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Regulation of the Cyclin Dependent Kinase Inhibitor p27 in Glioblastoma

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Master's in Biochemistry

Department of Biochemistry, Microbiology and Immunology

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Abstract

Glioblastoma Multiforme (GBM) is the most common and most malignant brain tumour. The cyclin dependant kinase inhibitor p27 is commonly inactivated in this and many other cancers. p27 is an important negative regulator of the cell cycle which acts by inhibiting cyclinE/cdk2 and cyclinA/cdk2 complexes. We identify PKCι, a pro-proliferative kinase which is activated by phosphoinositide 3 kinase (PI3K), as a regulator of p27 in GBM. siRNA-mediated depletion of PKCι in U87MG cells resulted in an increase in p27 protein levels, and p27 levels are extremely high in PKCι-null mouse embryonic fibroblasts. The mechanism by which PKCι regulates p27 is unclear, although it occurs at the post-transcriptional level, as QRTPCR analysis showed that PKCι depletion did not alter p27 mRNA levels. PKB is a known regulator of p27, but we show that PKCι does not regulate p27 through modulation of PKB activity. MEK/ERK signaling is also implicated in the regulation of p27. The combined inhibition of PKCι and MEK resulted in an increase in p27 levels which was greater than the added effects of inhibiting either kinase alone, suggesting some overlap in the function of these two pathways.

Additionally, we show that p27 is regulated by miRNAs, which are 20-25 nucleotide RNAs that repress gene expression by post-transcriptional silencing. In eukaryotes, this occurs predominantly at the level of translational inhibition and is mediated by defined sequences in the 3'UTRs of target genes. miRNAs are processed by dicer, and therefore the deletion of dicer abrogates miRNA expression. We show that siRNA-mediated depletion of dicer increases p27 levels and halts the cell cycle at G1 phase. The miRNA target site prediction

program Targetscan predicted 2 binding sites for miR-221/222 in the p27 3'UTR. Transfection of glioblastoma cells with inhibitors of miR-221 and/or miR-222 resulted in an increase in p27 protein levels. These results were corroborated by luciferase reporter assays in which dicer depletion or miR-221 inhibition increased activity from a luciferase reporter gene located immediately upstream of the p27 3'UTR. Site-directed mutagenesis of the predicted miR-221 binding sites in the reporter construct increased luciferase activity and abrogated the effect of miR-221 inhibition. Therefore, p27 is regulated by miR-221. We also show that PKCι and MEK alter p27 levels via an element in the 3'UTR. These results are consistent with a model in which oncogenic signaling pathways increase miR-221 expression to decrease translation of p27.

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I would like to thank Dr. Ian Lorimer for being the best supervisor a student could possibly ask for. His guidance, insight, encouragement and understanding during the preparation of this thesis will not be forgotten. I am grateful to Sylvie Brett for performing the site-directed mutagenesis of the p27 3' UTR. I would also like to thank the entire Lorimer lab for making my two years here so enjoyable. Anyone who walks by our lab at Christmas knows that the Lorimer lab is the best place to be! In particular, I am thankful to Jenn Hanson – there were a lot of well-needed coffee breaks that took place during the preparation of this thesis – and to Mitch Baldwin for helping to keep me ahead of the 8-ball. And finally I would like to thank my family, as I couldn't have done it without their continued support.

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List of Abbreviations

CDK: cyclin dependant kinase

CLL: chronic lymphocytic leukemia

CML: chronic myelogenous leukemia

GBM: glioblastoma multiforme

EGFR: epidermal growth factor receptor

ERK: extracellular regulated kinase

hKIS: human kinase-interacting stathmin

HER: human epidermal growth factor receptor

MEK: MAPK/Erk kinase

miRNA: microRNA

NES: nuclear export signal

PI3K: Phosphoinositide 3 kinase

PKB: Protein kinase B

PKCι: Protein kinase C iota

PTEN: phosphatase and tensin homolog

RISC: RNA-induced silencing complex

RNAi: RNA interference

siRNA: short interfering RNA

UTR: untranslated region

Chapter 1

Introduction

1.1 Glioblastoma

Glioma is the most common primary tumour of the central nervous system (1). Gliomas are heterogeneous tumours consisting of cells that resemble immature astrocytes, immature oligodendrocytes, or mixtures of the two cell types (2). They are graded on a scale of I to IV based on their degree of malignancy, with grade IV, also referred to as glioblastoma multiforme, being the most malignant type (3). Approximately half of gliomas are glioblastoma multiforme (GBM), and the median survival for such patients is 40-60 weeks from diagnosis, which ranks GBM among the most lethal of all cancers (4).

1.2 Treatment of Glioblastoma

Standard treatment for GBM consists of surgical resection followed by radiotherapy (5). Adjuvant chemotherapy may be added to this regimen, as it results in a modest survival benefit (6). However, virtually all patients have an inadequate response to treatment, and the tumour usually recurs (7). There is an urgent need for the development of new therapies.

Because of the limited success of systemic cytotoxic treatments, the newest cancer therapies focus on the modulation of the molecular pathways that are the cause of the disease. These targeted therapies have several potential advantages over conventional chemotherapies in that they may have high tumour specificity and low toxicity. This approach has been very successful in chronic myelogenous leukemia (CML), a cancer that results from the

deregulation of a single receptor tyrosine kinase (8). Inhibition of this kinase with a small molecule inhibitor resulted in complete remission in >80 % of patients (9, 10). Unlike CML, glioblastoma arises from an accumulation of many genetic alterations and this makes the development of targeted therapies particularly challenging.

1.3 Molecular Etiology of Glioblastoma

Glioblastomas may arise de novo (primary GBM), or from a lower grade glioma which acquires additional mutations (secondary GBM). The pathways to the development of each type of GBM are shown in figure 1.1. The pathways are similar in that they both begin with a precursor cell which sustains mutations of two types which cooperate to promote tumour formation. The first type activates receptor tyrosine kinases or alters their downstream signaling pathways, resulting in a persistant signal to proliferate. In glioblastoma, mutations of this type include EGFR, FGF, PDGF and PTEN (11). Cells have a built-in mechanism to detect persistant oncogenic signaling which results in the activation of cell cycle arrest pathways and/or apoptosis (12). Therefore, in order for a cell to become cancerous, it must also sustain mutations in genes which control the cell cycle. In GBM, commonly mutated genes of this type include CDK 4, CDK 6, cyclin D1, MDM2, $p16^{INK4a}$, $p19^{ARF}$, RB, and p53 (11).

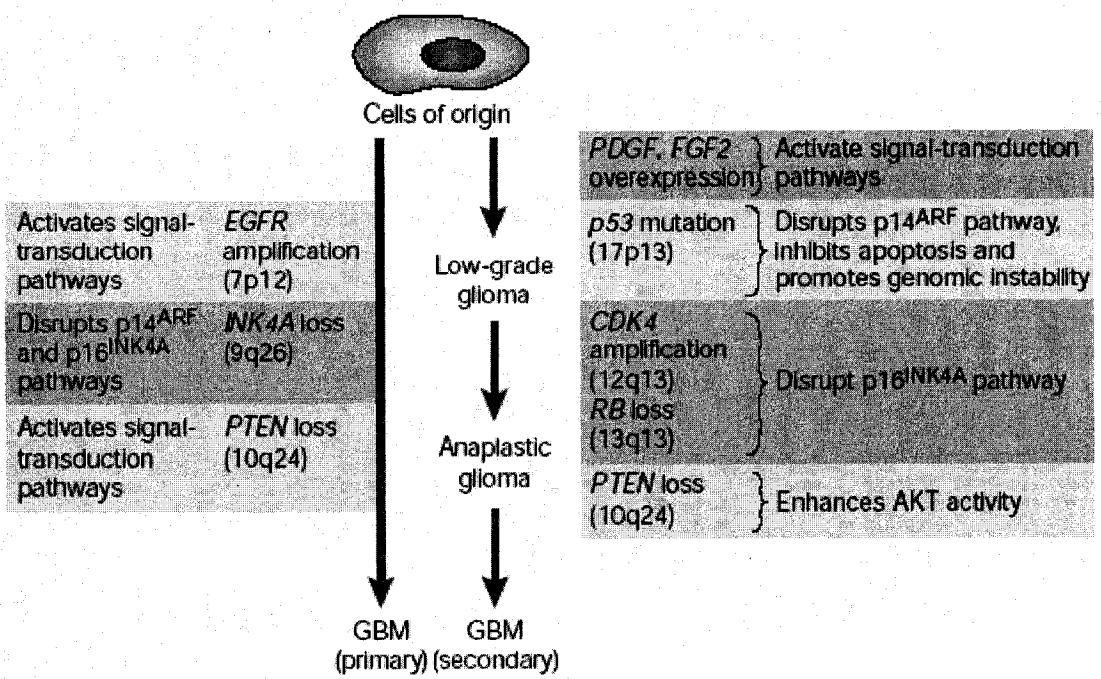
1.4 EGFR Signaling in Glioblastoma

Of the receptor tyrosine kinases, the most frequently altered gene in GBM is the epidermal growth factor receptor (EGFR). EGFR is a member of the human epidermal growth factor

Figure 1.1 Glioblastoma multiforme development occurs by one of two pathways.

Primary GBM develops de novo while secondary GBM develops from a lower-grade glioma due to the step-wise accumulation of multiple mutations. The mutations indicated are common mutations which have some correlation with clinical grade, although they represent only a subset of the mutations that occur in GBM. Figure from Holland, E.C. 2001. Gliomagenesis: genetic alterations and mouse models. *Nature Reviews Genetics* 2:120-9.

Figure 1.1


Cells of origin
Activates signal-transduction pathways
EGFR amplification (7p12)
Disrupts p14ARF and p16INK4A pathways
INK4A loss (9q26)
Activates signal-transduction pathways
PTEN loss (10q24)
Low-grade glioma
Anaplastic glioma
GBM (primary)
GBM (secondary)
PDGF, FGF2 overexpression
Activate signal-transduction pathways
p53 mutation (17p13)
Disrupts p14ARF pathway, inhibits apoptosis and promotes genomic instability
CDK4 amplification (12q13)
RB loss (13q13)
Disrupt p16INK4A pathway
PTEN loss (10q24)
Enhances AKT activity

receptor family, which also includes HER2, HER3 and HER4. In many cell types this family of receptors transduces signals from the cell surface to regulate normal cell growth and differentiation. EGFR is activated by the binding of one of its ligands, which include epidermal growth factor and transforming growth factor α. Ligand binding causes the receptor to homo- or heterodimerize, resulting in its autophosphorylation and generating multiple phosphorylated residues that serve as binding sites for signaling molecules. (4)

As EGFR signaling activates several pathways which promote cellular proliferation, it is not surprising that the EGFR gene alteration occurs frequently in many cancer types. In fact, EGFR is amplified in approximately 40-50% of GBM cases (13-15). This amplification results in a high density of receptors at the cell surface which is thought to induce dimerization in the absence of ligand (11). Gene amplification is often associated with EGFR mutations, the commonest of which is EGFRvIII (11). The mutant receptor, which is expressed in other cancers as well but not in normal tissues (11), has a deletion of exons 2-7 which truncates the extracellular ligand-binding domain, conferring upon it a ligand-independent constitutive activity (16). In vitro experiments have shown that activation of EGFR, whether it is via mutation or overexpression, confers enhanced tumourgenicity on glioblastoma cells (17-20). Interestingly, the mutant receptor preferentially activates the PI3K signaling pathway and can sensitize cells to inhibition with EGFR kinase inhibitors (21). In GBM patients, both overexpression of EGFR and EGFRvIII have been associated with shorter life expectancies (22). Therefore, EGFR would appear to be a good therapeutic target in GBM. Anti-EGFR antibodies as well as small molecule inhibitors have been tested in clinical trials with limited success. Although one study showed a survival benefit in non-

small cell lung cancer (23), little effect was seen in GBM (24, 25). It is thought that the lack of success of these drugs in GBM patients may be attributable to additional mutations which activate signaling downstream of EGFR so that its inhibition is rendered ineffectual. The next step, then, is to understand the mechanisms by which signaling downstream of EGFR promotes cellular proliferation, with the goal of identifying additional, more specific therapeutic targets.

Activated EGFR interacts with a host of signaling molecules which transduce its proliferative signal from the membrane to the nucleus (26). Both the MEK/ERK and PI3K signaling pathways, which are outlined in figure 1.2, have been shown to be indispensable for the ability of EGFR to promote proliferation (27, 28).

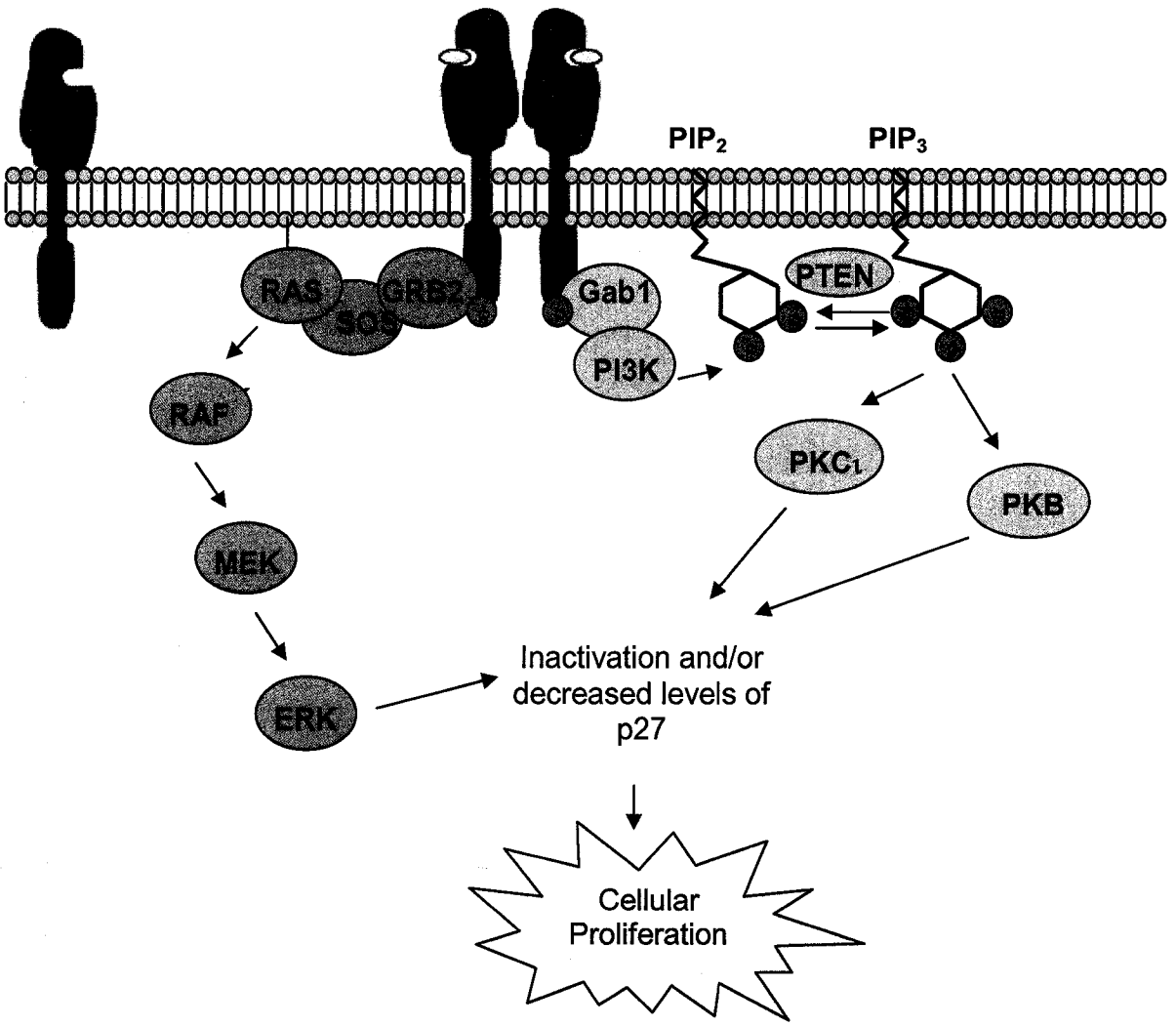
1.5 The MEK/ERK pathway

The MEK/ERK pathway was the first signal transduction cascade to be characterized from the cell membrane to the nucleus. After 15 years of intense study its role in a plethora of physiological functions including proliferation is well-established (29). The MEK/ERK pathway begins with the binding of the adaptor protein Grb2 to an activated receptor tyrosine kinase such as EGFR. Grb2 is associated with the guanine nucleotide exchange factor Sos, and its recruitment to the membrane allows it to activate the membrane-tethered GTPase Ras. Ras initiates a kinase cascade by recuiting and activating the kinase Raf, which phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. (30) ERK phosphorylates a wide range of substrates including membrane, and cytoskeletal and cytosolic proteins (29). Additionally, a fraction of activated erk translocates to the

Figure 1.2 Activation of the PI3K and MEK/ERK signaling pathways by EGFR.

