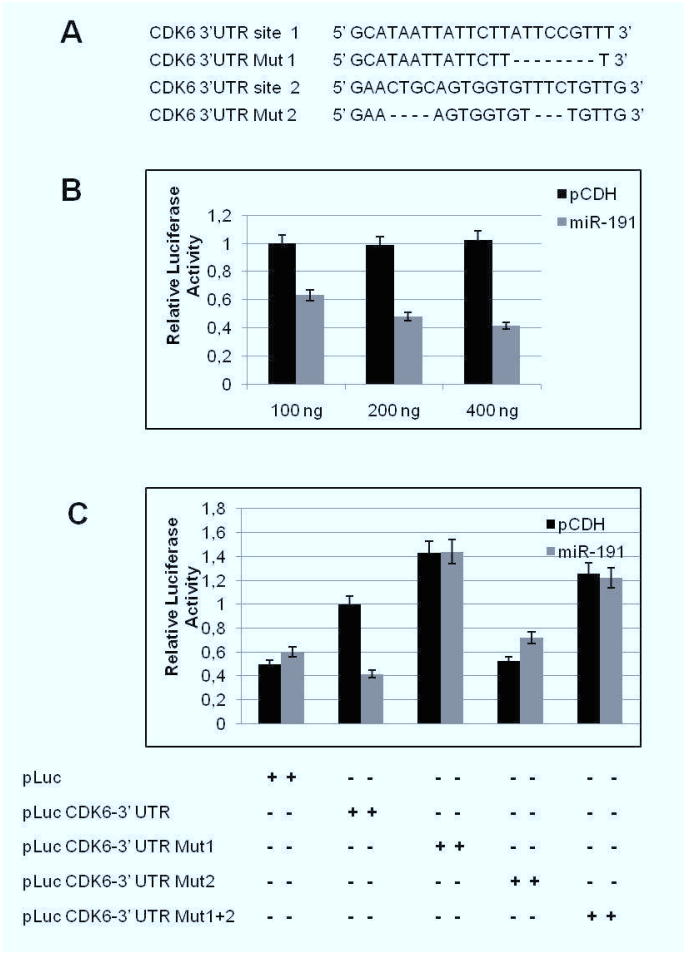
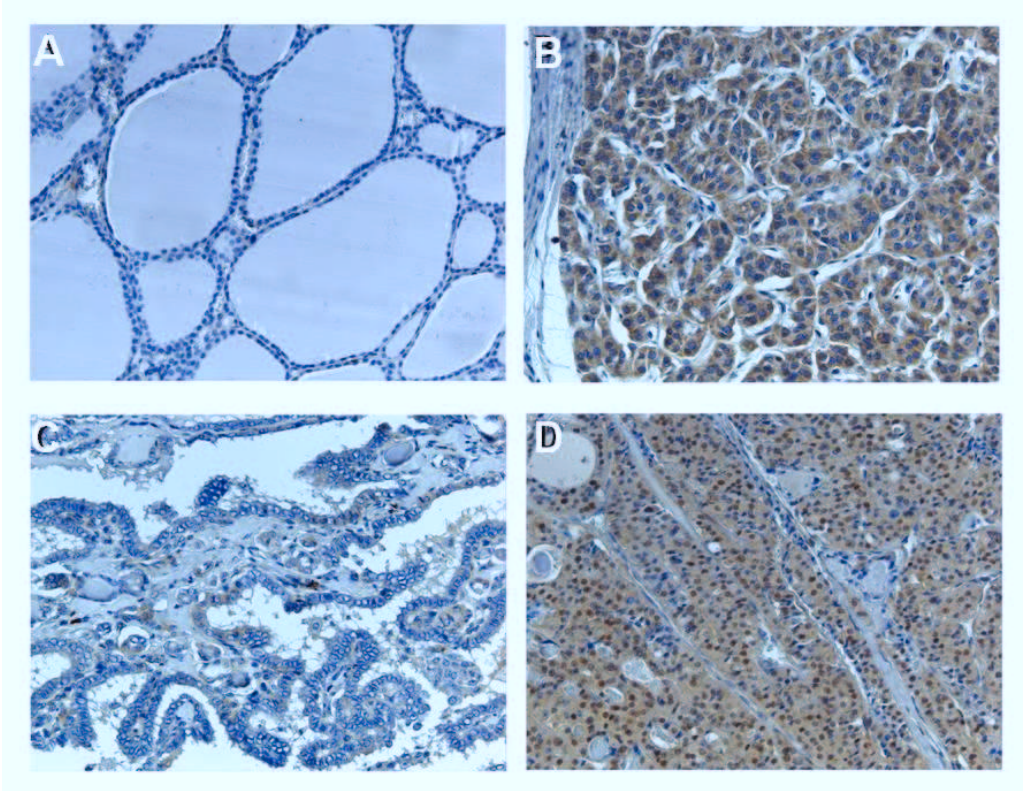


Figure 12: The 3'-UTR of CDK6 is a target of miR-191. A: The two seed sequences for miR-191 in CDK6-3'UTR, with the respective mutants (Mut1 and Mut2). Mut1+2 contained both modifications. B: Relative luciferase activity in WRO cells transiently transfected with increasing amounts of Lenti-miR-191 or the empty vector (pCDH). The relative activity of firefly luciferase expression was standardized to a transfection control using Renilla luciferase. C: Relative luciferase activity in WRO cells transiently transfected with the Lenti-miR-191, was assayed in presence of the wild-type CDK6 3'UTR sequence or with the mutants (Mut1, Mut2 and Mut1+2) and compared to the luciferase activity measured with the empty (pCDH) vector.

4.5 CDK6 overexpression inversely correlates with miR-191 in thyroid tumors

To understand if the CDK6 targeting by miR-191 has a pathological role in thyroid tumorigenesis, we evaluated the expression of the CDK6 protein in 20 thyroid tissue specimens (5 normal thyroid tissues, 5 follicular adenomas, 5 follicular carcinomas, and 5 papillary carcinomas) by immunohistochemical analysis. As shown in Figure 13, we found that CDK6 is not detectable in thyrocytes from normal glands (Figure 13A) (5 out of 5) and is weakly expressed only in 1 out of 5 PTCs (Figure 13C), with few focally distributed cytoplasmic-positive tumour cells (20%). In contrast, we detected clear cytoplasmic and nuclear CDK6 stainings in 4 out of 5 follicular adenomas (Figure 13B), in more than 50% cells and in 3 out of 5 follicular carcinomas (Figure 13D). The immunohistochemical results are summarized in the table of Figure 13. Thus, CDK6 expression is inversely related to miR-191 levels in thyroid carcinomas and this inverse correlation has, at least in part, a pathogenic role in thyroid cancerogenesis. Interestingly, PTCs showed very faint CDK6 immuostainings, identifying the miR-191 downreguation/CDK6 overexpression as peculiar to follicular thyroid cancers.



Histotype	Number of cases	Number of samples with the indicated grade of positivity			
		0	1+	2+	3+
Normal Thyroid tissues	5	5	0	0	0
Follicular Adenomas	5	0	0	1	4
Papillary Carcinomas	5	0	4	1	0
Follicular Carcinomas	5	0	0	2	3

Figure 13: Immunohistochemical analysis for CDK6 in normal and malignant thyroid tissues. Representative anti-CDK6 immunostainings of a normal thyroid (A), a follicular thyroid adenoma (B), a thyroid papillary carcinoma (C) and thyroid follicular carcinoma (D). (Magnification: 200 x).
E: Table summarizing the results of CDK6 immunohistochemistry on thyroid tumors. The proportion of malignant cells positively stained was scored from 0 to 3: 0, no positive cells; 1+, ≤10% of positive cells; 2+, 11–50% of positive cells; and 3+, >50% of positive cells.

5. Discussion

Mature functional miRNAs of approximately 22 nucleotides, that are generated from long primary miRNA (pri-miRNA) transcripts, control gene expression at the post-transcriptional level by degrading or repressing target mRNAs. Some miRNAs aberrantly expressed in cancer have been well documented (Chan et al., 2005; Pallante et al., 2006; Garzon et al., 2008). They were found to regulate the expression of signaling molecules, such as cytokines, growth factors, transcription factors, and proapoptotic and antiapoptotic genes.

In our study we have investigated the role of miR-191 in thyroid neoplasias. We have reported that miR-191 was significantly downregulated in TA and FTC with a fold change higher in FTCs. Conversely, miR-191 was upregulated in PTCs, whereas it was up- or downregualted in ATCs, suggesting that miR-191 downregulation is specific for the pathway leading to follicular neoplastic phenotype. Even though the opposite pattern of miR-191 expression in FTC and PTCs might appear contradictory, a similar situation has been already described by Aldred and colleagues (Aldred et al., 2004), which found that IGFBP6 is overexpressed in PTCs and downregulated in FTCs. Moreover, these Authors found that PTCs were characterized by the overexpression of CITED1 (Cbp/p300-interacting transactivator): a consensus sequence for p300 and cEBP/beta is found in the miR-191 promoter region (Battista S., unpublished results) and might be responsible of miR-191 upregulatio in PTCs. Downregulation of miR-191 in TA (thyroid adenoma) with respect to normal thyroid indicated that miR-191 downregulation may be an early event in thyroid carcinogenesis. This hypothesis is consistent with data showing that miR-191 is found downregulated in lungs of rats exposed to environmental cigarette smoke (ECS) (Izzotti et al., 2009) and in pre-cancerous azoxymethane-(AOM)-induced colon lesions in rats (Davidson et al., 2009). This precocious downregulation of miR-191 may be suggestive not just of an early event, but also, on the other hand, of a protective, anti-cancer effect associated to its expression. Such a role is supported by data showing that the chemoprotective effect of nutritional bioactives (such as fish oil) is mediated by the up-regulation of miR-191 (and other miRNAs) (Davidson et al., 2009).