Ligand binding results in EGFR to homo- or heterodimerization. This causes autophosphorylation and activation of the receptor. Activated EGFR initiates multiple signaling pathways, two of which are the PI3K pathway (blue) and the MEK/ERK pathway (orange). Both pathways inactivate p27 and/or decrease its protein levels via mechanisms which are not fully understood to promote cellular proliferation.

Figure 1.2


PIP2
PIP3
RAS
GRB2
SOS
Gab1
PI3K
PTEN
RAF
MEK
ERK
PKCι
PKB
Inactivation and/or
decreased levels of
p27
Cellular
Proliferation

nucleus where it phosphorylates transcription factors. One transcription factor which is particularly relevant to cellular proliferation is Elk-1; its phosphorylation results in the transcription of multiple genes involved in progression through the early phase of the cell cycle. (30)

1.6 The PI3K pathway

Phosphoinositide 3 kinase (PI3K) is activated by EGFR, via an adaptor molecule called Gab1 (31). The function of activated PI3K is to phosphorylate phosphoinositide (4,5) bisphosphate (PIP_2), which generates the second messenger phosphoinositide (3,4,5) triphosphate (PIP_3). PIP_3 creates a binding site for PH domain-containing proteins including phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB). (32) PDK1 activates both PKB and the atypical protein kinases C (aPKCs) (33). Considerably more is known about PKB than the aPKCs, although both have been implicated in the promotion of proliferation and survival. A large number of PKB substrates have been identified and the functional consequences of these phosphorylations have been elucidated in many cases. PKB is well known for its role in promoting survival by regulation of the FoxO and NFκB transcription factors, and phosphorylation of the anti-apoptotic protein BAD (34). PKB promotes proliferation by phosphorylating multiple cell-cycle-related targets including p27 and p21 (34). Humans have two highly similar aPKC isoforms: PKCι and PKCζ,, although only the ι isoform is expressed in glioblastoma cells (35). PKCι is considered an oncogene in non-small call lung cancer and ovarian cancer as it is frequently overexpressed and associated with decreased patient survival in both cancer types (36, 37). In vitro, expression of dominant negative PKCι significantly inhibits the transformed growth of lung cancer

cells (38). The activity of PI3K is antagonized by the tumour suppressor phosphatase and tensin homolog (PTEN), which dephosphorylates PIP_3 to convert it back to PIP_2 (39). PTEN is deleted in approximately half of glioblastomas (40), which underlines the importance of EGFR signaling and the PI3K pathway in GBM. In fact, activation of the PI3K pathway is significantly associated with increasing tumor grade, decreased levels of apoptosis, and with adverse clinical outcome in human gliomas (41).

1.7 Synergy in MEK/ERK and PI3K signaling

Although the MEK/ERK and PI3K pathways are often considered separately for the sake of simplicity, there is in fact a considerable amount of cross-talk between the two pathways. aPKCs have been shown to activate Raf, and Ras can also activate PI3K (42). In breast cancer cells, PKCζ, Ras and PI3K were found in a single complex (43). Furthermore, the transcription factor Elk-1, which is activated by ERK, induces expression of the PKCι gene (44). Synergy between these pathways has been observed, both in the prevention of apoptosis and in the stimulation of cellular proliferation (45). This type of synergy was also observed in one of the recent mouse models of malignant glioma. Expression of both PKB and activated Ras in neural progenitors resulted in a glioma with similar histological characteristics to GBM, although expression of either gene alone was not sufficient to cause glioma formation (46).

1.8 The cell cycle

The proliferative signal initiated by EGFR and transmitted through the PI3K and MEK/ERK pathways causes entry into the cell cycle. The cell cycle is divided into 4 phases as shown

in figure 1.3: G1 is the first gap phase, S is when DNA synthesis occurs, G2 is the second gap phase and M is when mitosis occurs. In the absence of proliferative signaling, cells will exit the cell cycle and enter a reversible growth arrest phase called quiescence (G_0). Progression through each phase is driven by cyclin dependent kinases (CDKs), which must be bound to their cyclin partners to be activated. There are four major cyclins are which are produced sequentially (D, E, A, and B) as the cell cycle progresses. (30) CDKs are additionally regulated by two families of inhibitors, all of which halt the cell cycle when overexpressed. The ink4 family is comprised of p15, p16 and p19, all of which specifically inhibit the activity of CDK4 and CDK6 to cause arrest in G1. The cip/kip family, which includes p21, p27 and p57 can inhibit all CDKs in vitro, although in vivo p27 arrests cells in G1 by inhibiting the activity of Cyclin E/CDK2. (47)

Growth factor signaling results in the transcription of multiple genes including cyclin D1, which binds to and activates CDK 4 and CDK 6. These kinases in turn phosphorylate multiple targets to promote progression through G1 phase. One such target is RB, which, when phosphorylated, releases the transcription factor E2F1 from sequestration so that it is free to activate the transcription of genes whose products are required for S phase activities such as nucleotide metabolism and DNA synthesis. E2F initiates two positive feedback loops, by stimulating its own expression and by inducing cyclin E expression, which binds to CDK2 to stimulate further Rb phosphorylation. At this point, passage through the cell cycle is independent of the activity of the Cdk4/6/ cyclin D complex, so that progression to S phase occurs even when growth signals are withdrawn and cyclin D levels fall. This "point of no return" is called the restriction point. (48) Because cancer cells can proliferate

in the absence of growth signals, deregulation of the restriction point is an important mechanism in the carcinogenic process (49).

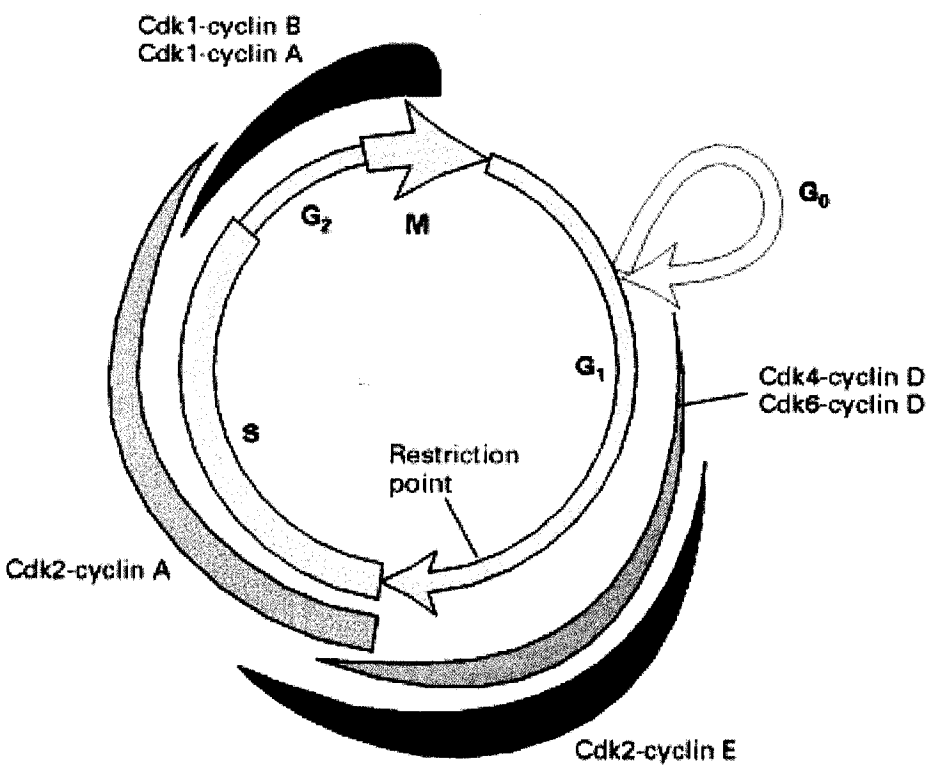
1.9 Cell cycle regulation in GBM

Accordingly, passage through the restriction point is tightly regulated by several CDK inhibitors which all have the ability to halt the cell cycle at G1 phase when overexpressed. Mutations in genes such as p53, MDM2 and $p14^{ARF}$ are very common in GBM and lead to an inability to induce expression of the CDK inhibitor p21 (49). The CDK inhibitors p15 and p16 are themselves often mutated in GBM (49). The CDK inhibitor p27 is also a key regulator of the restriction point, although the gene encoding p27 is rarely mutated in human cancers (50). Although it does not follow Knudson's classic two-hit hypothesis, p27 is considered a tumour suppressor because mice that are hemizygous for p27 are predisposed to tumour development in multiple tissues (51-53). The most compelling evidence for p27's role as a tumour suppressor comes from analysis of p27 protein levels in human cancers. Decreased p27 protein levels have been observed in up to 60% of human carcinomas and loss of p27 protein is considered an independent prognostic marker in many cancers including gliomas (50). Further, tumours which retain p27 expression often show mislocalization of the protein in the cytoplasm, which prevents it from binding to and inhibiting its nuclear cyclin/CDK targets (50). Therefore intense efforts have been undertaken to understand p27 regulation. Although several p27 regulators have been identified, many aspects of p27 regulation and the mechanisms causing its deregulation in cancer remain poorly understood.

Figure 1.3 The Cell Cycle.

Passage through each of the four stages of the cell cycle (G1, S, G2 and M) is regulated by the activity of cyclin-cdk complexes. The width of the colored bands is approximately proportional to the kinase activity of the indicated complexes. Following mitosis, cells can exit the cell cycle to enter a reversible state of growth arrest called quiescence (Go). Mitogenic stimulation allows quiescent cells to re-enter the cell cycle and results in the activation of the cdk4-cyclin D complex. Mitogens are required for passage through G1 until the restriction point, where cells are committed to completing the cell cycle even when mitogens are withdrawn. Figure from: Molecular cell biology, 4th edition. H. Lodish *et al*, 2000.

Figure 1.3


Cdk1-cyclin B
Cdk1-cyclin A
G2
M
G0
G1
Cdk4-cyclin D
Cdk6-cyclin D
S
Restriction
point
Cdk2-cyclin A
Cdk2-cyclin E

1.10 Regulation of p27 during the cell cycle

p27 regulation is complex; multiple signaling pathways alter its transcription, translation, degradation and subcellular location as the cell progresses through the cell cycle.

p27 levels are highest in quiescent cells, lower in G1-phase cells, and undetectable in S-phase cells (54). mRNA levels remain constant throughout the cell cycle, however, suggesting that post-transcriptional mechanisms are the major regulators of p27 levels (54). In S-phase cells, the degradation of p27 predominates as a regulatory mechanism (54) and is well understood. CDK2, coupled with either cyclin E or cyclin A, phosphorylates p27 at T187 which leads to its recognition by the ubiquitin ligase Skp2. Skp2 targets p27 for proteasome-mediated degradation, freeing cyclin/CDK2 complexes from inhibition by p27 in a positive feedback loop. (55) However, experiments with mice expressing a mutant p27 which cannot be phosphorylated at T187 revealed that although p27 degradation was prevented during S phase, it occurred normally as cells exited G_0 and progressed through G1 phase (56). This indicates that p27 degradation is controlled by a novel, Skp2-independent mechanism early in the cell cycle.

In G1 phase, p27 degradation is under the control of the cytoplasmic ubiquitin ligase KPC (57), although the events that cause p27 to be targeted for KPC-mediated degradation are poorly understood. In G_0 p27 is localized primarily in the nucleus where it binds and inhibits cyclinA/E/CDK2 complexes (58). As KPC is a cytoplasmic protein, one requirement for p27 degradation is its export to the cytoplasm (57). Upon entry into G1, growth factor stimulation activates a host of proteins which are implicated in p27 regulation, including hKIS (59). hKIS phosphorylates p27 at serine 10. This results in the nuclear

export of p27 which is dependent on the carrier protein CRM1 (59). As p27 does not harbour the nuclear export signal (NES) recognized by CRM1, interaction with the NES-containing protein Jab 1 is also required for export of p27 from the nucleus (60). Export of p27 to the cytoplasm is required for cell cycle progression, not just because this prevents its inhibition of cyclin E/cdk2, but because p27 associates with cyclin D/cdk/4/6 complexes to aid in their assembly (61). Nuclear export is associated with degradation of p27, as both S10 phosphorylation of p27 and overexpression of Jab 1 resulted in nuclear export coupled with accelerated p27 degradation (60, 62). A causative relationship between nuclear export and degradation has not been established, however, and it is likely that unknown cytoplasmic factors are responsible for directing p27 recognition by KPC. PKB plays a role in promoting progression through G1 phase by phosphorylating cytoplasmic p27 at T187 (63-65). This prevents p27 from re-entering the nucleus and exerting its inhibitory action, but it is not associated with increased p27 degradation (63-65).

As p27 half-life decreases fivefold with passage from G_0 to S phase, proteasome-mediated degradation is an important mechanism of p27 regulation in G1 (58). As mentioned above, sub-cellular localization is also important in p27 regulation. There is some evidence for the translational regulation of p27 as well, since p27 synthesis decreases with entry into G1 although mRNA levels remain constant (54). The decrease in p27 translation appears to be a consequence of decreased association of p27 with ribosomes (54).

1.11 Deregulation of p27 in cancer

Although transcriptional upregulation of p27 by FoxO transcription factors has been demonstrated, p27 mRNA levels are rarely altered in cancers (50). Proteolysis is an important regulator of p27 in cancer, as proteolytic degradation of p27 is increased in lysates from several types of cancers (66-68). Not surprisingly, Skp2 is over expressed in multiple cancers and associated with decreased p27 levels as well as decreased survival (69-71). In GBM, Skp2 is overexpressed as a result of amplification in 30% of patients and in several cell lines, although not those employed in this work (69). Cytoplasmic mislocalization is another common mechanism by which p27 is inactivated in cancer. This may in part be explained by the observation that Jab 1 is overexpressed in a variety of cancers where it is associated with decreased p27 levels and decreased patient survival. (72-74). Changes in stoichiometry of CDK complexes can cause functional inactivation of p27. Increases in components of the cyclinD/CDK4/6 complexes can lead to sequestration of p27 in this complex, titrating it away from the cyclinA/E/CDK2 complex that it is meant to inhibit (75). The contribution of this to carcinogenesis is difficult to evaluate, however. It has been shown that p16 loss was associated with poor prognosis in hepatocellular carcinoma when p27 levels were high, suggesting that p16 loss functionally inactivates p27 by increasing its association with cyclin D/CDK4/6 complexes (76). As p16, CDK4, CDK6 and cyclin D are commonly altered in GBM, sequestration of p27 into cyclinD/CDK4/6 complexes may be an important mechanism of p27 deregulation.

1.12 EGFR signaling inactivates p27 via the MEK/ERK and PI3K pathways

Many p27 regulators are downstream of EGFR family receptors, which themselves are commonly altered in GBM as mentioned above. Inhibition of HER2, the preferred dimerization partner of EGFR, causes p27-dependent G1 arrest and increases the stability of p27 as well as its phosphorylation at T187 and S10 (77).

The MEK/ERK and PI3K pathways are key effectors of EGFR signaling and are directly linked to p27 regulation. Inhibition of both MEK (78-81) and PI3K (82-84) has been shown to halt cellular proliferation by a mechanism that is dependent upon p27.

In the case of MEK/ERK signaling, there is no general consensus as to how this pathway regulates p27, although multiple mechanisms have been discovered. In breast cancer cells, ERK contributes to the S phase pathway of p27 degradation by directly phosphorylating p27 on T187 (80). However, MEK/ERK signaling induces p27 degradation in melanoma cells (78) and in fibroblasts in a manner that is independent of the S phase pathway (79). There is evidence that MEK signaling can cause export of p27 to the cytoplasm in epithelial cells (85) and in breast cancer cells in response to estrogens (81). Erk also upregulates transcription of cyclin D1 (86), which could contribute to p27 inactivation by changing the stoichiometry of the cyclin-cdk complexes. Finally, ERK was shown to decrease the stability of p27 mRNA in vascular smooth muscle cells in response to PDGF through an element in the p27 3' UTR (87). Therefore MEK/ERK signaling can affect p27 by multiple mechanisms which are likely to depend on cell type.

The mechanism by which the PI3K pathway negatively regulates p27 is even less clear. The PI3K-mediated decrease in p27 levels is an early event in G1, as overexpression of PTEN in GBM cells increased p27 levels before decreasing cdk2 activity (84). Therefore, although PI3K does cause upregulation of Skp2 which promotes p27 degradation (88), it also acts on p27 by an unknown mechanism in early G1 phase. Expression of PTEN or a small molecule PI3K inhibitor in glioblastoma cells does not alter p27 mRNA levels but does increase the stability of the protein (83). Thus transcriptional regulation of p27 by Forkhead box O transcription factors factors, which are regulated by PKB (89), can be ruled out. PKB contributes to PI3K-mediated regulation through its phosphorylation of p27, although this is not linked to p27 degradation (63-65). Additionally, the PKB phosphorylation site in p27 is not conserved, and mice show decreased p27 levels in G1 even though they lack this site (56). Thus it is likely that another PI3K-activated protein regulates p27 as well. It was recently shown in breast cancer cells that expression of dominant-negative PKCζ halted the cell cycle in G1 phase by increasing p27 protein levels (43). Thus the aPKCs may be responsible for PI3K-mediated regulation of p27, but the mechanism by which they exert this effect is entirely unexplored.

1.13 MicroRNAs as novel regulators of gene expression

An additional layer of complexity has been added to the study of gene expression by the recent discovery of microRNA (miRNA). miRNAs are 20-25 nucleotide RNAs which repress gene expression by post-transcriptional silencing (90). The first miRNA was discovered in the early 1990s (91), but was thought to be an oddity specific to worms. It wasn't until 2000 that a second miRNA was discovered in *C. elegans* (92) that was

conserved in a variety of animals (93). This discovery brought about an intensive effort to identify miRNA genes. To date, 474 human miRNAs have been cloned and verified (miR registry, www.sanger.ac.uk/Software/Rfam/mirna/index.shtml). Computational searches predict the existence of over 1000 miRNA genes, making miRNAs one of the largest classes of gene regulators (94, 95). miRNAs have been identified in diverse animals and plants and many are conserved among species (90). It now seems likely that all multicellular eukaryotes, and perhaps some unicellular eukaryotes, regulate gene expression by miRNAs (96). Each miRNA is predicted to regulate approximately 200 target genes, suggesting that approximately 1/3 of human genes might be subject to regulation by miRNAs (97).

Most miRNA genes are encoded in regions of the genome which are distant from previously annotated genes, implying that their expression is regulated independently. However, about one quarter of human miRNA genes reside in the introns of protein-coding genes, often in the same orientation as the mRNA, suggesting that their expression is controlled by the host gene promoter. miRNA genes may also be clustered for coordinated expression; this is true for over half of the known *Drosophila* miRNAs but relatively rare in humans. (90) miRNA expression is highly tissue-specific (98, 99), and relatively well conserved, with variation in miRNA expression patterns being more pronounced the greater the differences in physiology (100).

1.14 Biogenesis of miRNAs

The biogenesis of miRNAs is shown in figure 1.4. After being transcribed, the primary miRNA transcript is cleaved by the endonuclease drosha to generate a pre-miRNA. The

pre-miRNA is approximately 70 nt and folds into a stem loop structure with multiple bulges and mismatches. Pre-miRNAs are actively exported to the cytoplasm via Ran-GTP and exportin 5. In the cytoplasm the pre-miRNA is additionally processed by dicer to generate a 20 bp duplex. The duplex is in fact identical to an siRNA, which is also processed by dicer, and from this point on, the miRNA and siRNA pathways are indistinguishable. (90) The only distinction between miRNAs and endogenous siRNAs is their biogenesis; numerous siRNAs are generated from both strands of a long double stranded RNA, whereas a transcript forming a local hairpin structure is processed to give rise to a single miRNA molecule (101). The miRNA duplex is unwound by a helicase and one strand is preferentially incorporated into the RNA-induced silencing complex (RISC). The determination of which strand gets incorporated into the RISC is thought to be made by the helicase, which unwinds the miRNA duplex starting at the end which is less tightly paired, and incorporates the 5' end into the RISC. The complimentary strand, designated miRNA*, is thought to be degraded quickly, as miRNA*s have been cloned but with much less efficiency than their miRNA counterparts. (90)

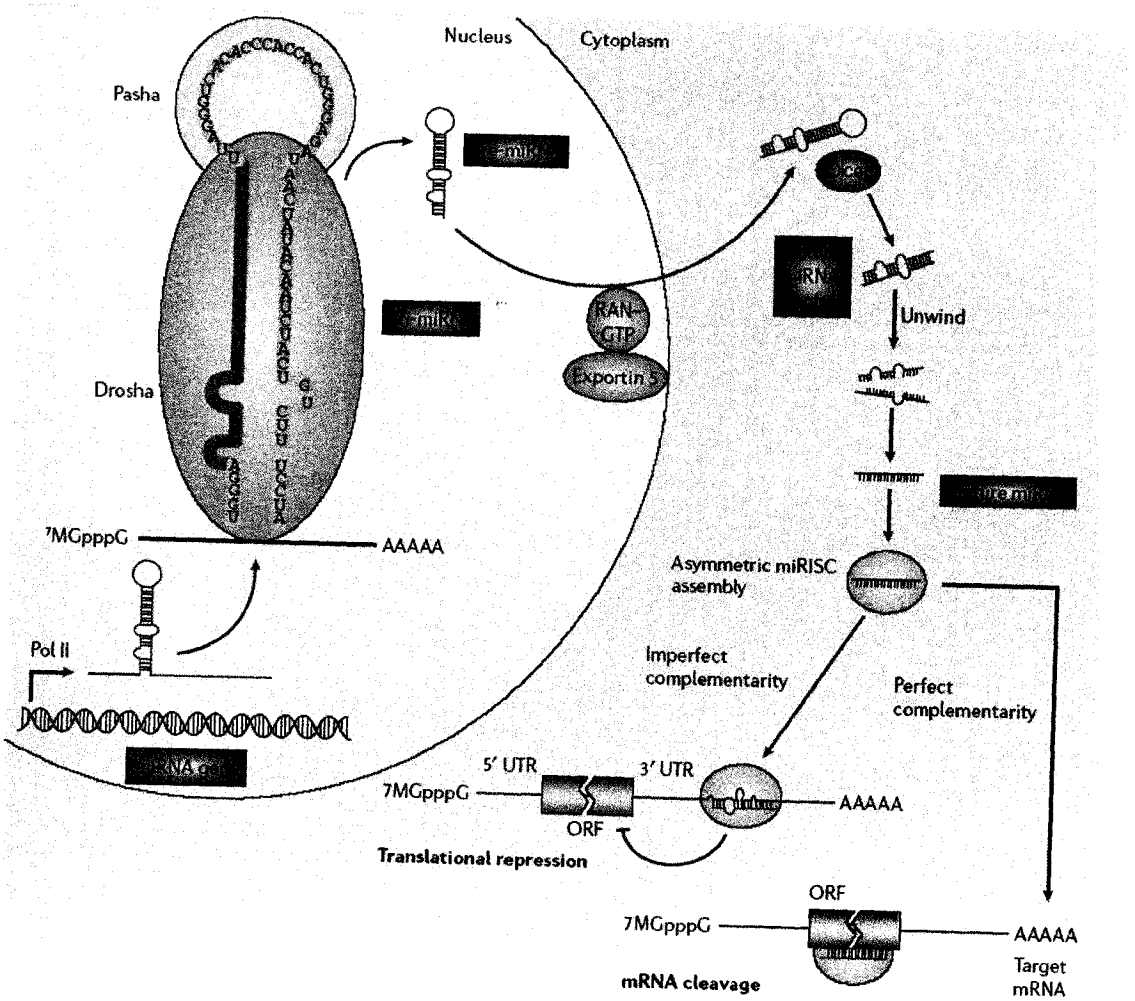
1.15 miRNA-mediated gene silencing

miRNAs silence gene expression by one of two mechanisms. If they are perfectly complimentary to their target mRNA sequences, the RISC will cleave the mRNA to cause its degradation. If the miRNA is imperfectly complimentary to its target, translation of the

Figure 1.4 MicroRNA Biogenesis and Function.

See text for details. Figure from: Esquela-Kerscher, A., and F.J. Slack. 2006. Oncomirs - microRNAs with a role in cancer. *Nat. Rev. Cancer.* 6:259-269.

Figure 1.4


Nucleus
Cytoplasm
Pasha
Drosha
pri-miRNA
pre-miRNA
RAN-GTP
Exportin 5
Unwind
Asymmetric miRISC assembly
Imperfect complementarity
Perfect complementarity
7MGpppG
AAAAA
Pol II
DNA
5′ UTR
3′ UTR
ORF
Translational repression
mRNA cleavage
Target mRNA

mRNA will be repressed without a change in levels of the mRNA. (90) There are three competing theories as to how translational repression by miRNAs is achieved. Most recently, it was shown that the let-7 miRNA interferes with growing polypeptides on actively translating polyribosomes (102). However, there is also evidence that miRNAs interfere with translation initiation, as they affected cap-dependent but not -independent translation (103). Finally, miRNAs may simply prevent translation by sequestering their target mRNAs away from ribosomes and initiation factors. miRNAs, their target mRNAs and RISC complex components have been shown to accumulate in cytoplasmic processing bodies (P bodies). (104) P bodies are cytoplasmic foci where mRNA degradation occurs, and they do not contain the components of the translation machinery (104). However, it has been suggested that their localization in these areas may be a consequence, rather than a cause, of inhibition of translation (103).

1.16 Prediction and validation of microRNA targets

As of October 2005, only 30 human miRNA target genes have been validated, owing to the lack of a high through-put method for target validation (105). All of the known miRNA binding sites reside in the 3'UTRs of their target mRNAs (105), and multiple binding sites seem to be important for efficient repression of translation (106). There is already evidence of binding sites for several different miRNAs in a single 3'UTR (90, 105) which hints at the complexity that could be achieved by the requirement for the cooperation of multiple miRNAs to repress translation. As miRNAs direct translational repression by binding with imperfect complimentarity, prediction of miRNA target sites is exceedingly difficult. The high degree of conservation of nucleotides 2-7 at the 5' end of the miRNA led to the

discovery that this "seed sequence" must be perfectly complimentary to its target mRNA, with imperfect binding at the 3' end (95). The importance of the 3' end is not fully understood, although there is some indication that the bulges resulting from imperfect complimentarity in this region may also be important for binding (107). The lack of validated miRNA targets to serve as a training set makes it difficult to design programs to predict miRNA target sites. However, several miRNA target prediction programs have been designed using complex algorithms which, at the simplest level, predict targets based on conservation of the seed sequence between multiple species, and the thermodynamic free-energy of binding (105).

One of the more sophisticated of these programs is Targetscan, which identifies miRNA target sites that are conserved among five species. The program is impressively successful, as twelve out of fifteen of its predicted targets were validated as bona fide miRNA binding sites (108). Targetscan identified 5300 human genes as miRNA targets which have a wide range of functions. Many of the predicted targets are involved in growth, cell cycle regulation and signal transduction (108), which raises the possibility that miRNAs could be involved in cancer.

1.17 MicroRNA and Cancer

Although miRNAs represent 1% of all human genes, more than 50 % them are located in regions of deletion, amplification, or translocation in cancer (109). This staggering statistic led to the identification of multiple miRNAs which act as tumour suppressor genes. The first indication that miRNAs can function as tumour suppressor genes came from analysis of

the 13q14 region, which is the most commonly deleted region in chronic lymphocytic leukemia (CLL). Intensive efforts to identify a protein-coding gene which was linked to CLL were met with failure (110). It was finally discovered that the region encoded 2 miRNAs, miR-16-1 and miR-15a, and that these miRNAs were mutated in 68 % of CLL cases (111). miR-16-1 and miR-15a were subsequently found to mediate the post-transcripitonal repression of the oncogene Bcl2, which may in part explain their tumour suppressor function (112), although, like most miRNAs, these miRNAs have hundreds of predicted targets (97). The study of miRNA deregulation in cancer has been greatly facilitated by the advent of miRNA microarrays, which allow for global analysis of miRNA expression. A recent study identified 26 overexpressed and 17 underexpressed miRNAs using samples from six different cancer types (113). Other oncogenes regulated by miRNA include ras and E2F1 (114, 115). miRNAs may also act as oncogenes by inhibiting tumour suppressors. PTEN is the only tumour suppressor gene to be identified as a miRNA target thus far (108), although there are several miRNAs which are upregulated in cancers (116), which suggests that these miRNAs may also inhibit tumour suppressor genes.

Although mutations causing deregulation of individual miRNAs is likely the main mechanism by which miRNAs contribute to tumourigenesis, there is some evidence that global regulation of miRNA may play a role as well. Alterations in the expression of components of the miRNA machinery have been observed in several cancers. Dicer expression is down-regulated in lung cancer and associated with decreased survival (117). As well, three human AGO genes, which form part of the RISC complex, are clustered together in a region frequently deleted in kidney and neuroectodermal tumours (118).

1.18 A potential role for miRNAs in p27 regulation

The deletion of dicer effectively abrogates miRNA expression (119), and is an excellent way to determine the involvement of miRNAs in cancer. Dicer-null mice die during embryogenesis with a lack of detectable multipotent stem cells (120). Conditional inactivation of dicer in murine embryonic stem cells compromised their proliferation, with an increased number of cells accumulating in G1 phase (119). A similar delay in progression from G1 to S phase was observed upon dicer deletion in *drosphila* germinal stem cells (121). This effect could be reversed by reducing the level of dacapo, the drosophila homologue of p21/p27. Additionally, the deletion of a region of the dacapo 3'UTR also delayed G1 to S phase progression by increasing dacapo levels. These data suggest that the miRNA pathway controls the cell cycle by reducing dacapo levels via regulation of the dacapo 3'UTR. (121)

1.19 Deregulation of miRNA expression in GBM and other cancers

As they were only recently discovered, very little is known about microRNAs in GBM; in fact only two papers have been published on the subject. Ciafre *et al* used microarrays to analyze the expression of 245 miRNAs in glioblastoma using both patient samples and cell lines. They found that a group of miRNAs comprised of miR-181a, miR-181b and miR-181c were all down-regulated in GBM, even though they are encoded on 3 different chromosomes, implying that they share a common biological function. miR-128 was also consistently downregulated. The only miRNA which was consistently upregulated in patient samples and cell lines (including those employed in this work) was miR-221 (122). This miRNA is also upregulated in papillary thyroid carcinomas (123, 124), and cancers of

the pancreas, stomach and colon (113, 125). Interestingly, there is a single instance where it was found to be down-regulated. Both miR-221 and miR-222, which are clustered on the X chromosome and share the same seed sequence (126), were found to be down-regulated through the erythropoietic pathway (127). Felli *et al* hypothesized that miR-221/222 downregulation unblocks the translation of proteins which promote erythropoiesis, and showed that one such protein was the kit receptor. MiR-221/222 inhibited normal erythropoiesis and erythroleukemic cell growth in part via kit receptor down-modulation, although they did not affect the proliferation of kit$^-$ hematopoeitic cell lines (127). Of course, the effect of miRNA expression is likely to vary somewhat from tissue to tissue, as different genes are expressed in different tissues, and the association of miR-221/222 downregulation with proliferation may be specific to kit-expressing cells of the hematopoeitic lineage. Overall, miR-221 is upregulated in multiple solid cancers, and an investigation into its potential role in cancer development is warranted.

Hypotheses

Part I

Activation of the PI3K pathway is often seen in cancer, and is especially common in glioblastoma. PI3K activates PKCι, which is also implicated in proliferation control, although the mechanism by which exerts its effect is unknown. I will test the hypothesis that PKCι controls the proliferation of glioblastoma cells by regulating the cyclin dependent kinase inhibitor p27.

Part II

MicroRNAs are novel regulators of post-transcriptional gene expression and are thought to play a role in cancer development. They control passage from G1 to S phase and in *Drosophila* they exerted this effect by regulation of the *Drosophila* homologue of p27. Thus p27 expression may be controlled by microRNA, and miR-221, as the sole upregulated miRNA in glioblastoma, is a good candidate to exert this effect. I will test the hypothesis that p27 is regulated by miR-221 in glioblastoma.

Specific Aims

Part I

1. Confirm that PKCι regulates p27
    a) By comparing p27 mRNA and protein levels in glioblastoma cell lines transfected with PKCι or control siRNA duplexes.
    b) By comparing p27 protein levels in PKCι-null vs. wild-type mouse embryonic fibroblasts.
2. Investigate the mechanism by which PKCι regulates p27 by examining the effect of PKCι depletion on known p27 regulatory pathways.

Part II

1. Deplete cells of the miRNA processing enzyme dicer and examining its effect on p27 protein levels to determine if p27 is a candidate for regulation by miRNA.
2. Determine whether miR-221 regulates p27 by
    a) Transfecting glioblastoma cells with miR-221 and control inhibitors and comparing p27 levels.
    b) Analyzing the effect of the miR-221 inhibitor on a reporter construct containing a wild type p27 3' UTR vs. a construct in which the putative miR-221 binding sites have been mutated.
3. Examine the effect of the MEK/ERK and PI3K pathways on the activity of the p27 reporter construct to determine if these pathways could potentially regulate p27 expression *via* a miR-221-dependent mechanism.

Significance

Glioblastoma is such a heterogeneous disease that treatment with a single agent alone is not likely to produce results in all patients. Although EGFR is mutated in about half of GBMs, EGFR-directed therapies have had little success in GBM. It was recently shown that response to EGFR inhibitors was strongly associated with co-expression of EGFRvIII and PTEN (128). This finding underlines the importance of understanding alterations in signaling downstream of EGFR. For the patients in whom PTEN expression has been lost, the best therapeutic strategy is a target downstream of PTEN. Unfortunately, the mechanisms by which the PI3K pathway promotes proliferation and resistance to apoptosis have not been entirely elucidated. This work shows that PKCι is a critical mediator of proliferation downstream of PI3K through its inhibition of p27.