The involvement of miR-191 downregulation in thyroid carcinogenesis seems validated by functional studies. Indeed, we show that miR-191 possesses anti-proliferative potential and regulates cell cycle transition from G1 to S in thyroid follicular carcinoma derived cell lines; in these cells, miR-191 also induced morphological changes and reduced migration. In order to identify the mechanism by which miR-191 may affect part or all these processes, we looked for possible targets. We identified CDK6 mRNA as a miR-191 target. Indeed, miR-191 overexpression reduces the CDK6 protein levels and the luciferase activity of about 50%. Moreover, the deletion of few nucleotides in either or both the seed sequences in CDK6 3'-UTR leads to the inhibition of the miR-191 activity.

Consistently, in this study and in others, an increased CDK6 expression is observed in thyroid carcinomas (Kebebew et al., 2006). Here we show that CDK6 overexpression is tightly related to miR-191 downregulation in human thyroid tumors, being PTCs almost negative. These data confirm the functional role of miR-191 in thyroid cancerogenesis, suggesting that the inverse correlation between miR-191 and CDK6 expression may be indicative of a follicular phenotype.

CDK6 has proto-oncogenic properties, and its activity is carefully regulated at multiple levels, as misregulation can induce unscheduled proliferation, and genomic and chromosomal instability. CDK4/CDK6, in complex with D-type cyclins (D1, D2, or D3), regulate the G1/S phase transition. Elevated activity of CDK6 may result in hyperphosphorylation of the retinoblastoma protein and, therefore, compromise its negative growth-regulatory activity (Harbour et al., 1999). Accordingly, restoration of miR-191 expression produces a cell phenotype similar to that induced by CDK6 inhibition, leading to retention in the G1 phase of the cell cycle and inhibition of cell migration on vitronectin (Alhaja et al., 2005).

The potential use of micro-RNAs in cancer therapy would take advantage of theitr target multiplicity. Indeed, our in vitro preliminary results suggest that replacement therapy with miR-191 might affect, beside the cyclins circle, the PTEN/PI3K/AKT pathway, a pathway identified as an important target for drug discovery (Carnero et al., 2008). In fact, we observed that, whereas WRO cells express low levels of PTEN mRNA and protein, transfection with a miR-191 expressing construct upregulates PTEN both at mRNA an protein level, compared to empty vector transfected cells. Loss of PTEN activity plays a role both in familiar (Cowden disease) and sporadic thyroid carcinomas (reviewed in Paes and Ringel 2008). Mechanisms for its inactivation include intragenic PTEN mutations, loss of heterozygosity (LOH) at the PTEN locus, promoter hypermethylation (mostly in follicular thyroid cancer), inappropriate subcellular compartimentalization.

Since the PTEN-PI3K-AKT pathway appears to play a prominent role in advanced forms of the disease, and hence likely represents an important focus for development of targeted therapies, future experiments will aim at investigating the mechanism of miR-191-induced PTEN expression. Bio-informatically predicted miR-191 targets include genes coding for transcription factors inhibiting PTEN trascription (such as pCAF and FOXO1), as well as protein kinases responsible of PTEN phosphorylation and degradation (Casein kinase 2 and GSK3). One or all of these targets, if validated, might mediate the mir-191 induced PTEN overexpression.

The association between miR-191 and PTEN expression has also important implication concerning lung cancerogenesis. In fact, miR-191 is downregulated in rats treated with environmental cigarette smoke (ECS) (Izzotti et al. 2009). Smoke induces PTEN downregulation both in vivo and in vitro (Shaykhiev et al., 2010) and loss of PTEN expression is a poor prognostic factor for patients with NSCLC (Tang et al 2006).It will be interesting to investigate if PTEN is a

downstream indirect target of miR-191 downregulation in lung cancerogenesis and if a miR-191 replacement therapy might have protective effects.

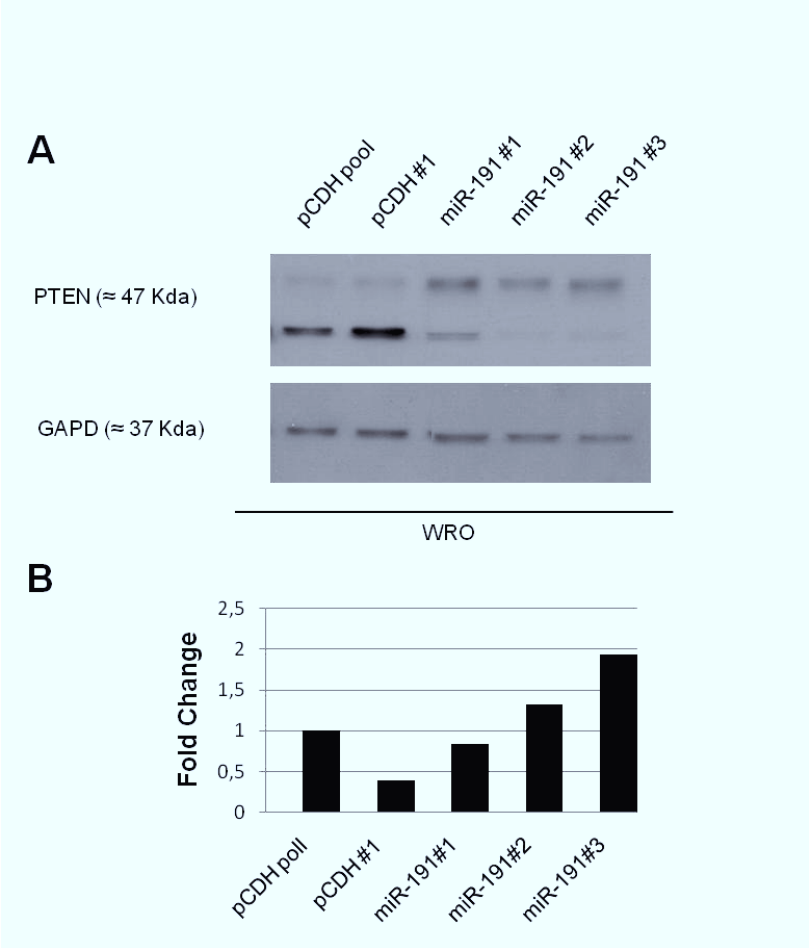


Figura 14: MiR-191 reintroduction induces PTEN overexpression. A: Immunoblots for PTEN in WROpCDH and WROmiR-191 cell clones. Immunoblot for GAPD has been used as loading control. B: qRT-PCR analysis of PTEN mRNA in samples as in A. The fold change values indicate the relative change in PTEN mRNA expression levels between control and miR-expressing cells, normalized with G6PD.

6. CONCLUSIONS

In conclusion the data reported here demonstrate that miR-191 is specifically downregulated in benign and malignant tumors of follicular histotype, with a fold-change that is significantly higher in follicular carcinomas with respect to adenomas. Conversely, in most PTCs and in 50% ATC miR-191 expression is upregulated, whereas, it is downregulated in FVPTCs, similarly to what observed for FTCs, suggesting that miR-191 downregulation may be a signature for a follicular-type behaviour and it could represent an ancillary diagnostic tool to discriminate the follicular neoplastic phenotype.

Consistent with a putative tumour suppressor role of miR-191 in the development of thyroid neoplasias of the follicular histotype, we demonstrate that restoration of miR-191 expression in a follicular thyroid cancer cell line reduces cell growth, migration and cell proliferation rate, retaining the cells in the G1 phase of the cell cycle.

Furthermore, we found that miR-191 negatively regulated the expression of CDK6, protein involved in cell cycle control, at the post-transcriptional level, without mRNA degradation. Therefore, CDK6 targeting might mediate the effect of miR-191 on cell-cycle.

Finally, in follicular adenomas and carcinomas, we immunohistochemically detected CDK6 over-expression and demonstrated that it is inversely related to miR-191 levels in thyroid carcinomas, suggesting that miR-191 may contribute to thyroid cancerogenesis by controlling CDK6 expression.

Moreover, our preliminary data suggest that the reintroduction of miR-191 induces PTEN expression

Even though the mechanism leading to PTEN upregulation needs to be further investigated, we can speculate that miR-191 might exert its anti-oncogenic effect also by targeting the PTEN/PI3K/AKT pathway.

If this double control is confirmed, miR-191 might represent an additional molecule for cancer thyroid therapy

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ACKNOWLEDGMENTS

In the first place I would like to thank my family and all the people who helped me to reach this goal.
This thesis is part out of my work carried out in Fusco's group. In this group, I have had the chance to work with many colleagues and it is a pleasure to express my gratitude to all of them.In particular I am grateful to Prof. Alfredo Fusco for his supervision and guidance in this project.
Very special thanks go to Dr. Sabrina Battista (my GURU!) because I owe her more than she knows.

miR-191 downregulation plays a role in thyroid follicular tumors through CDK6 targeting.

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Running title: miR-191 downregulation in thyroid neoplasias

Key words: miR-191, CDK6, thyroid, cell cycle, follicular carcinoma

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Abstract

miR-191 expression is frequently altered in several neoplasias, being upregulated in some cases, such as pancreatic, colon, lung and prostate carinomas, and downregulated in others, such as severe medulloblastomas and melanomas. In this study we have analysed the expression of miR-191 in thyroid neoplasias. We report that miR-191 is downregulated in follicular adenomas and carcinomas, with a reduction that is almost 2-fold higher in follicular carcinomas with respect to adenomas, but upregulated in thyroid carcinomas of the papillary histotype (PTCs), in comparison with the normal thyroid tissue. Consistent with a putative tumour suppressor role of miR-191 in the development of thyroid neoplasias of the follicular histotype, functional studies showed that restoration of miR-191 expression in a follicular thyroid cancer cell line reduces cell growth and migration rate. Furthermore, we found that miR-191 negatively regulated the expression of CDK6 protein, involved in cell cycle control, without changing the CDK6 mRNA level and decreased the activity of a luciferase reporter construct containing the CDK6-3'untranslated region. These results demonstrate that miR-191 regulates CDK6 at the post-transcriptional level, resulting in inhibition of cell proliferation and migration of a follicular thyroid carcinoma cell line. Finally, in follicular adenomas and carcinomas, we immunohistochemically detected CDK6 over-expression, suggesting that miR-191 may contribute to thyroid cancerogenesis by controlling CDK6 expression.