In addition to confirming the role of PKCι as a p27 regulator, this work also identifies a microRNA which regulates p27. This is a small but significant step in the monumentous task of validating the thousands of predicted miRNA targets. Many of the validated miRNA targets are cancer-related, and this work helps to substantiate the hypothesis that microRNAs have a crucial function in cancer progression, as well as identify miR-221 as a potential therapeutic target in GBM.

Chapter 2

Materials and Methods

2.1 Cell Culture

U87MG cells were obtained from Dr W Cavenee (Ludwig Institute for Cancer Research, La Jolla, CA, USA). A172 and U118 cells were from the American Type Culture Collection. U87MG and A172 are human glioblastoma cell lines mutated for PTEN and Ink4a/Arf but wild-type for p53, a set of mutations which is common in primary glioblastoma. The human glioblastoma cell line U118 is mutated for PTEN, Ink4a/Arf and also p53. The glioblastoma cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 7.5% donor bovine serum, 2.5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. Wild-type and PKCλ-deficient mouse embryonic fibroblasts (MEFs) were obtained from Dr S Hedrick (University of California at San Diego, La Jolla, CA, USA). MEFs were cultured at 37°C in 5 % CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 7.5% donor bovine serum, 2.5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μM non-essential amino acids, $5x10^{-5}$ M β-mercaptoethanol and 400 μg/ml G418.

2.2 RNA interference

Short interfering RNA duplexes (siRNAs) were chemically synthesized (Dharmacon research Inc., CO, USA). The target sequences and concentrations are listed below:

Duplex	Target sequence	Final concentration (nM)
PKCι A	GUGCAUCAACUGCAAACUC	5
PKCι B	UGAGGUUCGAGACAUGUGGU	20
Dicer A	CACUGGUCAGGGAAGACAUU	10
Dicer B	GAGUUUACUAAGCACCAGGUU	10
Non-specific control	AUUCUAUCACUAGCGUGACUU	20

siRNA duplexes were transfected using oligofectamine as outlined in the transfection section.

2.3 miRNA inhibition

miRIDIAN microRNA inhibitors targeting miRNA-221 and miRNA-222, as well as a negative control inhibitor were purchased from Dharmacon RNA technologies (Lafayette, CO, USA). They are single-stranded oligonucleotides which bind to and inhibit endogenous miRNAs. Inhibitors were transfected with oligofectamine as outlined in the transfection section. Analyses were performed 48 hours after inhibitor transfection.

2.4 Transfection

Glioblastoma cells were plated at a density of 100,000 cells per 35 mm plate the day before transfection. For transfection one 35 mm plate with an siRNA duplex or miRNA inhibitor: On the day of transfection, 10 μl of siRNA duplex or miRNA inhibitor was added to 175 μl

OptiMEM I (Invitrogen, CA, USA). In a separate tube, 4 µl of oligofectamine was mixed with 11 µl OptiMEM I (Invitrogen) and incubated at room temperature for 10 minutes. 15 µl of this mixture was then added to the diluted duplex/inhibitor, mixed gently and incubated at room temperature for 20 minutes. Meanwhile, the cells were washed with 1 ml warm OptiMEM I and then 800 µl OptiMEM I was added to each well. 200 µl of the combined oligofectamine/duplex solution was added dropwise to each plate, which was then rocked gently for 30 seconds. Cells were then incubated for 4 hours at 37°C with 5 % CO_2, after which 2 ml of warm antibiotic-free media was added. Analysis was performed 48 – 72 hours post-transfection.

For transfection of one 35 mm plate with luciferase reporter plasmids: On the day of transfection, 3 µl of Genejuice transfection reagent (Novagen, WI, USA) was mixed by vortexing with 100 µl serum-free DMEM and incubated 10 minutes at room temperature. Next, 0.1 µg of a renilla luciferase reporter plasmid (pRL-CMV, pRL-CMV-p27UTR, or pRL-CMV-mut) and 0.01 µg of the firefly luciferase normalizing control plasmid pGL3 were added to the serum mixture and mixed gently by pipetting. After a 15 minute incubation, 100 µl of this mixture was added dropwise to the plate, which was then rocked gently for 30 seconds. Analysis was performed 24 hours post-transfection.

2.5 Plasmids

The renilla luciferase reporter plasmid pRLp27 3'UTR was constructed as follows. The entire p27 3'UTR was PCR-amplified from the p27 clone MGC-5304 (ATCC) using the primers P27UTRF (ACTAGTGACGTCAAACGTAAACAGC) and P27UTRR

(GGATCCCTTTTATTGATTACTTAATGTGTAACAA). The A-Addition and PCR cloning kits (Qiagen) were used to subclone the 3' UTR of p27 into pDrive (Qiagen). The 3' UTR was then sequenced and subcloned into the renilla luciferase reporter vector pRL-CMV. To do this, pRL-CMV was digested with Bam HI and Xba I to remove a 252 bp section containing the SV40 late poly (A) signal immediately behind the luciferase gene. The p27 3' UTR (which contains its own poly (A) signal) was cloned into this site following digestion of pDrive with Spe I (which generates ends compatible with Xba I) and Bam HI. After cloning into pRL-CMV, the p27 3'UTR was sequenced using the primers pRLF (ATGCTATTGTTGAAGGTGCC) and pRLR (TTATTGAAGCATTTATCAGG).

The predicted miRNA target sites within the p27 3' UTR, were mutated using the Quickchange XL site-directed mutagenesis kit (Stratagene). The first site was mutated using the primers p27mut1a (GCCTCTAAAAGCGTTGGAGGGATCATTATGCAATTAGG) and p27mut1rev (CCTAATTGCATAATGATCCCTCCAACGCTTTTAGAGGC). The second site was mutated using the primers p27mut2b (CCTGTGTATATAGTTTTTACCTTTTAGGGATCACATAAACTTTGGG) and p27mut2rev (CCCAAAGTTTATGTGATCCCTAAAAGGTAAAAACTATATACACAGG). The mutated constructs were sequenced using the pRL primers described above.

2.6 Luciferase Assays

Glioblastoma cells were plated at a density of 100,000 cells per 35 mm plate. The next day, siRNA duplexes or miRNA inhibitors were transfected. 24 hours later, cells were co-transfected with 0.1 μg of the renilla luciferase reporter plasmid pRLp27 3'UTR and 0.01μg of the firefly luciferase reporter plasmid pGL3. 48 hours after the initial transfection, cells were harvested for analysis of luciferase activity. Luciferase assays were performed using the Dual Luciferase Assay System (Promega) according to the manufacturer's instructions. Briefly, cells were lysed by scraping in 200 μl 1X passive lysis buffer. After 3 freeze-thaw cycles, luciferase activity was measured in 20 μl of the lysate using a luminometer. The luminometer dispensed 100 μl of Luciferase assay reagent II (LarII), took a 10 second measurement of firefly luciferase luminescence, and then dispensed 100 μl of Stop & Glo reagent, the renilla luciferase substrate which also quenches the firefly luciferase signal. After a 2 second delay to allow for quenching of the firefly signal and inactivation of firefly luciferase, renilla luminescence was measured for 10 seconds. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the p27 3'UTR-containing plasmid to the firefly luciferase activity of pGL3.

2.7 Western Blotting

Cells were washed twice with PBS and then lysed with an appropriate amount (*ie.* 75 μl per 35 mm plate) of 2X boiling western blot lysis buffer containing 5 mM Tris, pH 6.8, 4 % sodium dodecyl sulphate and 2 % glycerol. Lysates were scraped into microcentrifuge tubes, boiled for 5 minutes, cooled on ice for 5 minutes, and then sonicated for 5 minutes. The protein concentration of each sample was determined using the BCA assay kit (Pierce,

Rockford, IL, USA). 4 X Laemmli buffer (0.004 % bromophenol blue, 1 % sodium dodecyl sulfate, 2.5 g glycerol, 0.83 M tris pH 6.8, 14.3 M β-mercaptoethanol) was added to 80 – 100 μg of protein for a final concentration of 1 X Laemmli buffer. Samples were boiled 5 minutes before loading onto a NuPAGE 4-12 % Bis-Tris poly-acrylamide gel (Invitrogen). Samples were electrophoresed using 1 X NuPAGE running buffer and transferred to Hybond-P PVDF membrane (Amersham). To ensure equal protein loading and transfer, membranes were stained for 3 minutes with 0.025 % amido black in 10 % acetic acid and 50 % methanol, then rinsed to remove excess stain and photographed. Membranes were then incubated overnight with the following antibodies in 5 % skim milk in TBST (10 mM Tris pH 7.6, 150 mM NaCl, 0.05 % Tween-20).

Antibody	Dilution	Supplier
Phospho-Akt (S473) Mouse mAb	1:1000	Cell Signaling Technology
Dicer mouse mAb, CHIP grade	1:200	Cedarlane Laboratories Ltd, Hornby, ON, Canada
Phospho-p44/42 Map Kinase (Thr202/Tyr204) Antibody	1:1000	Cell Signaling Technology
Pan Erk mAb	1:5000	BD Transduction Laboratories
Kip1/p27 mAb	1:1000	BD Transduction Laboratories
PKCι mAb	1:1000	BD Transduction Laboratories
β-Tubulin mAb	1:5000	Sigma Aldrich

Blots were washed 3 times for 10 minutes in TBST and then incubated for 1 hour with a 1:5000 dilution of secondary goat anti-rabbit or goat anti-mouse HRP conjugated antibodies (Cedarlane). After washing 3 times for 10 minutes with TBST, blots were visualized with the Supersignal West Pico Chemiluminescent kit (Pierce). The image was captured using the GeneGnome System (Syngene) and Genesnap software (Syngene). Band quantification was also performed using Genesnap software.

2.8 Quantitative RT-PCR Analysis

Total RNA was extracted from U87MG cells 48 hours after transfection with PKCι or control siRNA duplexes using the RNeasy Kit (Qiagen). 4 μg of RNA was used as a template for first strand cDNA synthesis using M-MLV Reverse Transcriptase and random hexamer primers (First Strand cDNA Synthesis kit (Fermentas Life Sciences) as per the supplier's protocol. DNase digestion was performed using Turbo DNase (Ambion). RT-PCR was performed using the Lightcycler 1.5 instrument and the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Molecular Biochemicals, Mannheim, Germany) with 2 mM $MgCl_2$ and 1 μM primers. The primer set used to amplify p27 was P27F (AGATGTCAAACGTGCGAGTG) and P27R (TCTCTGCAGTGCTTCTCCAA). β-2 microglobulin was used as a reference gene and was amplified with the primers B2MFOR (TGCTGTCTCCATGTTTGATGTATCT) and B2MREV (TCTCTGCTCCCCACCTCTAAGT). The following LightCycler conditions were used: (i) denaturation program (94° for 10 minutes); (ii) amplification and quantification programs were repeated 45 times (94° for 2 s; 56° for 10 s; 72°C for 7 s with a single fluorescence measurement); (iii) melting curve program (65 – 99°C continuous fluorescence measurement); (iv) elongation program (72°C for 2 minutes) and (v) a cooling step to 40°C. The LightCycler software package version 5.3.2 was used to determine relative expression levels of p27. Quantification of p27 mRNA levels was extrapolated from a standard curve constructed from a serial dilution of control RNA.

2.9 Flow cytometry

U87MG cells were plated in 100 mm culture plates at 5 x 10^5 cells per dish. The following day the cells were mock-transfected or transfected with Dicer A or control duplexes. 48 hours post-transfection, both adherent and non-adherent cells were collected, washed twice with PBS and centrifuged at 1500 rpm for 5 minutes between washings. The cells were then fixed in 1 ml of ice-cold 70 % (v/v) ethanol/PBS at -20° C for 24 hours. To stain the nuclei, cells were incubated in 1 ml of a solution containing 18 μg/ml propidium iodide in PBS and 40 μg/ml RNase A for 24 hours in the dark at 4°C. Cells were resuspended and analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter, Mississauga, Canada). Data acquisition was done using Expo 32 AVC software (Beckman Coulter). DNA histograms were obtained and analyzed by Mod Fit LT software (Verity Software House Inc., Sopsham, ME, USA)

Chapter 3

Results

Part I: Regulation of p27 by PKCι

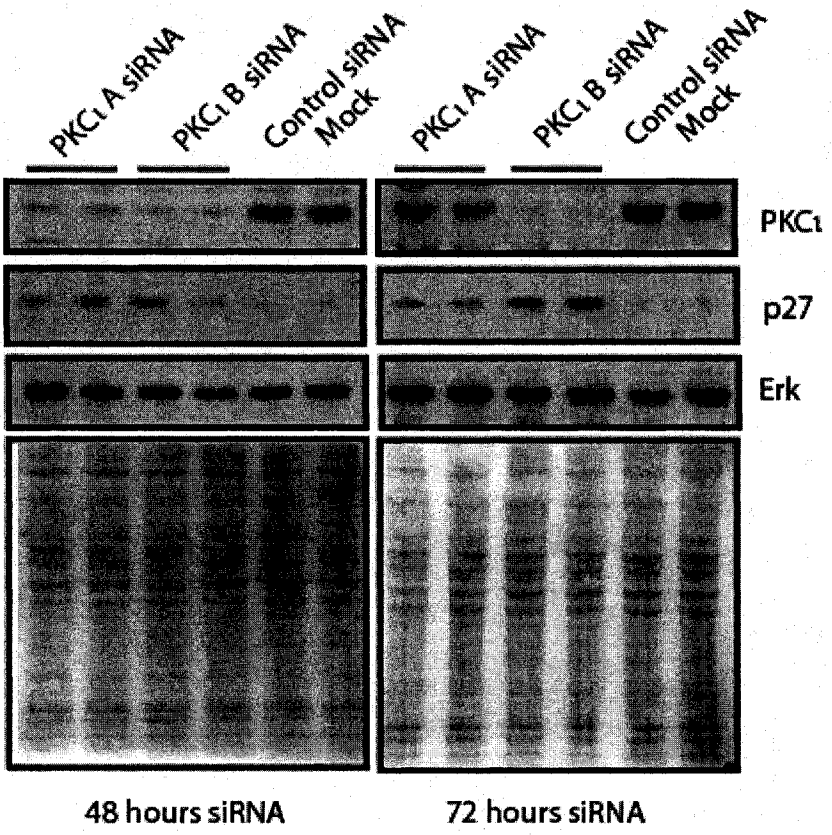
3.1 PKCι regulates p27 protein levels

Preliminary results from our lab have shown that siRNA-mediated depletion of U87MG cells of PKCι results in an increase in p27 levels (129). However, off-target effects of RNAi are already well documented at the transcriptional level (130) and the recent discovery that silencing at the translational level requires only a short, partially complimentary sequence suggests that off-target effects of exogenous siRNAs may be more prevalent than originally thought. Therefore, I confirmed that PKCι regulates p27 protein levels using a second siRNA. U87MG cells were mock-transfected or transfected with control or PKC ιA or PKCιB duplexes, and PKCι and p27 levels were analyzed by western blotting. **Figure 3.1** shows that both PKCιA and PKCιB duplexes, but not the control duplex, substantially decrease PKCι protein levels at 48 and 72 hours after their transfection. A strong increase in p27 levels can be seen in the cells transfected with PKCι duplexes, but not those transfected with the control duplex. The increase in p27 levels corresponds to the degree of PKCι knockdown. Although the use of two siRNA duplexes decreases the possibility that the effect of PKCι on p27 is an off-target effect of RNAi, it does not rule it out entirely. Therefore, the relationship between PKCι and p27 was investigated in mouse embryonic fibroblasts (MEFs). PKCι-null and wild-type MEFs were harvested for western blot analysis of p27 levels at 70% and 90% confluence. **Figure 3.2** shows that p27 levels are

Figure 3.1 PKCι depletion causes an increase in p27 protein levels.

U87MG cells were mock-transfected, transfected with 20nM random control siRNA, 5nM PKCι A siRNA, or 20 nM PKCι B siRNA. Cells were harvested 48 or 72 hours post-transfection and analyzed by western blotting with the indicated antibodies. The Erk antibody as well as amido black staining of the membrane were used to confirm equal protein loading.

Figure 3.1


PKCι A siRNA
PKCι B siRNA
Control siRNA
Mock
PKCι A siRNA
PKCι B siRNA
Control siRNA
Mock
PKCι
p27
Erk
48 hours siRNA
72 hours siRNA

higher in the PKCι-null MEFs. The experiment was performed in rapidly proliferating cells (70 % confluence) and cells approaching contact inhibition (90 % confluence) since it is known that p27 levels vary with cell density. **Figure 3.2** shows that although p27 levels were much higher in the denser cells, p27 levels increased a similar amount at both densities, showing that the relationship between PKCι and p27 is not affected by cell density. This experiment eliminates the possibility that the effect of PKCι on p27 is the result of off-target effects of RNAi and shows that regulation of p27 by PKCι is conserved in mice and humans.

3.2 Regulation of p27 by PKCι does not occur at the transcriptional level

Having shown that PKCι is a regulator of p27, I next investigated the mechanism by which PKCι exerts its effect. p27 regulation is thought to occur primarily at the post-translational level; however regulation of p27 transcription (131) and mRNA stability (87, 132) have been documented. I therefore began by investigating whether PKCι depletion alters levels of p27 mRNA. U87MG cells were transfected with the PKCιB or control siRNA duplexes and harvested 72 hours later for QRTPCR or western blot analysis of p27 levels. **Figure 3.3** shows that p27 mRNA levels do not change with PKCι depletion, although a western blot of samples from siRNA transfections done in parallel show increased p27 protein levels in response to PKCι depletion. Therefore, regulation of p27 by PKCι must occur at the translational or post-translational level, as p27 transcription and mRNA stability are unaltered by PKCι depletion.

Figure 3.2 PKCλ protein levels are increased in PKCλ$^{-/-}$ MEFs.

PKCλ$^{-/-}$ or wild type mouse embryonic fibroblasts were seeded at $3x10^5$ cells/ 60 mm plate and harvested 24 or 48 hours later, at 70% and 90% confluence. Cell lysates were analyzed by western blotting with the indicated antibodies. The actin antibody indicates protein loading.

Figure 3.2

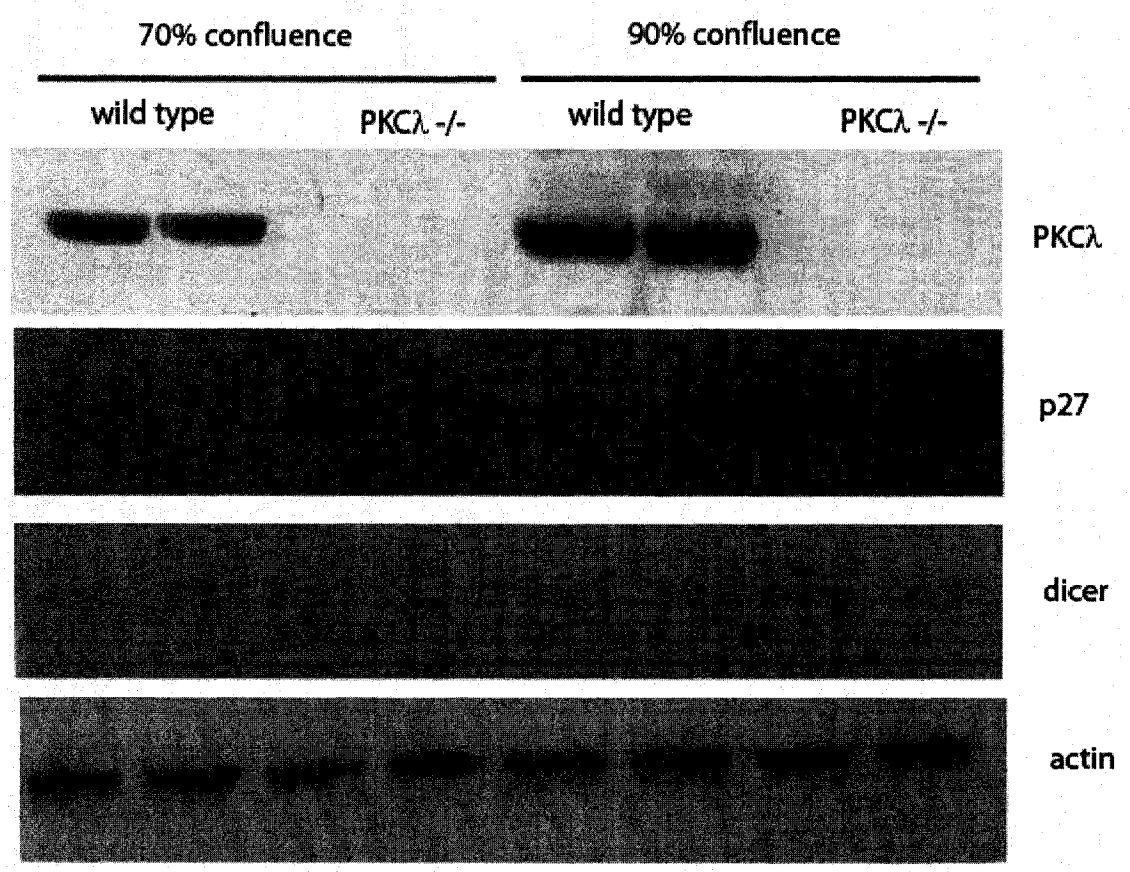
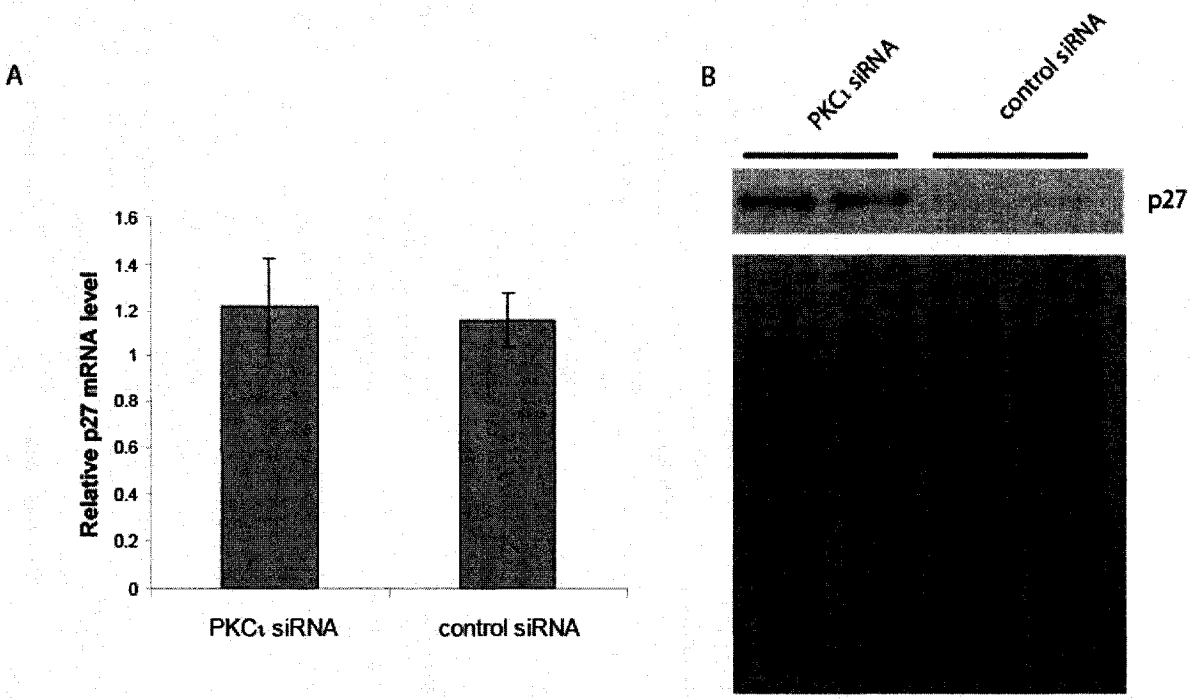

70% confluence
90% confluence
wild type
PKCλ -/-
wild type
PKCλ -/-
PKCλ
p27
dicer
actin

Figure 3.3 p27 mRNA levels are unaltered by depletion of PKCι. U87MG cells were transfected with 20 nM random control siRNA or 20 nM PKCι B siRNA. 72 hours later, cells were either harvested for western blot to confirm an increase in p27 protein levels or total RNA was isolated and cDNA was synthesized for QRTPCR analysis. **A.** No change in p27 mRNA levels occurs with PKCι depletion. p27 mRNA levels were analyzed by quantitative PCR with β2-microglobulin mRNA levels as a normalizing control. **B.** Western blot confirming that PKCι depletion increased p27 protein levels.

Figure 3.3


A
Relative p27 mRNA level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PKCι siRNA
control siRNA
B
PKCι siRNA
control siRNA
p27

3.3 Regulation of p27 by PKCι does not occur via modulation of PKB activity

There are multiple signaling pathways that are known to regulate p27, and PKCι could act on one or more of these pathways to decrease p27 levels. Therefore I began by examining the effect of siRNA-mediated depletion of PKCι on the activity PKB, a well-known regulator of p27. Western blotting with an antibody recognizing activated PKCι showed no change in PKB activity, although a substantial increase in p27 levels was observed in the PKCι-depleted cells (**figure 3.4**). Therefore, PKCι does not decrease p27 levels by modulation of PKB activity.

3.4 MEK/ERK and PKCι have a synergistic effect on p27

The next pathway that I investigated as a potential mechanism by which PKCι regulates p27 is the MEK/ERK pathway. The small molecule inhibitor U0126 is used as a specific inhibitor of MEK 1/2, although it has been reported that the third member of the MEK family, MEK 5, is also inhibited by U0126. MEK 1/2 phosphorylate and activate ERK 1/2; thus EKR activity is commonly used as a read-out for activity of the pathway. **Figure 3.5** shows a western blot of U87MG cells treated with U0126 and PKCι siRNA alone as well as in combination. Treatment of cells with U0126 resulted near-complete inhibition of ERK 1/2 activity, as shown by western blotting with an antibody to activated ERK. MEK/ERK inhibition resulted in a robust increase in p27, an observation that is consistent with several other reports (42, 43, 78, 87, 133). A slight but reproducible decrease in ERK activity is seen in cells treated with PKCι siRNA. This is consistent with the idea that cross-talk between MEK/ERK and PI3K signaling exists. Interestingly, when cells were depleted of PKCι and subsequently treated with U0126, an increase in p27 levels was observed which

Figure 3.4 PKB activation is unaltered by depletion of PKCι.

U87MG cells were mock-transfected, transfected with 20nM random control siRNA, 5nM PKCι A siRNA, or 20 nM PKCι B siRNA. Cells were harvested 72 hours post-transfection and analyzed by western blotting with antibodies against activated PKB and Erk as a loading control. Amido black staining of the membrane was used to confirm equal protein loading and effective transfer.

Figure 3.4

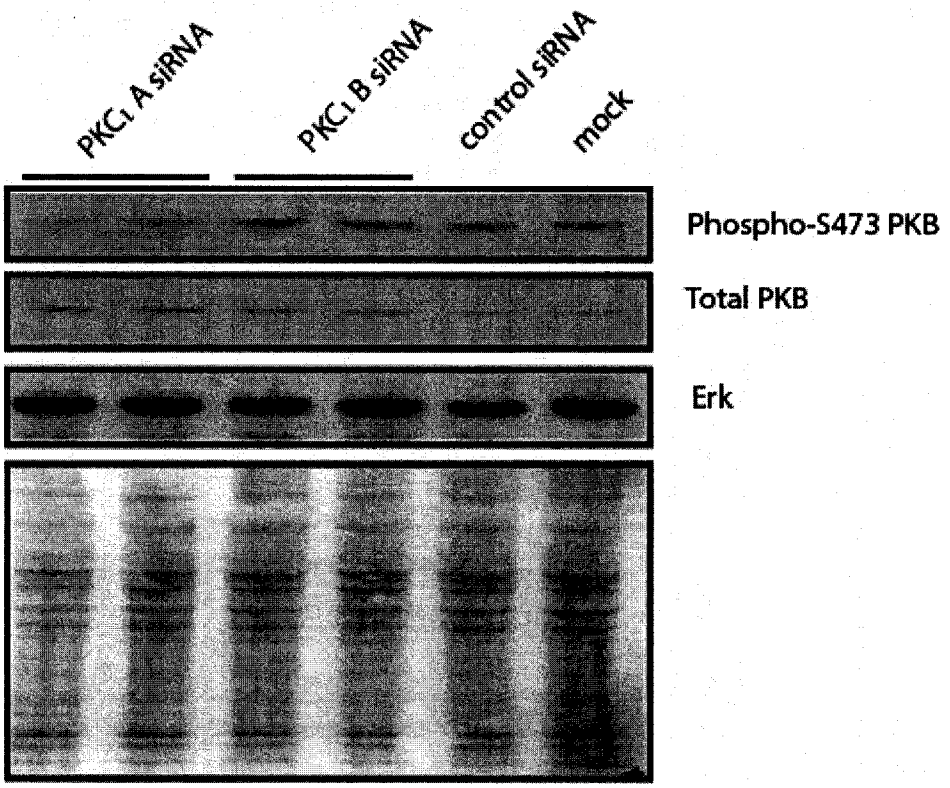
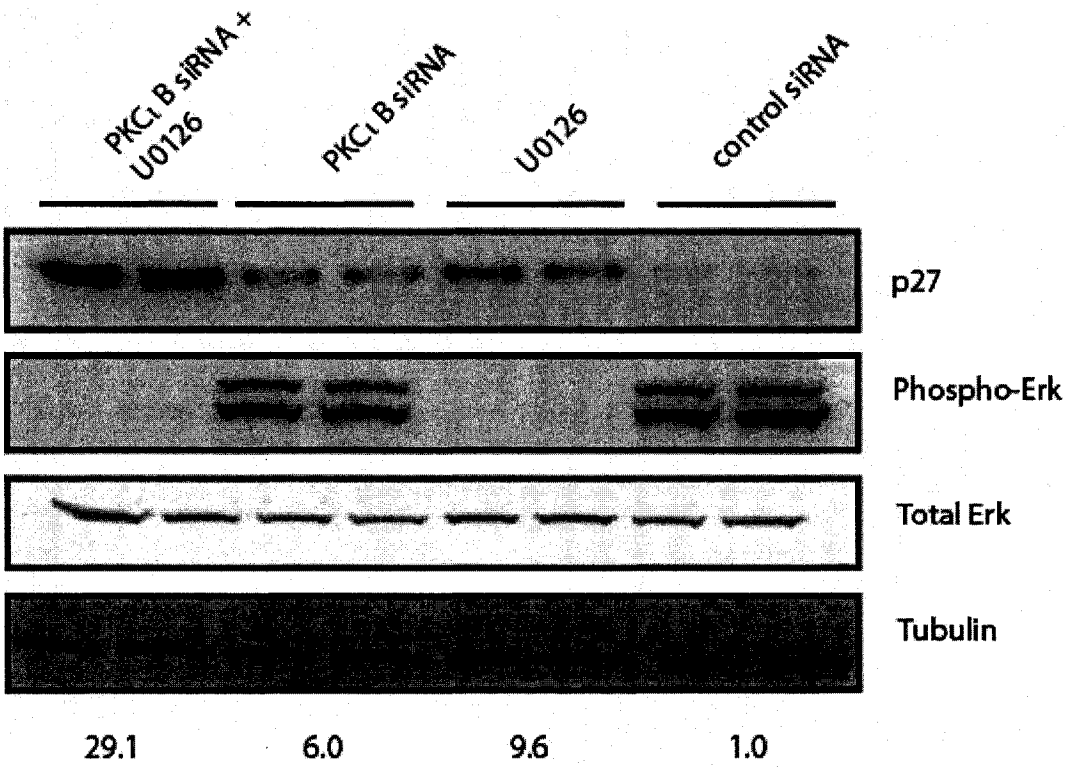
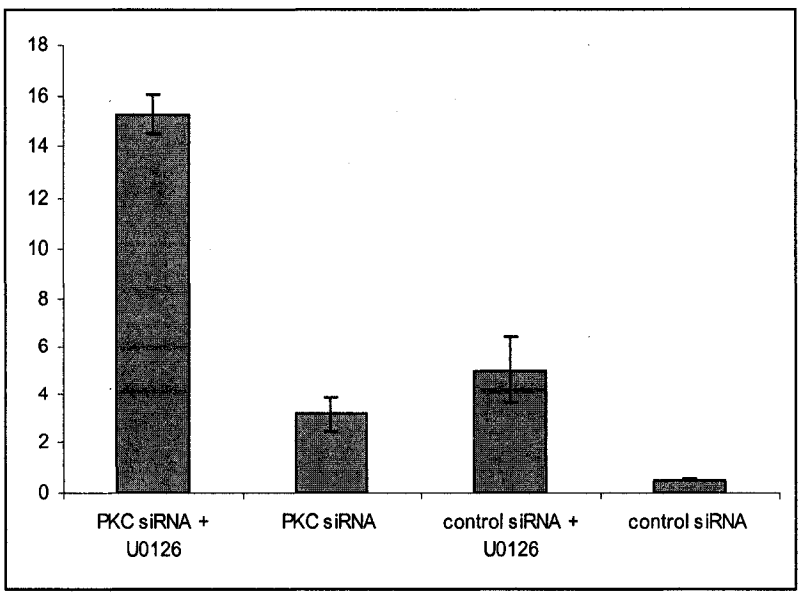

PKCι A siRNA
PKCι B siRNA
control siRNA
mock
Phospho-S473 PKB
Total PKB
Erk

Figure 3.5 PKC and MEK have a synergistic effect on p27 levels.