Introduction

Thyroid tumors are of two types: benign or malignant. Benign tumors are principally represented by adenomas, while malignant tumors are, in most cases, carcinomas. Thyroid carcinomas are one of the most common malignancies of the endocrine system, and accounts for approximately 1% of all newly diagnosed cancer cases (Aldred et al., 2004), with an increasing incidence all over the world (reviewed in Wartofsky, 2010). Well-differentiated thyroid carcinomas include papillary (PTC) and follicular (FTC) carcinomas. FTC generally comprises about 10% of all diagnosed thyroid cancers and is usually a more aggressive form of cancer than the more common papillary type. It is likely that FTCs can develop from pre-existing benign follicular adenomas or directly by passing the stage of adenoma. Unlike papillary thyroid cancer, it can be difficult to diagnose FTC without performing surgery because there are no characteristic changes in the way the thyroid cells look. Therefore, the only way to tell if a follicular neoplasm is a cancer, is to look at the entire capsule around the nodule and see if there is any sign of invasion. This difficulty in diagnosis is one of the most frustrating areas for physicians who study thyroid disease, because its surgery is often the only way of definitively diagnosing a thyroid nodule (Lin and Chao, 2006). The follicular variant of papillary thyroid carcinoma (FVPTC) is believed to behave in a clinical manner similar to usual or classical papillary cancer and to follow a similar indolent course. However, these innocuous-appearing lesions, although sharing nuclear features of papillary cancer, may behave clinically in an unexpectedly malignant fashion, giving rise to bone metastases and vascular invasion (Baloch and LiVosli, 2000).

The aim of the scientific community is to set up molecular assays to analyze clinical tissues in the diagnosis and management of thyroid cancer then allowing far more accurate characterization of individual tumors and maybe the early diagnosis of recurrence.

MicroRNAs (miRNAs or miRs) have emerged as an important class of short endogenous RNAs that act as post-transcriptional regulators of gene (Bartel et al., 2000). One miRNA is capable of regulating several distinct mRNAs and, altogether, the human miRNAs identified so far are believed to modulate more than one-third of the mRNA species encoded in the genome (Lewis *et al*. 2003; John *et al*. 2004; Kiriakidou *et al*. 2004). Moreover, each gene may be regulated by more than one miRNA. Therefore, the potential regulatory circuitry afforded by miRNAs is enormous. The analysis of microRNA expression profile in several types of cancer has shown an altered microRNA expression in cancer compared to the respective normal tissues, and several studies and/or meta-analyses have delineated their role in predicting cancer progression and prognosis. Previous work has demonstrated an aberrant miRNA expression profile that distinguishes unequivocally among PTCs, ATCs and normal thyroid tissue (Pallante et al., 2006; Visone et al., 2007; Pallante et al., 2010).

Recent results show that miR-191 expression is up-regulated in breast, colon and lung cancers, (Volinia et al., 2006; Xi et al., 2006), as well as in leukemic cell lines, including HL60 (Kasashima et al., 2004) and those expressing ALL1 fusion proteins (Nakamura et al., 2007), whereas it appears downregulated in severe medulloblastoma (Ferretti et al., 2008), in AML with normal karyotype (Garzon e al., 2008) and in melanomas where the low miR-191 expression correlates with a poor prognosis (Caramuta et al., 2010). Genetic variants of miR-191 have been also recently described in patients with familial breast (Shen et al 2009) and ovarian (Shen et al., 2010) cancer. However, no targets for miR-191 have been so far identified.

The aim of our work has been to investigate the role of miR-191 in thyroid carcinogenesis. First, we report that miR-191 is downregulated in follicular adenomas and carcinomas, but not in PTCs, in comparison with the normal thyroid tissue, with a fold-change that is significantly higher in FTC with respect to TA. Restoration of miR-191 expression in thyroid carcinoma cell lines reduces cell proliferation leading to an increased number of cells in the G1 phase of the cell cycle. The identification of

the CDK6 gene, coding for a protein involved in cell cycle control, as target for miR-191, accounts for the effects of miR-191 on the cell cycle, and, ~~then,~~ for the role of miR-191 in thyroid cancerogenesis. Immunohistochemisty showing the overexpression of CDK6 proteins in follicular-type tumors validate this hypothesis.

Materials and methods

Thyroid tissue samples

Human neoplastic thyroid tissues and normal adjacent tissue or the controlateral normal thyroid lobe were obtained from surgical specimens and immediately frozen in liquid nitrogen. Thyroid tumors were collected at the Service d'Anatomo- Pathologie, Centre Hospitalier Lyon Sud, Pierre Benite, France. Tissue from patients with follicular adenoma (n = 25), follicular thyroid cancer (n = 24), papillary thyroid cancer (n = 15), anaplastic thyroid cancer (n = 8) and follicular variant of papillary thyroid cancer (n = 6) was studied. All thyroid tissue diagnoses were confirmed histologically.

RNA extraction and quantitative reverse transcription (qRT-PCR)

Total RNA isolation from human tissues and cells was performed with Trizol (Invitrogen) according to manufacturer's instructions. RNA was extracted from paraffin-embedded specimens after pulverizing the tumors by using dry-ice-chilled stainless steel mortar and pestles. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis. qRT-PCR analysis was performed on a panel of FTC samples of human thyroid origin and on cells by using miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse-transcribed. After a 5-fold dilution, 2 µl of the cDNA were amplifyed by using miScript SYBR® Green PCR Kit (Qiagen) following manufacturer's instructions. Primers (miScript Primer Assays; Qiagen) were: Hs_miR-191_1 (amplifying human miR-191) and Hs_RNU6B_2 (amplifying U6, used as loading control). For CDK6 mRNA detection, we reverse transcribed total RNA from cell lines by using QuantiTect ® Reverse Transcription Kit and then qRT-PCR for CDK6 was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Charleroi, Belgium) and the following primers: CDK6_for 417: 5'-

CCGAGTAGTGCATCGCGATCTAA and CDK6_rev 823: 5'-CTTTGCCTAGTTCATCGATATC and G6PD_for: 5'-ACAGAGTGAGCCCTTCTTCAA and G6PD_rev: 5'-ATAGGAGTTGCGGGCAAAG. G6PD was used as an internal standard for data calibration. The $2^{-\Delta\Delta Ct}$ formula was used for the calculation of differential gene expression.

Cell cultures and stable transfections

The human thyroid carcinoma WRO cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories) containing 10% fetal bovine serum (GibcoLaboratories),1% glutamine (Gibco Laboratories), and 1% ampicillin/streptomycin (Gibco Laboratories) in a 5% CO_2 atmosphere.

For stable transfections, $5x10^5$ cells were plated in 100 mm plates and 1.8 μg of the pre-miR-191 expressing vector or the empty vector were co-transfected with 0.2 μg CMV-neomicin construct, using FuGENE® HD Transfection Reagent (Roche Diagnostics GmgH, Mannheim, Germany), following manufacturer's instructions.

Plasmids

The Lentivector-based Micro-RNA Precursor construct expressing pre-miR-191 (PreMiR-191) and the corresponding empty vector (pCDH-CMV-MCS-EF1-copGFP) were purchased by System Biosciences (Mountain View, CA) and stably transfected in WRO cells.

For the CDK6-3'UTR luciferase reporter construct, the 1133 bp 3'-UTR region of CDK6 gene, including 2 the putative binding sites for miR-191, was amplified from WRO cells DNA by using the following primers:

FwCDK6: 5'-CCACCACTCCCAACTTGAC and RevCDK6 5'-ATGCAATCTAGACTAGCACCCAGTAAGACATCC. The amplified fragment was cut with XbaI and cloned into pGL3-Control firefly luciferase reporter vector (Promega, Madison, Wisconsin, USA) at the XbaI site (pGL3-CDK6-3'UTR).

Deletions of 8 and 7 bp into each of the seed regions of the CDK6 3'-UTR construct were introduced by site directed mutagenesis (Eurofins MWG/Operon, Ebersberg Deutschland), in order to generate the mutant constucts Mut1 and Mut2, respectively (Fig. 4B). A mutant construct, in which both sites were deleted (Mut1+2) was also generated. Renilla luciferase vector (pRL-CMV), for transient transfection efficiency, was from Promega.

Proliferation and colony assay

Proliferation was assayed by using the Cell Titer (Promega) colorimetric assay, following manufacturer's instructions. Briefly, 1X 103 cells were plated in each well of a 96-well. The following day, 20 ul of Cell Titer were added to the medium, and the 96-well has been incubated at 37°C for 1 hour and then read ina 96-well plate reader at 450nm. The experiment has been performed in triplicate and assayed for 10 days, every other day.

Colony assay was performed as previously described (Pallante et al., 2008).

Flow cytometric analysis

WROpCDH and WRO miR-191 stable clones were serum starved and analyzed by flow cytometry after 48 h. Cells were harvested in PBS, washed once with PBS, and fixed for 2 h in cold ethanol (70%). Fixed cells were washed once in PBS and treated with 25 µg/ml RNase A in PBS for 30 minutes, then washed again in PBS and stained with 50 µg/ml propidium iodide (Roche, Basel Switzerland) and 0,1% Nonidet®P 40 Substitute (Sigma-Aldrich, St. Louis, MO). Stained cells were analyzed with a fluorescence activated cell sorter (FACS) Calibur (Becton-Dickinson, Franklin Lakes, NJ, USA), and the data were analyzed using a mod-fit cell cycle analysis program. The measurements were performed in triplicate.