U87MG cells transfected with 20nM random control siRNA, or 20 nM PKCι B siRNA. 65 hours post-transfection, cells were treated with 20 μM of the MEK inhibitor U0126 or with vehicle alone. 17 hours later, cells were harvested and analyzed by western blotting with the indicated antibodies. The fold change in p27 levels with each treatment was quantified using Genesnap software and is indicated in the graph at the bottom of the figure.

Figure 3.5


PKCι B siRNA + U0126
PKCι B siRNA
U0126
control siRNA
p27
Phospho-Erk
Total Erk
Tubulin
29.1
6.0
9.6
1.0


18
16
14
12
10
8
6
4
2
0
PKC siRNA + U0126
PKC siRNA
control siRNA + U0126
control siRNA

was greater than the additive effects of U0126 or PKCι depletion (quantified at the bottom of the figure). Thus PKCι and MEK/ERK signaling appear to synergize in the negative regulation of p27.

Part II: Regulation of p27 by microRNA

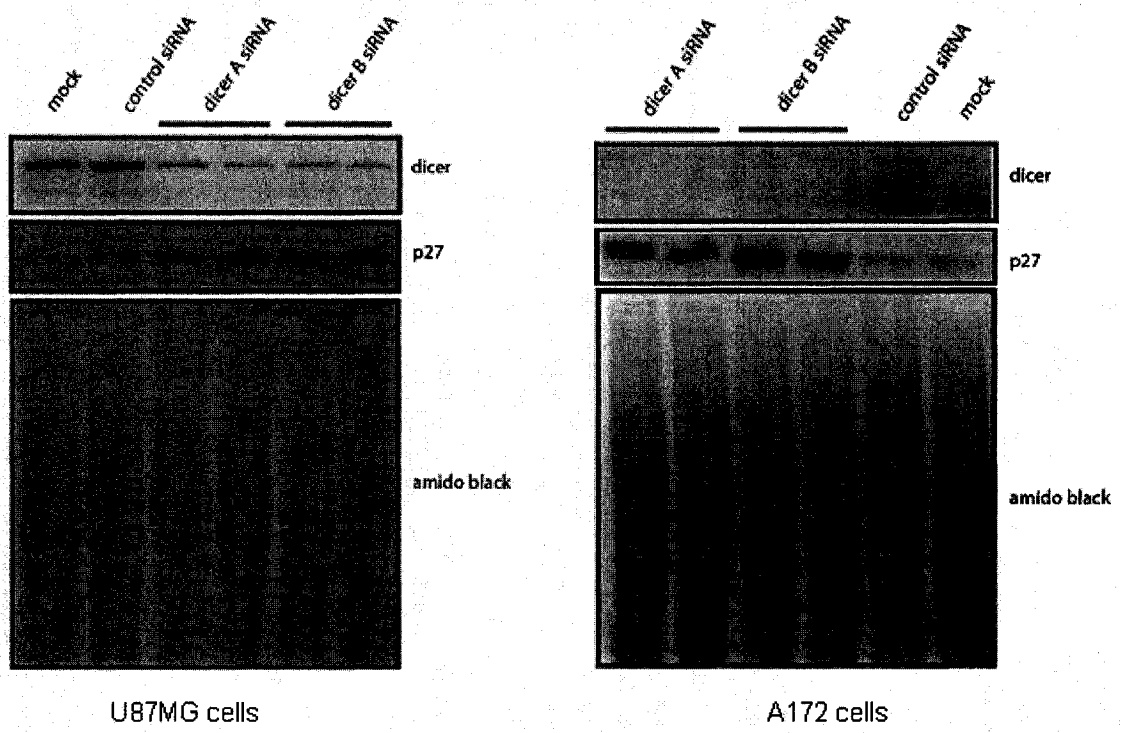
3.5 Dicer regulates p27 expression and cell cycle progression

Dicer is required for the processing of miRNAs and its deletion abrogates miRNA expression (119). Thus if a gene is regulated by miRNA, its protein levels should be increased in cells depleted of dicer. I used two different siRNAs to deplete glioblastoma cells of dicer. **Figure 3.6** shows that both siRNAs effectively reduced dicer levels in both U87MG and A172 cells. The reduction in dicer protein resulted in an increase in p27 protein levels in both cell lines. These data indicate that p27 is regulated by dicer but do not show that p27 is a direct target of miRNA. To rule out the possibility that the effect of dicer on p27 could be indirect, ie. *via* the miRNA-mediated down-modulation of another p27 regulator, a luciferase assay was conducted. The entire p27 3'UTR was cloned into pRL directly behind a renilla luciferase reporter gene which is controlled by a constitutive promoter. This construct, called pRL-p27 3'UTR, was co-transfected into U87MG cells with a firefly luciferase-expressing plasmid as a control for transfection efficiency. The cells were lysed and firefly and renilla luciferase activities were measured. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the p27 3'UTR-containing plasmid to the firefly luciferase activity.

Figure 3.6 Dicer depletion increases p27 protein levels.

The indicated glioblastoma cell lines were mock-transfected or transfected with a random control siRNA, 10 nM dicer A siRNA or 10 nM dicer B siRNA. Cells were harvested 48 hours post-transfection for western blot analysis with antibodies against dicer and p27.

Figure 3.6

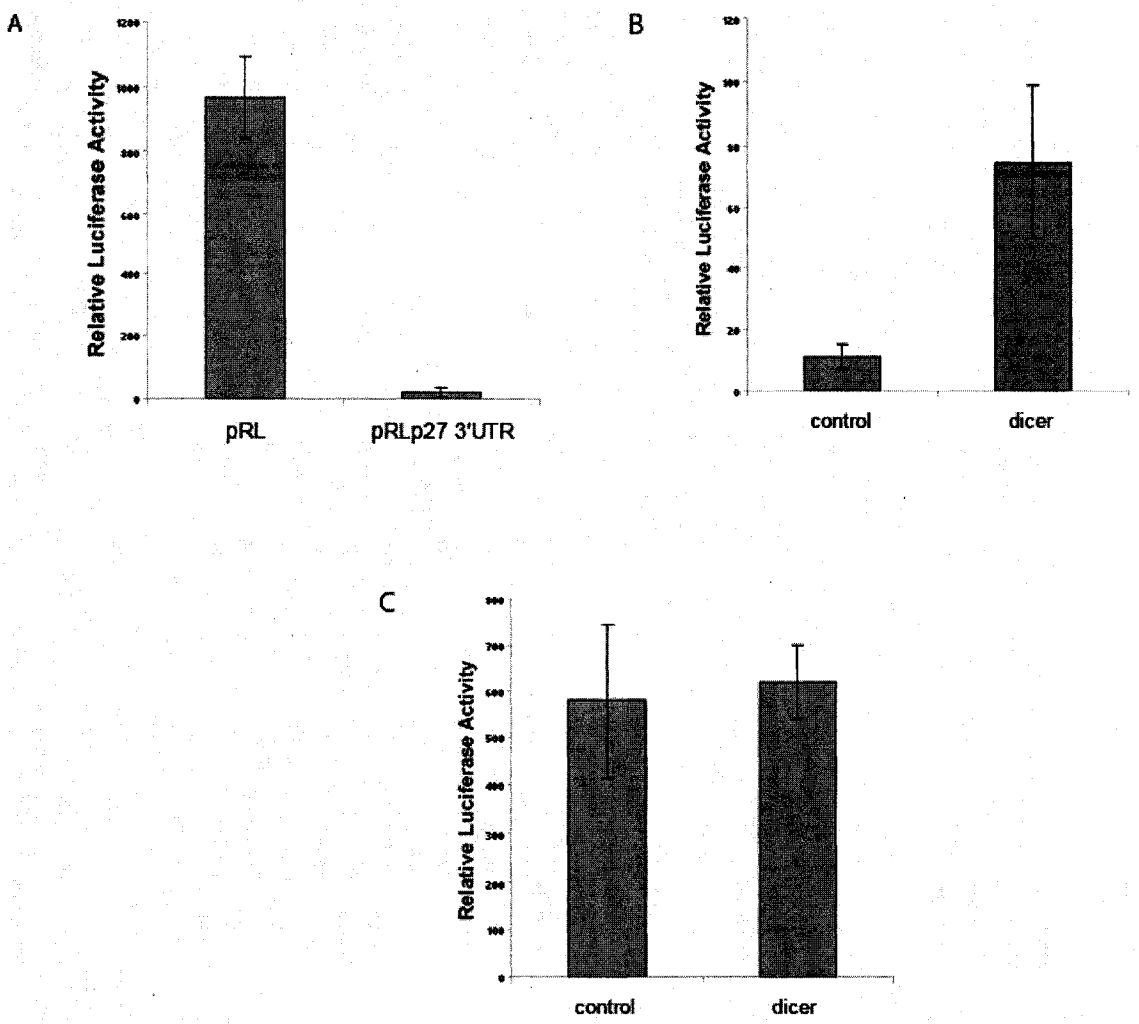

mock
control siRNA
dicer A siRNA
dicer B siRNA
dicer
p27
amido black
U87MG cells
dicer A siRNA
dicer B siRNA
control siRNA
mock
dicer
p27
amido black
A172 cells

Using this system, the effect of the p27 UTR on the expression of the reporter gene was determined. **Figure 3.7A** shows pRL-p27 3'UTR has much less luciferase activity than pRL. Therefore, the p27 3' UTR negatively regulates expression of the renilla luciferase gene, and, it can be inferred, of p27 itself. If this negative regulation is mediated by miRNA (or another dicer-dependent silencing pathway), then depletion of dicer should return the luciferase activity of pRL-p27 3'UTR to that of pRL. Therefore the same luciferase assay was performed with U87MG cells transfected with control and dicerA siRNAs. **Figure 3.7B** shows that the depletion of dicer results in an increase in relative luciferase activity as expected, although the magnitude of the effect is small. The failure of dicer depletion to increase the luciferase activity of the pRL-p27 3'UTR plasmid to that of the pRL plasmid indicates that there are probably other regulatory elements within the 3' UTR of p27 which are not controlled by dicer. Unlike the pRL-p27 3'UTR plasmid, the luciferase activity of the pRL plasmid was not affected by dicer siRNA (**figure 3.7C**), verifying that the dicer knockdown increases luciferase activity *via* the p27 3' UTR.

Figure 3.7 Dicer regulates the p27 3'UTR.

U87MG cells were mock-transfected or transfected with 20 nM random control siRNA, 10 nM dicer A siRNA. 24 hours later, cells were co-transfected with 0.1 μg of either pRL or pRL-p27 3'UTR and 0.01μg of pGL3. 24 hours later, luciferase activity was analyzed. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the pRL plasmids to the firefly luciferase activity of pGL3. The p27 3'UTR decreases basal activity of the luciferase reporter **(A).** dicer siRNA increases reporter activity from pRLp27 3'UTR compared to a the control siRNA **(B)** although it has no effect on pRL **(C).**

Figure 3.7


A
Relative Luciferase Activity
pRL
pRLp27 3'UTR
B
Relative Luciferase Activity
control
dicer
C
Relative Luciferase Activity
control
dicer

Next, to determine whether dicer depletion has an effect on cell cycle progression, U87MG cells transfected with dicer B and control siRNAs and subsequently analyzed by flow cytometry. **Figure 3.8** shows that dicer depletion causes an increase in the proportion of cells in G1 phase, whereas the control siRNA had no effect. Thus dicer function is important for passage from G1 to S phase of the cell cycle.

Altogether, these data show that dicer controls p27 expression via an element in its 3' UTR. Because dicer is essential for miRNA processing, they also suggest that p27 may be regulated by miRNA. However, dicer does not function solely in the processing of miRNAs; it processes endogenous siRNAs as well and was recently found to be involved in another small-RNA-mediated silencing pathway which causes transcriptional silencing via heterochromatin formation (134). Nonetheless these results provide a strong rationale for investigating the potential role of the miRNA pathway in the regulation of p27.

3.6 The p27 3'UTR contains 2 conserved binding sites for miR-221/222

The Targetscan program was used to identify potential miRNA binding sites within the 3' UTR of p27. 10 sites in total were identified as conserved binding sites for conserved miRNAs and are shown in **figure 3.9A**. Of particular interest are the two miR-221/222 binding sites (shown complexed with their predicted target sites in **figure 3.9B**), as this miRNA is upregulated in several cancers and in U87MG cells (122). When applied to all of the 3' UTRs in the human genome, Targetscan predicted 219 conserved targets of miR-221/222, with p27 being the third-best match. The cell cycle inhibitor $p57^{kip2}$ was also a predicted target, but in total the predicted targets did not include an overwhelming

Figure 3.8 Dicer depletion alters the cell cycle distribution of U87MG cells.

U87MG cells were mock-transfected or transfected with 10 nM dicerB or 20 nM control siRNAs. Cells were harvested 48 hours later for flow cytometric analysis.

Figure 3.8

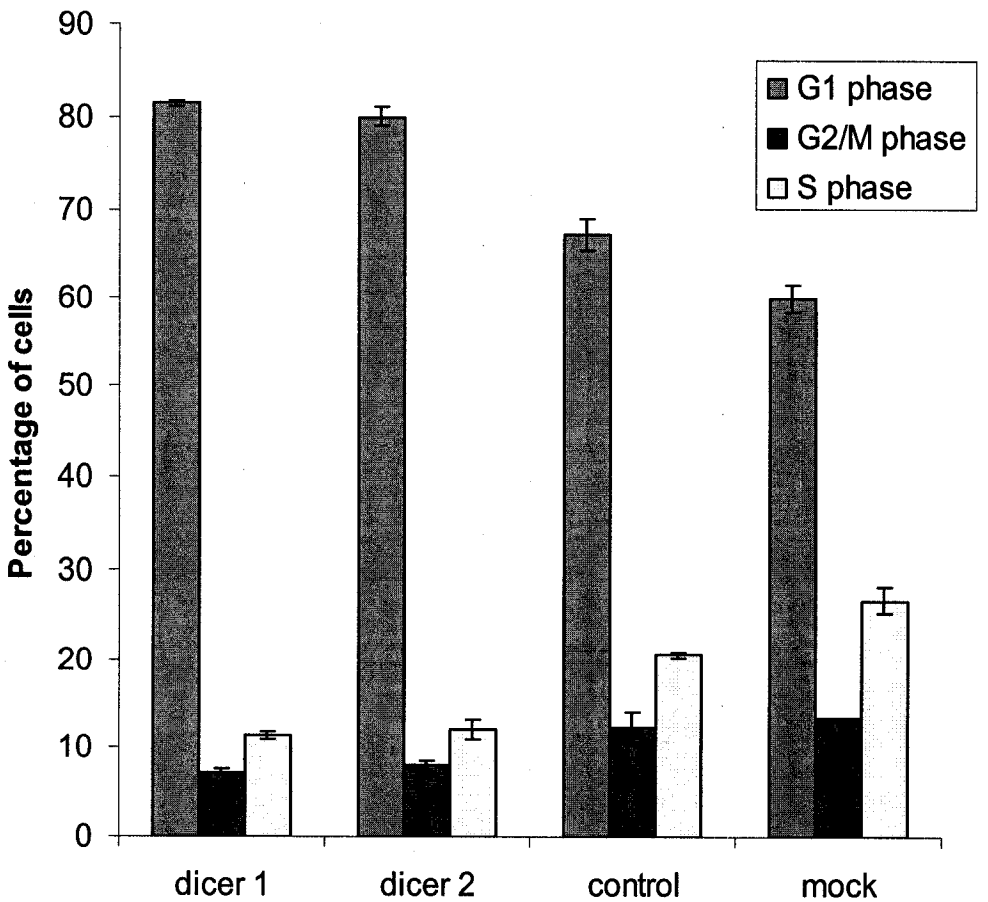
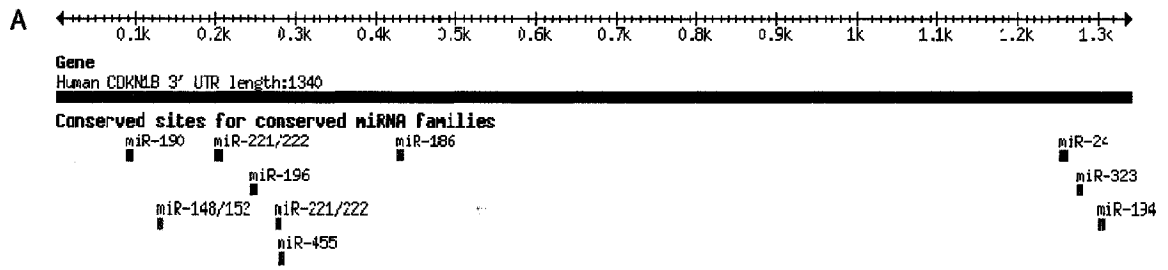

90
80
70
60
50
40
30
20
10
0
Percentage of cells
G1 phase
G2/M phase
S phase
dicer 1
dicer 2
control
mock

Figure 3.9 miRNA binding sites in the p27 3'UTR identified by Targetscan.

A. 9 conserved miRNAs have conserved binding sites within the 3' UTR of p27.
B. miR-221 and miR-222 are shown complexed with their binding sites in the p27' 3'UTR. They bind to the same 2 sites within the p27 3'UTR as they have the same seed sequence, although binding at the 3' end differs. **C.** Site-directed mutagenesis of pRL-p27 3'UTR was used to mutate 3 residues within the seed sequence of the each of miR-221/222 binding sites. Mutated residues are shown in red.

Figure 3.9



B Position 172-207 of CDKN1B 3' UTR

hsa-miR-222 5' ...AAAUGAUCUGCCUCUAAAAG-CGUUGGAUGUAGCA...
3' CUCUGGG----------UCAUCGGUCUACAUCGA

Position 177-207 of CDKN1B 3' UTR

hsa-miR-221 5' ...GAUCUGCCUCUAAAAGCGUUGGAUGUAGCA...
3' CUUUGGG---------UCGUCUGUUACAUCGA

Position 249-281 of CDKN1B 3' UTR

hsa-miR-222 5' ...UGUGUAUAUAGUUUUUACCUUUUAUGUAGCAC...
3' CUCUGGGUCA-----UCGG----UCUACAUCGA

Position 255-281 of CDKN1B 3' UTR

hsa-miR-221 5' ...UUUUUACCU---------UUUAUGUAGCAC...
3' CUUUGGGUCGUCUGUUACAUCGA

C 5' ...GAUGUAGCA... p27 3'UTR
3' ...CUACAUCGA miR-221

5' ...GAGGGATCA... p27 3'UTR
3' ...CUACAUCGA miR-221

abundance of tumour suppressor genes. Other predicted target genes were involved in a wide array of functions including apoptosis, activation of transcription and translation initiation, and inducing differentiation.

3.7 miR-221/222 regulate p27

miRNA inhibitors were used to determine whether p27 is regulated by miR-221/222. They are single-stranded oligonucleotides which bind to and inhibit endogenous miRNAs. Transfection of glioblastoma cell lines with these miR-221 and miR-222 inhibitors alone or in combination resulted in an increase in p27 levels while the negative control inhibitor had no effect (**figure 3.10**). The increase in p27 levels was dose-dependent, with a maximal increase in p27 levels occurring with 400 nM of inhibitor. Quantification of the p27 bands indicates that treatment of cells with 400 nM miR-221 inhibitor causes a 10-fold increase in p27 levels relative to the mock-transfected cells. These results show that miR-221/222 regulate p27 expression.

To show that the regulation occurs via binding of miR-221 to the p27 3' UTR, luciferase assays were performed. **Figure 3.11A** shows that transfection of U87MG cells with the miR-221 inhibitor causes a significant increase in luciferase activity from the RLp27 3'UTR reporter compared mock-transfected cells or cells transfected with a negative control inhibitor. The effect of miR-221 inhibition was dependent on the p27 3' UTR, as it was not seen with a control plasmid which does not contain the p27 3'UTR (**figure 3.11B**). To determine whether miR-221 exerts its effect *via* the two predicted miR-221 binding sites, site-directed mutagenesis was employed to mutate 3 out of 7 residues of the seed sequence

Figure 3.10 Inhibition of miR-221 increases p27 protein levels.

Synthetic inhibitors of miR-221 and miR-222 were transfected into U87MG cells alone or in combination at the indicated concentrations. Cells were harvested 48 hours post-transfection and analyzed by western blotting.

Figure 3.10

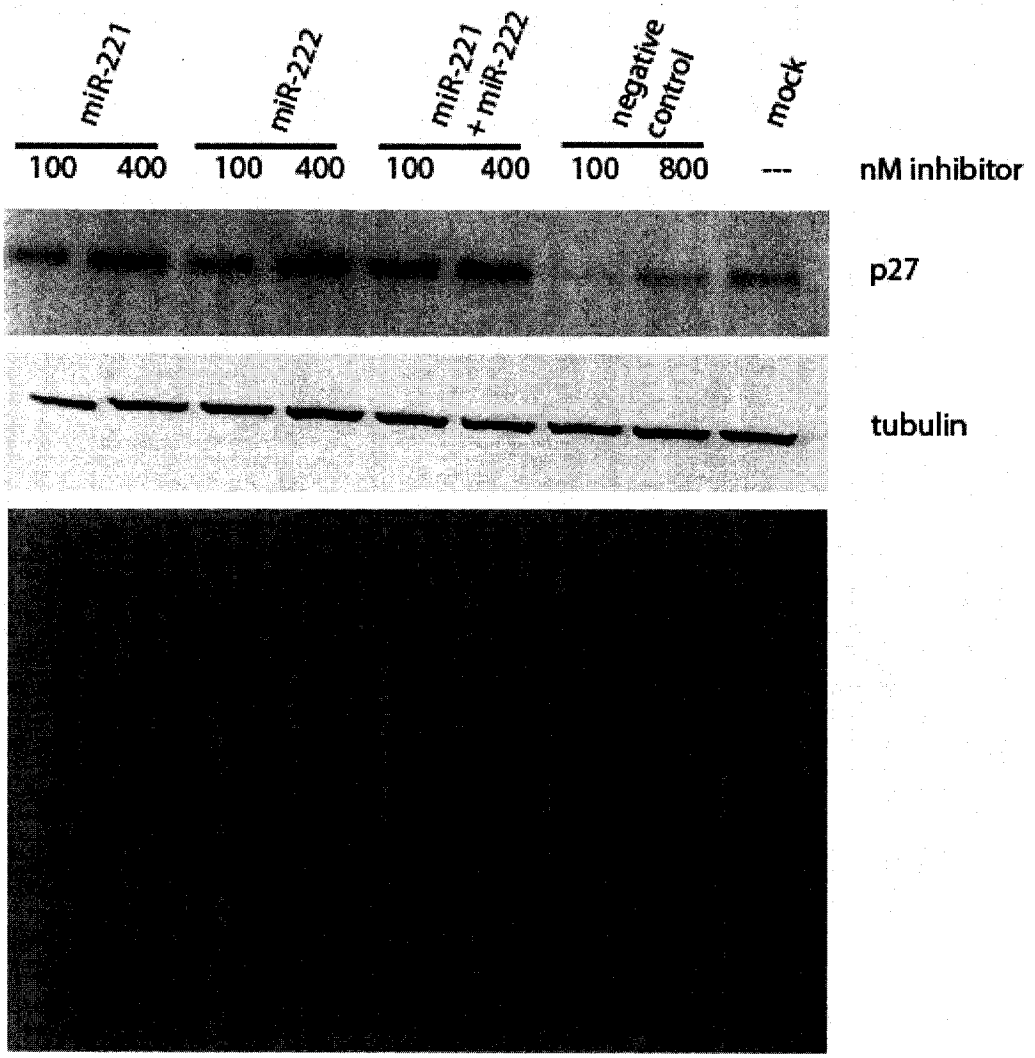
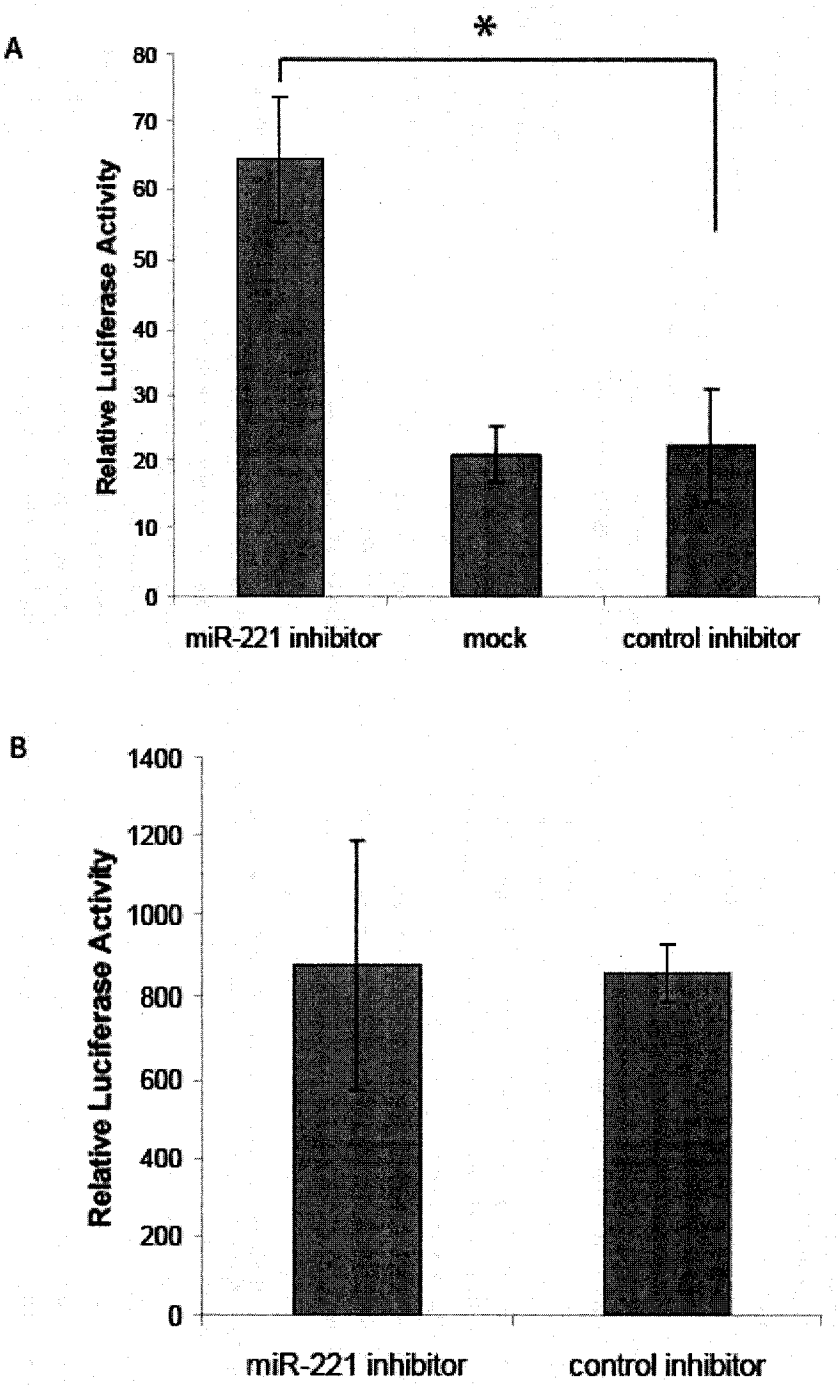

miR-221
miR-222
miR-221 + miR-222
negative control
mock
100 400 100 400 100 400 100 800 ---
nM inhibitor
p27
tubulin

Figure 3.11 Luciferase activity from the pRL-p27 3'UTR reporter construct is increased by inhibition of miR-221.

U87MG cells were co-transfected with 0.01μg of the firefly luciferase reporter plasmid pGL3 and 0.1μg of either one of the following renilla luciferase reporter constructs: pRLp27, which contains the wild-type p27 3'UTR; pRLp27-M1M2, in which both of the miR-221 binding sites are mutated. 24 hours later, luciferase activity was analyzed. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the p27 3'UTR-containing plasmid to the firefly luciferase activity of pGL3. miR-221 inhibition increased luciferase activity of the pRLp27 3'UTR reporter **(A)** but not of pRL alone **(B)**. This represents one of several experiments, each performed in triplicate; error bars indicate standard deviation. * P= 0.05, student's T test.

Figure 3.11


A
*
80
70
60
50
40
30
20
10
0
Relative Luciferase Activity
miR-221 inhibitor
mock
control inhibitor
B
1400
1200
1000
800
600
400
200
0
Relative Luciferase Activity
miR-221 inhibitor
control inhibitor

in each of the miR-221 target sites in pRL-p27 3'UTR (**figure 3.9**). This approach has been used by others to eliminate miRNA binding (127). **Figure 3.12A** shows that an increase in luciferase activity was observed when both miR-221 binding sites were mutated. Mutation of the first site (M1) resulted in a modest increase in luciferase activity, while mutation of the second site (M2) had no effect. Importantly, mutation of both sites had the greatest effect on luciferase activity, which is in agreement with the hypothesis that multiple miRNA binding sites are required for optimal inhibition of translation.

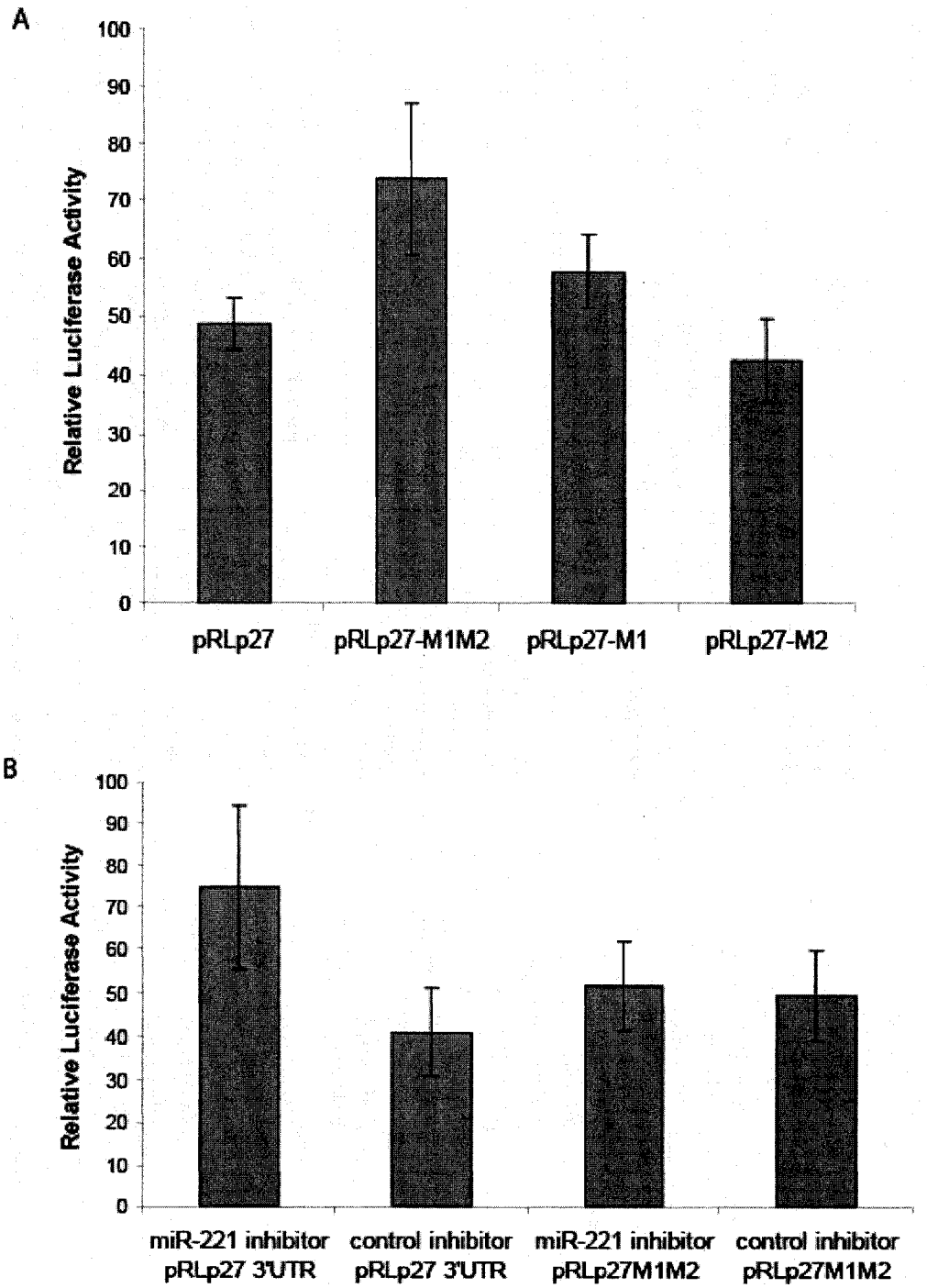
Next, the effect of miR-221 inhibitors on the activity of the wild-type and mutated p27 3'UTR reporter constructs was compared. Inhibition of miR-221 resulted in a significant increase in luciferase activity in cells expressing the unmutated reporter construct as shown in **figure 3.12B**. In those cells expressing the reporter construct mutated at both miR-221 binding sites, miR-221 inhibitors had no effect on luciferase activity. Surprisingly, the mutation of the two miR-221 binding sites did not return luciferase activity to that which was obtained with miR-221 inhibition. This could indicate that the mutagenesis of the seed sequence was not sufficient to completely abrogate miR-221 binding. Nonetheless, these results show that the predicted miR-221 binding sites are required for miR-221's inhibitory effect on p27 levels.

In conclusion, these results identify miR-221 as a negative regulator of p27 expression. I have shown that miR-221 inhibition increases endogenous p27 levels by western blot, and then using a luciferase reporter construct, that the effect occurs directly, *via* the p27 3' UTR.