Protein extraction, western blotting and antibodies

Cells were scraped in ice-cold PBS, and then lysed in ice-cold NP40 lysis buffer (0.5% NP40, 50 mM HEPES (pH 7), 250 mM NaCl,5 mM EDTA, 50 mM NaF, 0.5

mM Na3VO4, 0.5 mMphenylmethylsulfonyl fluoride, Complete inhibitor (Roche)). For Western blotting, 30 micrograms of protein from WROpCDH and WRO miR-191 were electrophoresed in 10% SDS–PAGE minigels and transferred onto Hybond C nitrocellulose membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). After incubating with primary antibodies anti-CDK6 (sc-177, Santa Cruz Biotechnology Inc.) and anti-actin (sc-1616, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C overnight, the nitrocellulose membranes were washed three times with Tris-Buffered Saline Tween-20 (TBS 0,1% Tween) and then incubated with horseradish peroxydase (HRP)-conjugated donkey anti-rabbit (GE Healthcare UK Ltd, Buckinghamshire, England) (dilution 1: 3000) or donkey anti-goat IgG (Santa Cruz Biotechnology Inc.) (dilution 1:6000), respectively, for 1 hr at room temperature. After washing with TBST, blots were visualized by using the Western blotting detection reagents (GE Healthcare UK Ltd).

Dual-luciferase reporter assay

For dual-luciferase reporter assay, $3x10^5$ WRO cells were co-transfected in 6-well plates with the pGL3-CDK6-3'UTR luciferase vector (400 ng) or with the mutated 3′-UTR of CDK6 (Mut1, Mut2 and Mut1+2), together with the Renilla luciferase plasmid (4 ng) and the Pre-MiR-191 expressing constructs or the corresponding empty vector (1.6 μg), by using FuGENE® HD Transfection Reagent (Roche Diagnostic Corporation, Indianapolis, IN). The pRL-TK control vector expressing Renilla luciferase (Promega) was used for normalization of cell number and transfection efficiency. Firefly and Renilla luciferase activity was measured 48h after transfection using the Dual-Luciferase Reporter Assay System (Promega) by a Lumat LB 9507 apparatus (Berthold Technologies, Bad Wildbad, Germany).

Cell migration assays

A wound-induced migration assay (scratch assay) was performed as described previously (Angers-Loustau et al., 1999), with some modifications. Briefly, WROpCDH or WROmiR-191 cell clones were plated to form a monolayer, in

presence of serum. The cells were then trypsinized, washed in PBS and allowed to attach on vitronectin-coated 6-well plates. Using a sterile yellow tip, a scratch was made in triplicate. Detached cells were removed, and the scratches were monitored for 24 hours under serum-free conditions. Migration rate was quantified by measuring the distance between the edges of wound and compared at t=0 to t=24h. In addition, a more quantitative haptotactic migration assay was performed essentially as described previously (Keely et al., 1995). Briefly, transwell inserts (8 µm pore size) in triplicates were coated on the underside with vitronectin (5 µg/ml). 2 x 10^4 cells were placed in the upper chamber in absence of serum (DMEM + 1%BSA) and allowed to migrate across inserts for 24 hours at 37°C. Unmigrated cells from the upper chamber were removed, and migrated cells in the lower side of the membrane were stained with crystal violet (0,5% in 25% methanol); after washing with PBS, cells were lysed in 1% SDS and the absorbance at 570 nm was measured. Each set of experiments was performed in triplicate. Statistical analysis of the data was performed using the paired Student's *t*-test.

Immunohistochemistry

Five µm-thick paraffin sections were cleared in xylene and alcohol-rehydrated. After microwave antigen retrieval with citrate buffer, slides were treated with a 0,3% solution of hydrogen peroxidase in methanol to block the endogenous peroxidase activity for 30 min and then washed in phosphate buffer solution before immunoperoxidase staining. The slides were then incubated overnight at 4°C in a humidified chamber with the primary antibody (diluted 1:50 in PBS). Tissue sections were covered with biotinylated anti-rabbit/anti-mouse immunoglobulins *(Dako LSAB2 System)* for 20 min, and then stained with streptavidin labeled with peroxidase; the signal was developed by using as substrate the diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS pH 7.6 for 5 min. After chromogen development, slides were washed,

dehydrated with alcohol and xylene, and mounted with cover slips using a permanent mounting medium. Negative controls were performed by omitting the first antibody. The proportion of malignant cells positively stained was scored from 0 to 3: 0, no positive cells; 1+, ≤10% of positive cells; 2+, 11–50% of positive cells; and 3+, >50% of positive cells.

Results

MicroRNA-191 expression is downregulated in thyroid follicular adenomas and carcinomas

In order to investigate the role of miR-191 in thyroid cancer, we analyzed mir-191 expression in 6 normal thyroid tissues and in a panel of thyroid neoplastic samples, including 25 follicular adenomas, 24 follicular carcinomas, 15 PTCs, 8 ATCs and 6 follicular variant of PTCs (FVPTCs), by performing quantitative stem-loop RT-PCR. As shown in Figure 1, miR-191 is significantly downregulated in all the follicular adenomas (Figure 1A) and carcinomas (Figure 1B) compared to normal thyroid. However, the miR-191 expression levels were significantly lower in the carcinoma samples with respect to the adenomas. Conversely, in most PTCs, miR-191 expression was upregulated (Fig. 1C) and it was upregulated just in 50% ATCs (Fig. 1D) in comparison with normal thyroid tissues; interestingly, miR-191 expression in FVPTCs was downregulated (Figure 1E), similarly to what observed for FTCs, even though at a lower extent. This result is suggestive of miR-191 downregulation as a signature for a follicular-type behaviour (such as metastasis and blood invasion).

MiR-191 is downregulated in human thyroid carcinoma and retrovirally transformed rat thyroid cell lines

Subsequently, we analysed miR-191 expression in a panel of thyroid carcinoma cell lines of different histotypes, including WRO (from follicular carcinoma), FB2 and BCPAP (from papillary thyroid carcinoma), FRO and FB1 (from anaplastic carcinoma), compared to normal thyrioid. As shown in Figure 2A, apart from the BCPAP cell line, a drastic reduction in miR-191 expression with respect to normal thyroid tissue was observed in the thyroid carcinoma cell lines independently from the histotype.

In order to verify whether miR-191 is a more general event in thyroid neoplastic cell transformation, we analysed miR-191 expression in panel of rat thyroid cells deriving from the transformation of normal differentiated thyroid cells, PC Cl 3 (Fig 2B) and FRTL5 (Figure 2C), with the following viral oncogenes: *v-mos* (PC 663), *v-raf* (PC raf), E1A plus v-raf (PC R15 raf), Ki-ras (PC kiki and FRTL5 Ki MSV), v-ras-Ha (PC Ha-ras) and Polyoma Middle T (PC Py), previously generated in our laboratory (Berlingieri et al., 1995). As shown in Figure 2B and 2C, all the transformed cell lines showed a drastic reduction in miR-191 expression in comparison to the normal thyroid cells PC Cl3 and FRTL5, suggesting that miR-191 downregulation may be a general event in thyroid cell transformation.

miR-191 expression affects cell shape, cell proliferation and migration of WRO cells

To define the role of miR-191 downregulation in the process of thyroid carcinogenesis we restored miR-191 expression levels in WRO cells by transfecting them with the PreMiR-191 Lentivector-based Micro-RNA Precursor construct, expressing preMiR-191, or with the corresponding empty vector (pCDH-CMV-MCS-EF1-copGFP), together with a plasmid confering neomycin-resistence. After selection, several GFP-positive clones were picked up, amplified and checked for miR-191 expression (Fig. 3B), by quantitative RT-PCR. The selected WROmiR-191 cell clones (2 representative clones are showed in Figure 3A) showed an epithelial-like morphology, being more tightly packed, with respect to mock transfected WRO cells and to WRO cells transfected with the control vector (WRO-pCDH), which had a scattered distribution (Fig. 3A). The acquisition of an epithelial phenotype is in agreement with a role of tumor suppressor, hypothesized for miR-191.

In order to demonstrate the tumor suppressive activity of miR-191, we assessed the proliferation rate in miR-191 and empty vector-transfected WRO cells. The analysis of cell growth revealed a lower proliferation rate of the WRO-miR-191 cell clones in comparison with the cells carrying the empy vector (Figure 4A) or with mock

transfected cells (Fig. 4A). Moreover, we carried out a colony-forming assay performed on WRO cells after transfection with a vector carrying the miR-191 or the backbone vector. As shown in Figure 4B the WRO cells transfected with miR-191 generated fewer colonies than cells transfected with the backbone vector.

To better define the effect of miR-191 expression on cell cycle progression, we used flow cytometric analysis (FACS) to measure the DNA content of WRO miR-191 and WROpCDH cell clones. Transition from G1 to S phase was significantly lower in the WROmiR-191 cell clones than in the WROpCDH cells (Figure 4C). In fact, 19% of WROmiR-191 cells were in S phase and 52% in G0/G1 phase, whereas 24% of WROpCDH cells were in S phase and 45% in G0/G1 phase (Figure 4C).

These results indicate that miR-191 overexpression decreases cell proliferation rate of a thyroid carcinoma cell line, retaining the cells in the G1 phase.

Since WROmiR-191 cells appeared less scattered on the cell culture plate, we evaluated cell motility in WROmiR-191 and control cells, by performing “scratch” and transwell chamber assays. As shown in Figure 5A, the migration (% wound closure) was comparable in WROmiR-191 and control cells, when assayed on plastic cell culture plates; conversely, migration was reduced in WROmiR-191 cells compared to control, when the scratch was performed on vitronectin (VN)-treated plates, the motility of the WROmiR-191 cells was reduced (about 31%) compared with control cells (Figure 5A).

Furthermore, we measured the invasive ability of the the WRO-mir191 and WRO-pCDH cells by performing a transwell migration assays. As shown in Figure 5B, the ability of WRO cells to invade and cross the polycarbonate Nucleopore filters was slightly reduced by the restoration of the miR-191 expression in WRO cells. This effetc was more evident when the transwell underside was covered with VN (Fig 5B): in thic case, the ability of WRO-mir191 cells to cross the membrane was reduced by 30% compared to WROpCDH cells. This differential behaviour in presence or absence of vitonectin suggest that at least one miR-191 target has to be implicated in the $\alpha_v\beta_3$–integrin-mediated inhibition.

miR-191 downregulates CDK6 expression

MicroRNAs act on many cellular processes by modulating the expression of mRNA targets. Therefore, to investigate the mechanism by which miR-191 may affect tumorigenesis, cell proliferation and migration, we used bioinformatic tools (mirGen, Target Scan, Pictar and miRanda), to search for potential mRNA targets. We identified several genes involved in DNA replication, cell cycle control and cell death regulation that were potentially targeted by miR-191. Among them we selected the CDK6 gene, which encodes a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition (Serrano et al., 1993). We selected this gene also because the activity or amount of Cdk6 is elevated in some cancer cells and lymphomas (Costello *et al.*, 1997; Timmermann *et al.*, 1997; Corcoran *et al.*, 1999). Two sites matching the miR-191 seed sequences were predicted in the 3'UTR of the CDK6 gene (Figure 6A). To validate the influence of miR-191 on the CDK6 target, we evaluated the expression of CDK6 protein levels in WRO, WROpCDH and WROmiR-191 cell clones, by Western blot analysis (Figure 6B). The introduction of miR-191 significantly decreased by almost 50%) CDK6 protein level.

Interestingly, there were no significant changes in *CDK6* mRNA levels in cells transfected with the miR-191 or with the empty vector (Figure 6C). These results are consistent with post-transcriptional regulation of the CDK6 protein by miR-191 and exclude its role in *CDK6* mRNA degradation.

To determine whether direct interaction between miR-191 and *CDK6* mRNA caused the decrease in CDK6 protein expression, we performed luciferase assays. To this aim, we inserted a sequence corresponding to the CDK6 3'UTR downstream of the luciferase gene, by cloning into the pGL3-control vector. This reporter vector was co-transfected into WRO cells, together with the miR-191 expressing construct or the corresponding empty vector. Luciferase activity, measured 48 h later, resulted significantly reduced by miR-191 expression, in a dose-dependent manner, as

compared with cells transfected with the control vector (Fig.7B). These results indicate that miR-191 interferes with CDK6 mRNA translation by directly interacting with the 3'UTR. This conclusion is supported by similar experiments in which we used a reporter construct carrying target sites modified by 8 nucleotides-deletion in one or both sites.

MiR-191 ability of downregulating luciferase activity was lost when either or both sites were mutated, suggesting that miR-191 acts through interaction with both sites (Fig. 7B).

These data show that miR-191 directly downregulate CDK6 protein expression, by interacting with two seed sequences in its 3'-UTR.

CDK6 overexpression inversely correlates with miR-191 in thyroid tumors

To understand if the CDK6 targeting by miR-191 has a pathological role in thyroid tumorigenesis, we evaluated the expression of the CDK6 protein in 20 thyroid tissue specimens (5 normal thyroid tissues, 5 follicular adenomas, 5 follicular carcinomas, and 5 papillary carcinomas) by immunohistochemical analysis.

As shown in fig. 8, we found that CDK6 is not detectable in thyrocytes from normal glands (Fig. 8A) (5 out of 5) and is weakly expressed only in 1 out of 5 PTCs (Fig. 8C), with few focally distributed cytoplasmic-positive tumour cells (20%). In contrast, we detected clear cytoplasmic and nuclear CDK6 stainings in 4 out of 5 follicular adenomas (Fig. 8B), in more than 50% cells and in 3 out of 5 follicular carcinomas (Fig. 8D). The immunohistochemical results are summarized in the table of Fig 8 E.

Thus, CDK6 expression is inversely related to miR-191 levels in thyroid carcinomas and this inverse correlation has, at least in part, a pathogenic role in thyroid cancerogenesis. Interestingly, PTCs showed very faint CDK6 immuostainings, identifying the miR-191 downreguation/CDK6 overexpression as peculiar to follicular thyroid cancers.

Discussion

Mature functional miRNAs of approximately 22 nucleotides that are generated from long primary miRNA (pri-miRNA) transcripts control gene expression at the post-transcriptional level by degrading or repressing target mRNAs. Some miRNAs aberrantly expressed in cancer have been well documented (Chan et al., 2005; Pallante et al., 2006; Garzon et al., 2008). They were found to regulate the expression of signaling molecules, such as cytokines, growth factors, transcription factors, and proapoptotic and antiapoptotic genes.

In this study we investigated the role of miR-191 in thyroid neoplasias. We report that miR-191 was significantly downregulated in TA and FTC with a fold change higher in FTCs. Conversely, miR-191 was upregulated in PTCs whereas it was up- or downregualted in ATCs, suggesting that miR-191 downregulation is specific for the pathway leading to follicular neoplastic phenotype. Even though the opposite pattern of miR-191 expression in FTC and PTCs might appear contradictory, a similar situation has been already described by Aldred and colleagues (Aldred et al., 2004), which found that IGFBP6 is overexpressed in PTCs and downregulated in FTCs. Moreover, these Authors found that PTCs were characterized by the overexpression of CITED1 (Cbp/p300-interacting transactivator): a consensus sequence for p300 and cEBP/beta is found in the miR-191 promoter region (Battista S., unpublished results) and might be responsible of miR-191 upregulatio in PTCs.

Downregulation of miR-191 in AT with respect to normal thyroids indicated that miR-191 downregulation may be an early event in thyroid carcinogenesis. This hypothesis is consistent with data showing that miR-191 is found downregulated in lungs of rats exposed to environmental cigarette smoke (ECS) (Izzotti et al., 2009) and in pre-cancerous azoxymethane-(AOM)-induced colon lesions in rats (Davidson et al., 2009). This precocious downregulation of miR-191 may be suggestive not just of an early event, but also, on the other hand, of a protective, anti-cancer effect associated to its expression. Such a role is supported by data showing that the

chemoprotective effect of nutritional bioactives (such as fish oil) is mediated by the up-regulation of miR-191 (and other miRNAs) (Davidson et al., 2009).

The involvement of miR-191 downregulation in thyroid carcinogenesis seems validated by functional studies. Indeed, we show that miR-191 possesses anti-proliferative potential and regulates cell cycle transition from G1 to S in thyroid follicular carcinoma derived cell lines; in these cells, miR-191 also induced morphological changes and reduced migration. In order to identify the mechanism by which miR-191 may affect part or all these processes, we looked for possible targets. We identified CDK6 mRNA as a miR-191 target. Indeed, miR-191 overexpression reduces the CDK6 protein levels and the luciferase activity of about 50%. Moreover, the deletion of few nucleotides in either or both the seed sequences in CDK6 3'-UTR leads to the inhibition of the miR-191 activity. Consistently, in this study and in others, an increased CDK6 expression is observed in thyroid carcinomas (Kebebew et al., 2006). Here we show that CDK6 overexpression is tightly related to miR-191 downregulation in human thyroid tumors, being PTCs almost negative. These data confirm the functional role of miR-191 in thyroid cancerogenesis, suggesting that the inverse correlation between miR-191 and CDK6 expression may be indicative of a follicular phenotype. CDK6 has proto-oncogenic properties, and its activity is carefully regulated at multiple levels, as misregulation can induce unscheduled proliferation, and genomic and chromosomal instability. CDK4/CDK6, in complex with D-type cyclins (D1, D2, or D3), regulate the G1/S phase transition. Elevated activity of CDK6 may result in hyperphosphorylation of the retinoblastoma protein and, therefore, compromise its negative growth-regulatory activity (Harbour et al., 1999). Accordingly, restoration of miR-191 expression produces a cell phenotype similar to that induced by CDK6 inhibition, leading to retention in the G1 phase of the cell cycle and inhibition of cell migration on vitronectin (Alhaja et al., 2005).

Our preliminary data suggest that loss of miR-191 may predispose to thyroid cancer not just by CDK6 upregulation, but also by PTEN reduction. In fact, levels of Pten, the loss of which renders the thyroid highly susceptible to neoplastic transformation

through mechanisms that include increased thyrocyte proliferation (Yeager et al., 2007), are increased in miR-191 transfected WRO cells.

Therefore, our data confirm a role of miR-191 in carcinogenesis. Indeed, its expression has been recently found altered in many kind of malignancies. Moreover, genetic variants of miR-191 (as well as of other six selected microRNA) have been described in patients with familial breast cancer (Shen et al., 2009). The C/G substitution affecting the pre-miRNA sequence, but not the mature miR-191 sequence, is possibly translated into conformational changes in the predicted secondary structure, thereby blocking processing into the functional mature miRNA.

In conclusion the data reported here demonstrate a that miR-191 is specifically downregulated in benign and malignant tumors of follicular histotype, and the targeting of the CDK6 gene might account for a role of miR-191 downregulation in thyroid carcinogenesis.

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Legend to figures

Figure 1: miR-191 expression in thyroid tumors. Quantitative RT-PCR analysis of miR-191 expression in a panel of FAs (A), FTCs (B), PTCs (C), ATCs (D) and FVPTCs (E) of human thyroid origin. The fold change values indicate the relative change in the expression levels between tumor samples and the normal thyroid tissue, assuming that the value of the normal control sample was equal to 1.

Figure 2: miR-191 expression in human and rat thyroid cell lines. Quantitative RT-PCR analysis of miR-191 expression in (A) human thyroid cell lines compared to normal thyroid and in (B) normal rat thyroid cell lines (PC Cl3 and FRTL5) and cells transformed by various oncogenes (PC 663, PC RAF, PC R15 RAF, PC kiki, PC Ha. PC Py and FRTL5 ki-MSV).

Figure 3: WRO cells transfected with Lenti-miR-191 plasmid or with the empty vector. A: Phase contrast (a, c, e, g) and fluorescence (b, d, f, h) images of mock transfected WRO cells (a, b), WRO cells tansfected with the empty vector (c, d) and cells transfected with the miR-191 expressing construct (representative clones) (e,f: WROmiR-191#1; g,h: WRO miR-191#3). B: Quantitative RT-PCR analysis of miR-191 expression in clones stably transfected with the pre-miR-191-carrying vector or with the empty vector, compared to mock transfected cells.

Figure 4: miR-191 reduces proliferation and slows down the G1/S transition in WRO cells. A: The effect of miR-191 on WRO cells proliferation was assayed by Cell Titer. The diagram showes a reduction in proliferation in miR-191 transfected cells compared to cells transfected with the empty vector and to mock transfected cells. B: The effect of miR-191 on cell cycle was assayed by Flow cytometric analysis of WRO cells stably transfected with the miR-191 or the control vector.

Figure 5: miR-191 expression reduces cell migration on Vitronectin. A: A wound-induced migration assay, in presence or absence of a vitronectin matrix coating, was performed using WROpCDH and WROmiR-191 cell clones in the absence of serum or growth factors. The extent of cell migration into the wounded area was photographed under phase-contrast microscopy at 0 hour and after 24 hours. Magnification, x100. Data shown are representative of three separate experiments and are diagrammed in the lower panel. Migration rate was quantified by measuring the distance between the edges of wound and compared at *t*=0 to *t*=24h. The migration rate is represented as percentages in graphics illustrating the reduction of wound closure in WROmiR-191 cell clones, plated on VN. B: Diagram of transwell assays, performed in presence (right panel) or absence (left panel) of VN coating. Migration is expressed as percent of adsorbance at 570 nm, compared to control cells.

Fig. 6: MiR-191 expression downregulates CDK6 protein, but not mRNA expression. A: Alignment of miR-191 with the putative seed sequences in the CDK6 3'-UTR. B: Immunoblots for CDK6 in WRO, WROpCDH and WROmiR-191 cell clones. Immunoblot for α-actin has been used as loading control. C: qRT-PCR analysis of CDK6 mRNA in samples as in B. The fold change values indicate the relative change in CDK6 mRNA expression levels between control and miR-expressing cells, normalized with G6PD.

Figure 7: **The 3'-UTR of CDK6 is a target of miR-191.** A: The two seed sequences for miR-191 in CDK6-3'UTR, with the respective mutants (Mut1 and Mut2). Mut1+2 contained both modifications. B: Relative luciferase activity in WRO cells transiently transfected with increasing amounts of Lenti-miR-191 or the empty vector (pCDH). The relative activity of firefly luciferase expression was standardized to a transfection control using Renilla luciferase. C: Relative luciferase activity in WRO cells transiently transfected with the Lenti-miR-191, was assayed in presence of the wild-type CDK6 3'UTR sequence or with

the mutants (Mut1, Mut2 and Mut1+2) and compared to the luciferase activity measured with the empty (pCDH) vector.

Fig. 8: Immunohistochemical analysis for CDK6 in normal and malignant thyroid tissues.

Representative anti-CDK6 immunostainings of a normal thyroid (A), a follicular thyroid adenoma (B), a thyroid papillary carcinoma (C) and thyroid follicular carcinoma (D). (Magnification: 200 x).

E: Table summarizing the results of CDK6 immunohistochemistry on thyroid tumors. The proportion of malignant cells positively stained was scored from 0 to 3: 0, no positive cells; 1+, ≤10% of positive cells; 2+, 11–50% of positive cells; and 3+, >50% of positive cells

Figure 1

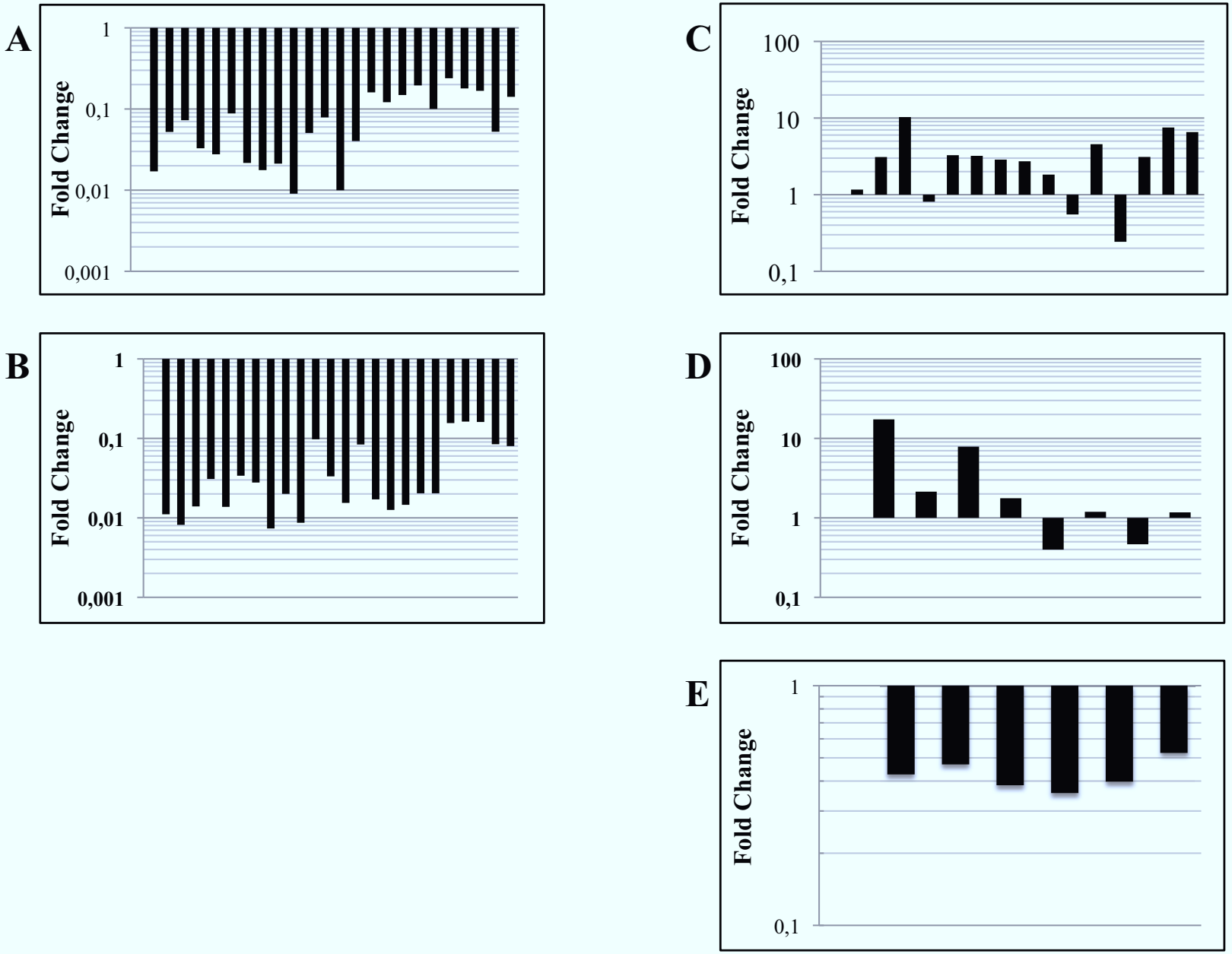
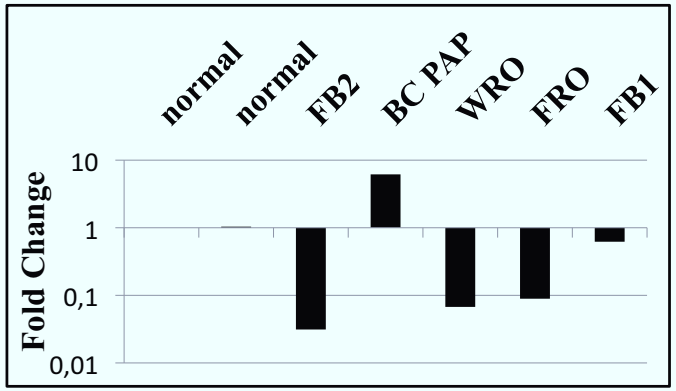

A
1
0,1
0,01
0,001
Fold Change
B
1
0,1
0,01
0,001
Fold Change
C
100
10
1
0,1
Fold Change
D
100
10
1
0,1
Fold Change
E
1
0,1
Fold Change

Figure 2

A


normal
normal
FB2
BC PAP
WRO
FRO
FB1
Fold Change
10
1
0,1
0,01

B

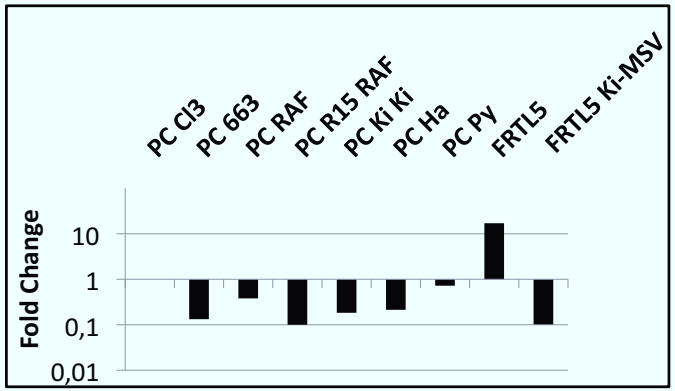
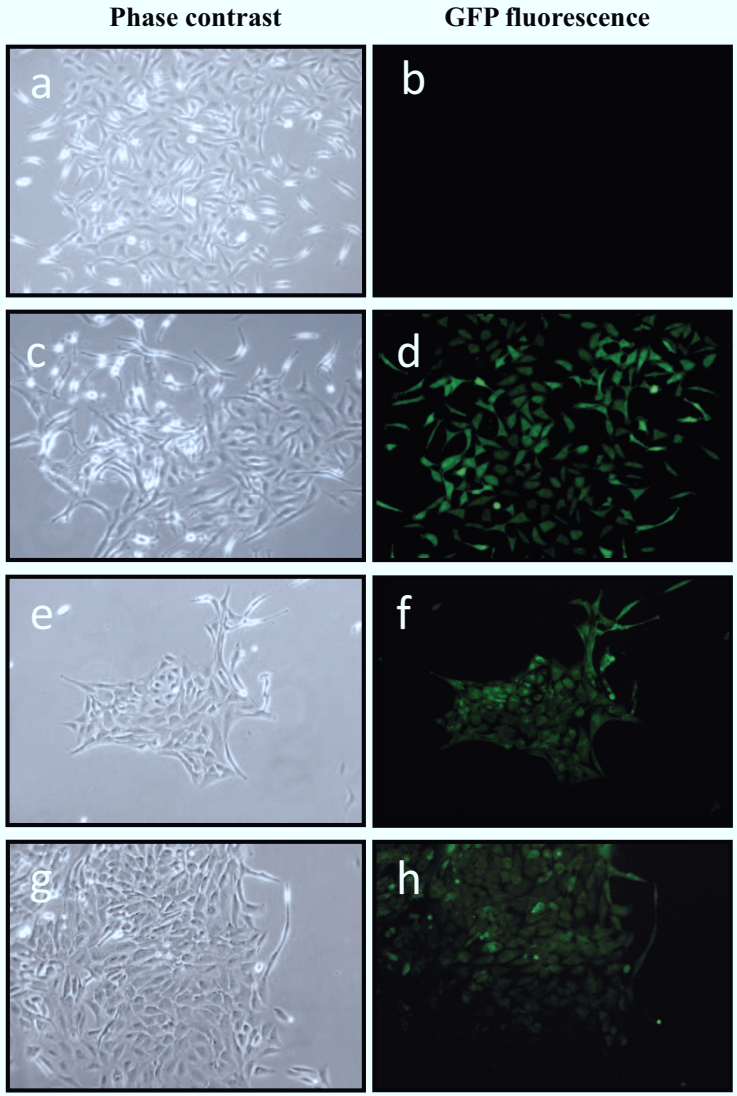

PC Cl3
PC 663
PC RAF
PC R15 RAF
PC Ki Ki
PC Ha
PC Py
FRTL5
FRTL5 Ki-MSV
Fold Change
10
1
0,1
0,01

Fig. 3

A


Phase contrast
GFP fluorescence
a
b
c
d
e
f
g
h

B

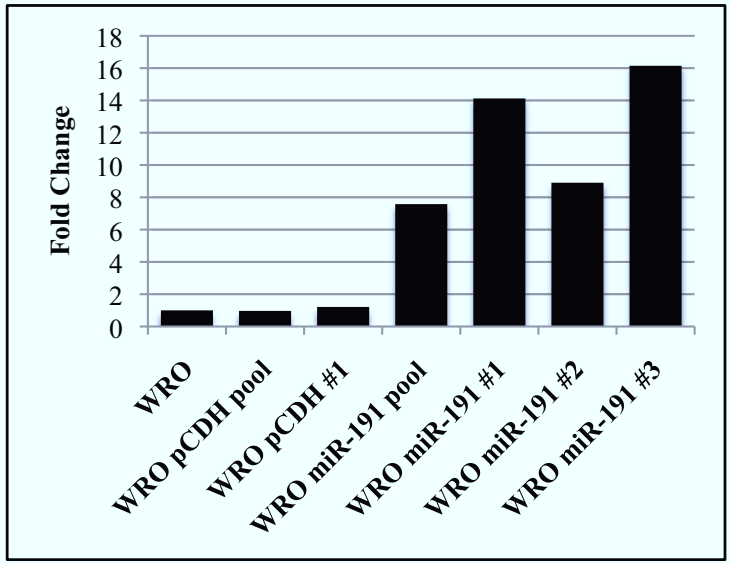
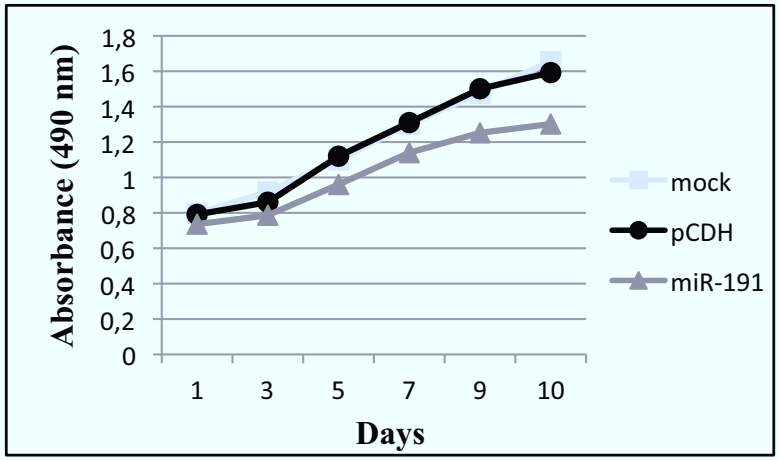

Fold Change
18
16
14
12
10
8
6
4
2
0
WRO
WRO pCDH pool
WRO pCDH #1
WRO miR-191 pool
WRO miR-191 #1
WRO miR-191 #2
WRO miR-191 #3

Fig. 4

A


Absorbance (490 nm)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1
3
5
7
9
10
Days
mock
pCDH
miR-191

B

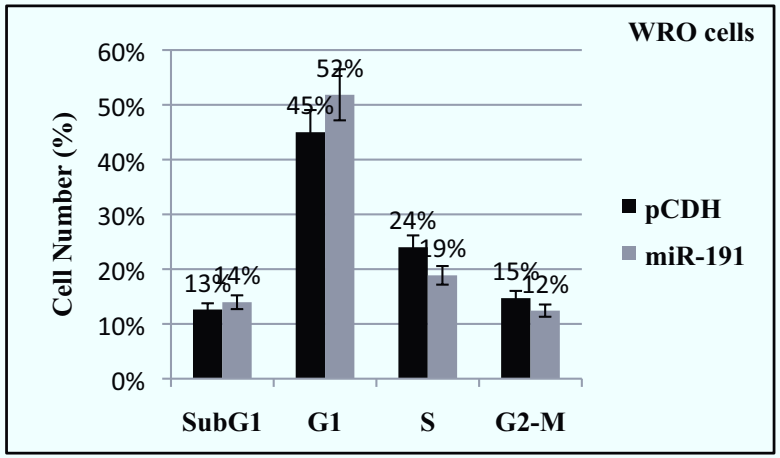

WRO cells
Cell Number (%)
60%
50%
40%
30%
20%
10%
0%
13%
14%
45%
52%
24%
19%
15%
12%
SubG1
G1
S
G2-M
pCDH
miR-191

Figure 5

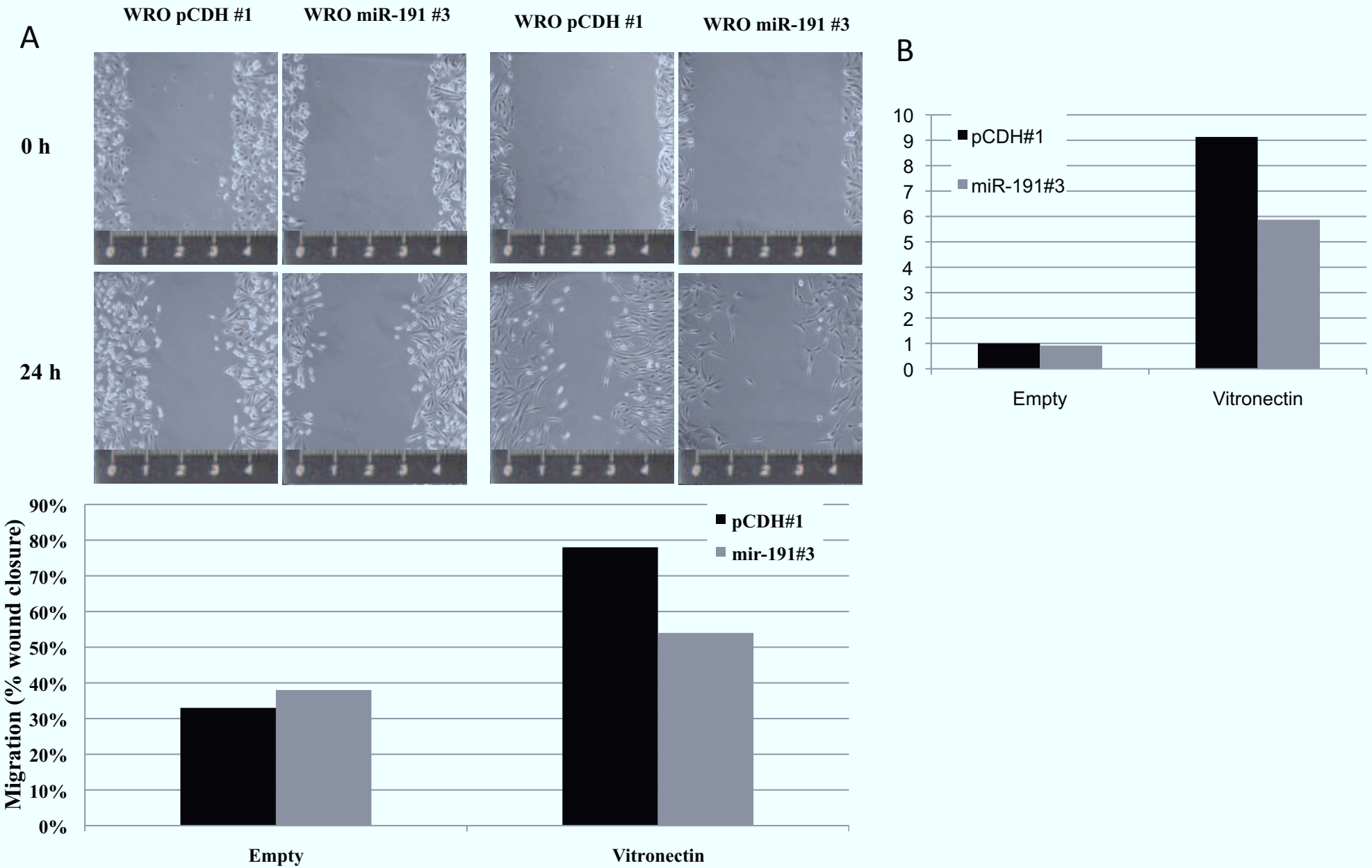
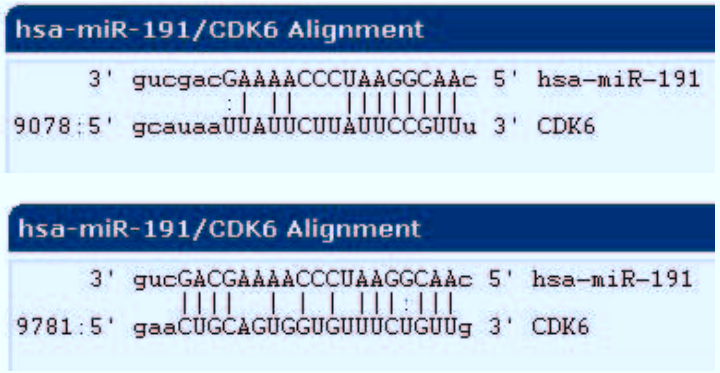
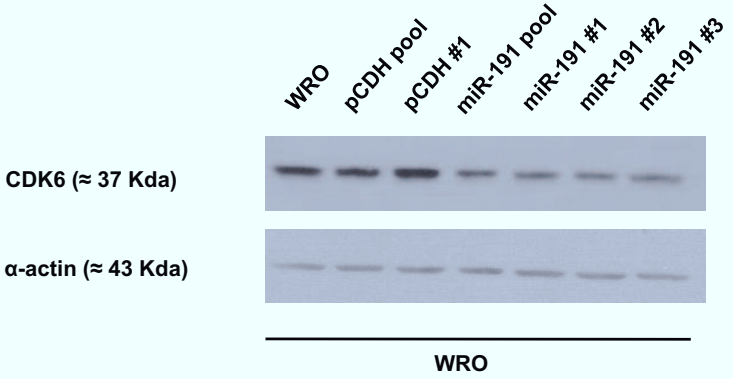

A
WRO pCDH #1
WRO miR-191 #3
WRO pCDH #1
WRO miR-191 #3
0 h
24 h
Migration (% wound closure)
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
pCDH#1
mir-191#3
Empty
Vitronectin
B
10
9
8
7
6
5
4
3
2
1
0
pCDH#1
miR-191#3
Empty
Vitronectin

Fig. 6

A


hsa-miR-191/CDK6 Alignment
3' gucgacGAAAACCCUAAGGCAAc 5' hsa-miR-191
9078:5' gcauaaUUAUUCUUAUUCCGUUu 3' CDK6
hsa-miR-191/CDK6 Alignment
3' gucGACGAAAACCCUAAGGCAAc 5' hsa-miR-191
9781:5' gaaCUGCAGUGGUGUUUCUGUUg 3' CDK6

B


WRO
pCDH pool
pCDH #1
miR-191 pool
miR-191 #1
miR-191 #2
miR-191 #3
CDK6 (≈ 37 Kda)
α-actin (≈ 43 Kda)
WRO

C

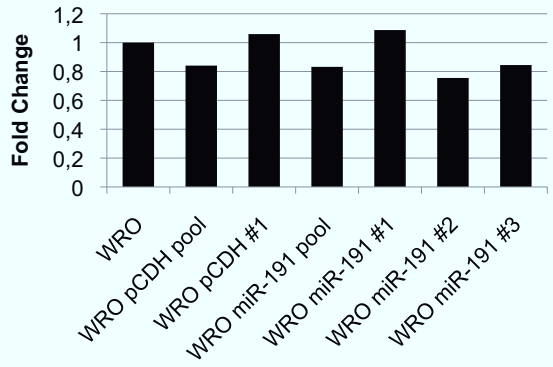
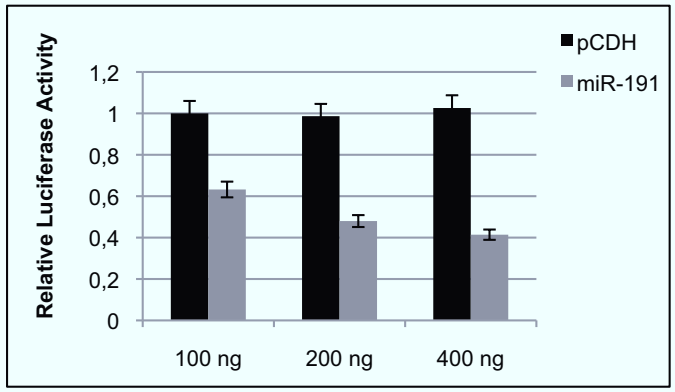

Fold Change
1,2
1
0,8
0,6
0,4
0,2
0
WRO
WRO pCDH pool
WRO pCDH #1
WRO miR-191 pool
WRO miR-191 #1
WRO miR-191 #2
WRO miR-191 #3

Fig. 7

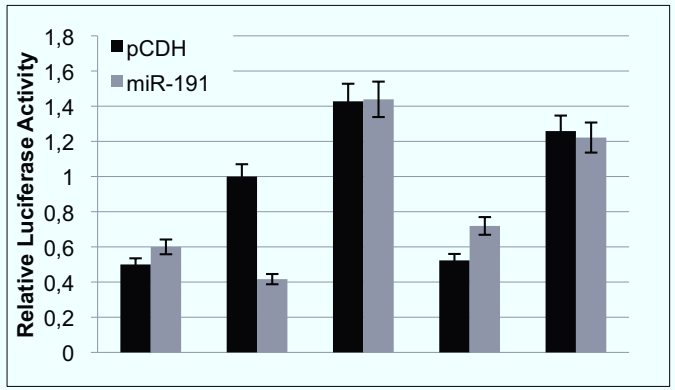
A

CDK6 3'UTR site 1	5' GCATAATTATTCTTATTCCGTTT 3'
CDK6 3'UTR Mut 1	5' GCATAATTATTCTT - - - - - - - - T 3'
CDK6 3'UTR site 2	5' GAACTGCAGTGGTGTTTCTGTTG 3'
CDK6 3'UTR Mut 2	5' GAA - - - - AGTGGTGT - - - TGTTG 3'

B

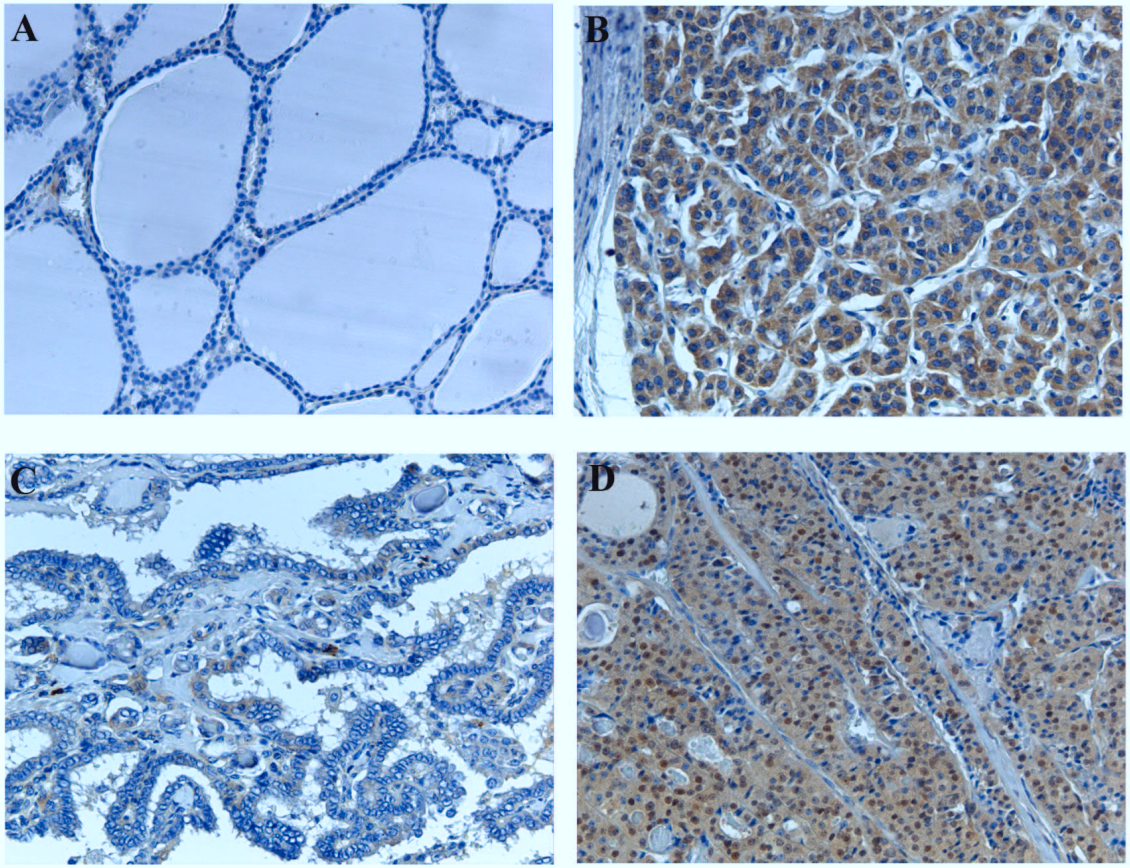


C



pLuc	+ +	- -	- -	- -	- -
pLuc CDK6-3' UTR	- -	+ +	- -	- -	- -
pLuc CDK6-3' UTR Mut1	- -	- -	+ +	- -	- -
pLuc CDK6-3' UTR Mut2	- -	- -	- -	+ +	- -
pLuc CDK6-3' UTR Mut1+2	- -	- -	- -	- -	+ +

Fig. 8



E

Histotype	Case	Percent of cdk6 positive cells			
		0	1+	2+	3+
Normal Thyroid tissues	5	5	0	0	0
Follicular Adenomas	5	0	0	1	4
Papillary Carcinomas	5	0	4	1	0
Follicular Carcinomas	5	0	0	2	3