Figure 3.12 The predicted miR-221 binding sites are required for the effect of miR-221 on the p27 3'UTR.

A. U87MG cells were co-transfected with 0.01μg of the firefly luciferase reporter plasmid pGL3 and 0.1μg of one of the following renilla luciferase reporter constructs: pRLp27, which contains the wild-type p27 3'UTR; pRLp27-M1M2, in which both of the miR-221 binding sites are mutated; and pRL-p27-M1 and -M2, which contain mutations in the first and second miR-221 binding sites, respectively. 24 hours later, luciferase activity was analyzed. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the p27 3'UTR-containing plasmid to the firefly luciferase activity of pGL3. Mutation of both miR-221 binding sites (M1M2) increased luciferase activity, as did mutation of the second site (M2), although mutation of the first site (M1) had no effect. The experiment was performed in triplicate; error bars indicate standard deviation. **B.** U87MG cells were transfected with miR-221 or negative control inhibitors. 24 hours later, the cells were co-transfected with 0.01μg of the firefly luciferase reporter plasmid pGL3 and 0.1μg of either pRLp27 (unmutated UTR) or pRLp27-M1M2 (mutated UTR). 24 hours later, luciferase activity was analyzed. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the p27 3'UTR-containing plasmid to the firefly luciferase activity of pGL3. * $P < 0.05$, student's T-test.

Figure 3.12


A
Relative Luciferase Activity
100
90
80
70
60
50
40
30
20
10
0
pRLp27
pRLp27-M1M2
pRLp27-M1
pRLp27-M2
B
Relative Luciferase Activity
100
90
80
70
60
50
40
30
20
10
0
miR-221 inhibitor pRLp27 3'UTR
control inhibitor pRLp27 3'UTR
miR-221 inhibitor pRLp27M1M2
control inhibitor pRLp27M1M2

Finally, I identified two miR-221 binding sites which are required for miR-221-mediated regulation of p27.

3.8 The role of oncogenic signaling in the miRNA-mediated regulation of p27 in GBM

Because miRNAs are implicated in cancer, I wished to explore the possibility that oncogenic signaling through the MEK/ERK and PI3K pathways might regulate miRNA expression. There is some evidence for global regulation of miRNA via modulation of dicer activity. A microarray performed in our lab showed that PKCι depletion caused a decrease in dicer mRNA levels. Thus activation of dicer, and consequently increased miRNA processing could be a mechanism by which PKCι decreases p27 levels. To determine whether PKCι regulates dicer, a western blot was performed to compare dicer levels in U87MG cells transfected with PKCι and control siRNAs. **Figure 3.13A** shows that PKCι depletion did not cause a change in dicer levels. Dicer levels were also examined in $PKC^{-/-}$ and wild type MEFs and no difference was observed (**figure 3.13B**).

Although PKCι does not regulate dicer protein levels, it is perhaps more likely that it could be involved in miRNA-dependent regulation of p27 by upregulating miR-221/222 expression. I began to test this hypothesis by examining the effect of PKCι depletion and MEK inhibition on the pRL-p27 3'UTR reporter construct. **Figure 3.14A** shows that transfection of cells with PKCι siRNA increases luciferase activity, while the control siRNA does not. Similar results were obtained with MEK inhibition. **Figure 3.15A** shows that addition of the MEK inhibitor U0126 increased luciferase activity well above that of the vehicle control. Both PKCι depletion and MEK inhibition decreased luciferase activity

Figure 3.13 PKCι does not regulate dicer protein levels.

(A) U87MG cells were mock-transfected or transfected with 5 nM of PKCιA, 20 nM PKCιB, or 20 nM control siRNAs. Cells were harvested 48 hours later for western blot analysis of dicer and PKCι protein levels. Amido black staining (bottom) indicates equal protein loading. **(B)** Wildtype or PKCλ-/- MEFs were harvested for western blot analysis of dicer and PKCι protein levels. Actin levels indicate equal protein loading.

Figure 3.13

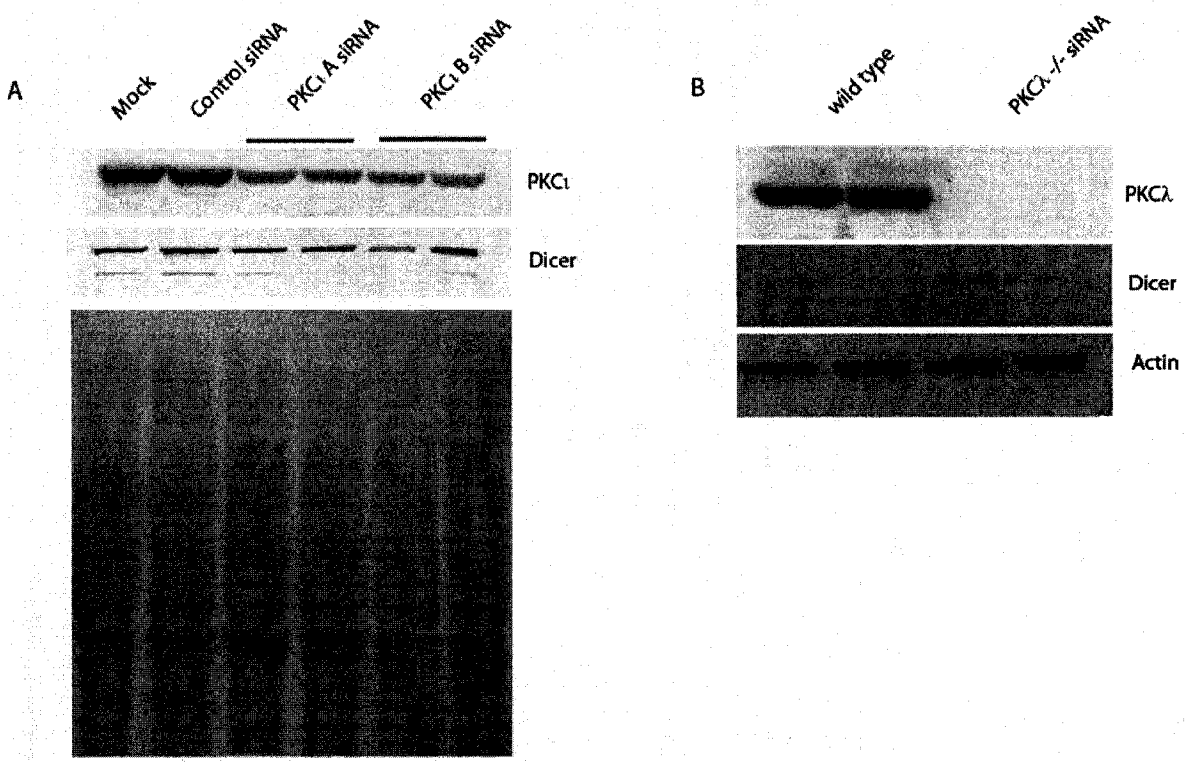

A
Mock
Control siRNA
PKCι A siRNA
PKCι B siRNA
PKCι
Dicer
B
wild type
PKCλ -/- siRNA
PKCλ
Dicer
Actin

Figure 3.14 Depletion of PKCι increases activity of the p27 3'UTR reporter construct.

U87MG cells were mock transfected or transfected with 20 nM random control siRNA or 20 nM PKCι B siRNA. 24 hours later, cells were co-transfected with 0.1 μg of the renilla luciferase reporter plasmid pRLp27 3'UTR and 0.01μg of the firefly luciferase reporter plasmid pGL3. 24 hours later, luciferase activity was analyzed. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the p273'UTR-containing plasmid to the firefly luciferase activity of pGL3. PKCι depletion increases luciferase activity from the pRLp27 3'UTR plasmid **(A)** and decreases luciferase activity from the pRL plasmid **(B).** These data represent one of several experiments; each one was performed in triplicate. * P = 0.005, student's T-test; error bars indicate standard deviation.

Figure 3.14

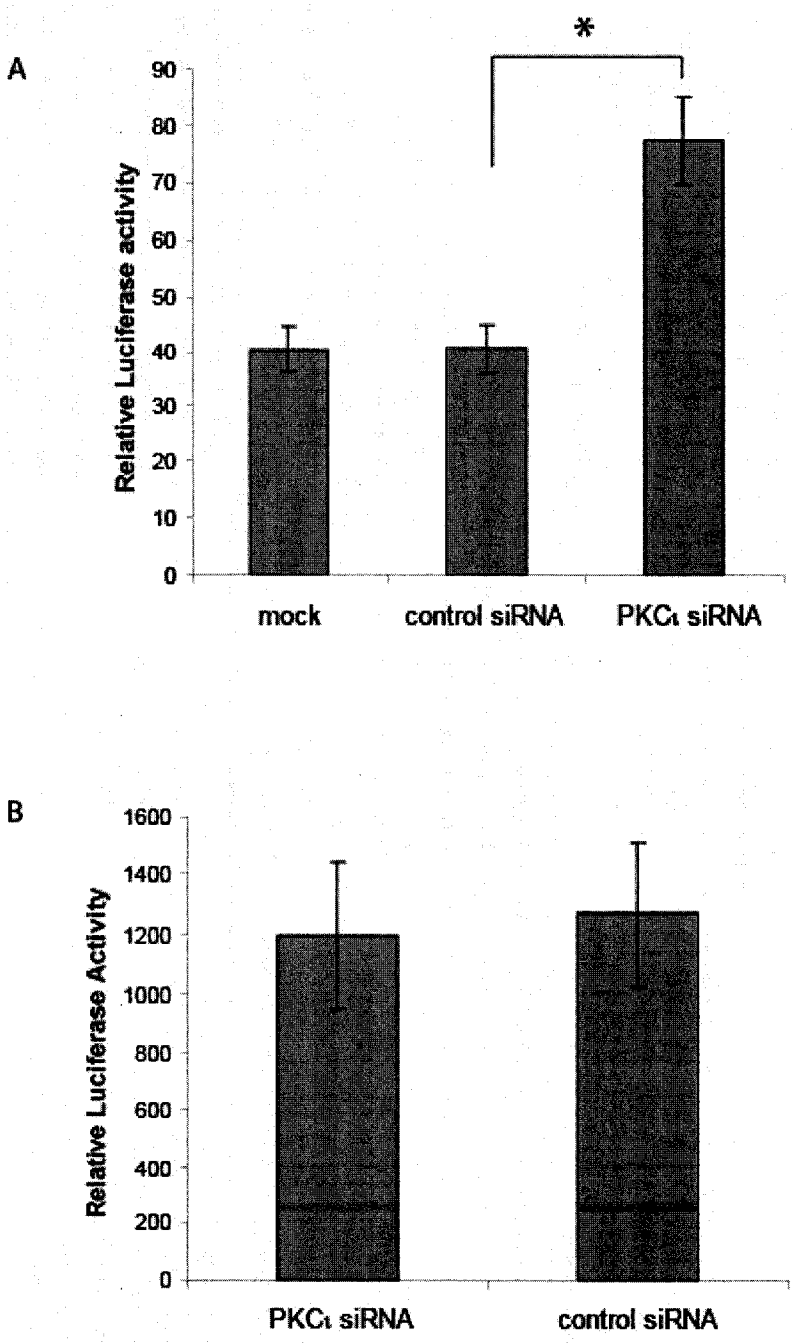
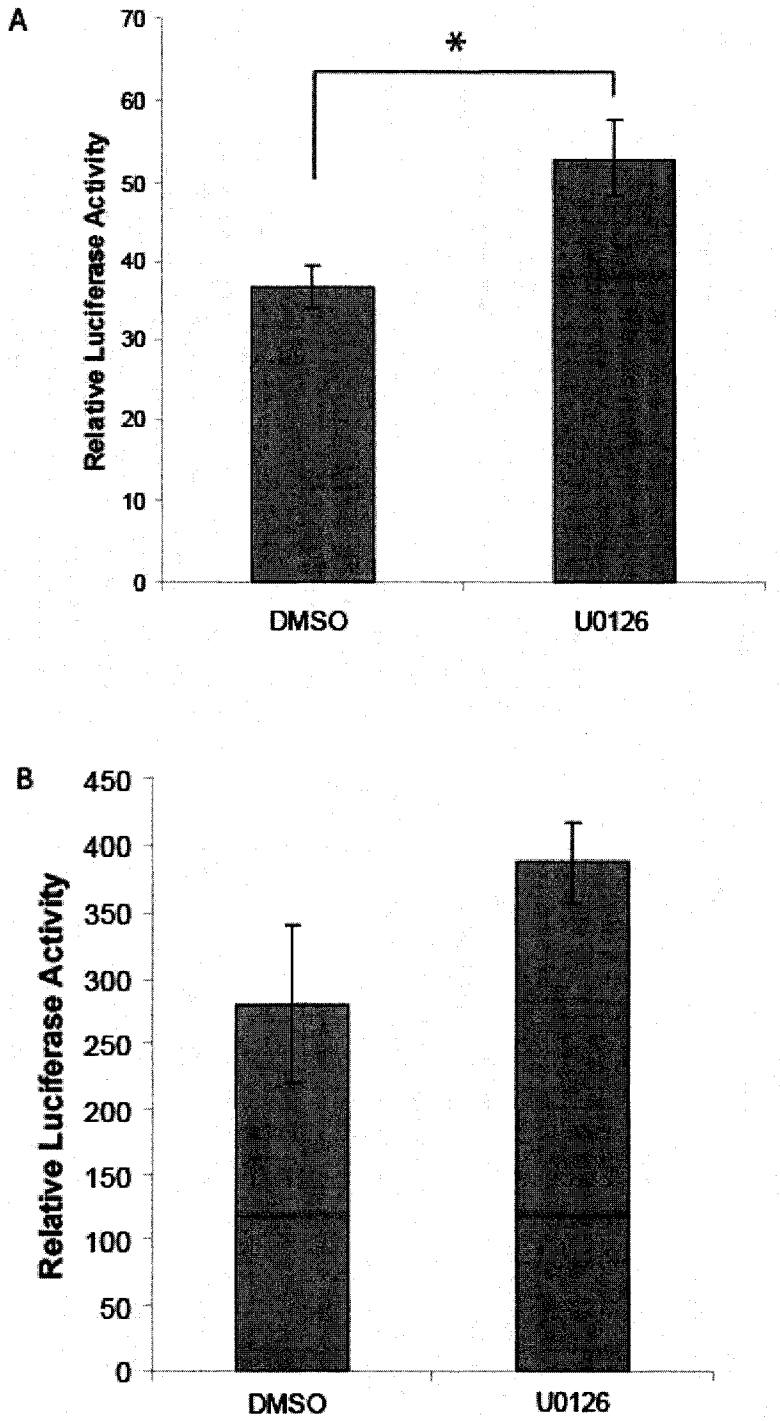

A
*
Relative Luciferase activity
90
80
70
60
50
40
30
20
10
0
mock
control siRNA
PKCι siRNA
B
Relative Luciferase Activity
1600
1400
1200
1000
800
600
400
200
0
PKCι siRNA
control siRNA

Figure 3.15 Inhibition of MEK increases activity of the p27 3'UTR reporter construct.

U87MG cells were co-transfected with 0.1 μg of the renilla luciferase reporter plasmid pRLp27 3'UTR and 0.01μg of the firefly luciferase reporter plasmid pGL3. Cells were then treated with 20 μM of the MEK inhibitor U0126 or vehicle alone (DMSO) 16 hours before harvesting. Cells were harvested 24 hours after transfection with the reporter plasmids for analysis of luciferase activity. The relative luciferase activity was obtained by normalizing the renilla luciferase activity of the p27 3'UTR-containing plasmid to the firefly luciferase activity of pGL3. U0126 treatment increased luciferase activity from pRLp27 3'UTR **(A)** but decreased activity from pRL **(B).** These data represent one of several experiments; each one was performed in triplicate. * P = 0.01, student's T-test; error bars indicate standard deviation.

Figure 3.15


A
Relative Luciferase Activity
70
60
50
40
30
20
10
0
*
DMSO
U0126
B
Relative Luciferase Activity
450
400
350
300
250
200
150
100
50
0
DMSO
U0126

from the pRL plasmid (**figures 3.14B and 3.15B**). This indicates that these signaling pathways have effects on the pRLp27 3'UTR plasmid which are independent of the p27 3'UTR. However, the effect of PKCι depletion and MEK inhibition on the p27 3'UTR is still valid as the p27 3'UTR-independent effects caused a decrease, rather than an increase, in luciferase activity. This may mean that the effect of these two signaling pathways on the p27 3'UTR is in fact larger than it appears. Therefore both PKCι and MEK/ERK signaling control p27 levels, at least in part via an element in the p27 3'UTR. These results are consistent with the hypothesis that PKCι and MEK/ERK could control p27 levels by increasing expression of miR-221, although further experiments are required to confirm this hypothesis.

Chapter 4

Discussion

Glioblastoma is an aggressive brain tumour for which therapeutic options are limited and provide little survival benefit. The identification of new therapeutic targets for this disease will require an in-depth understanding of the proliferative signaling networks and how they are altered in GBM. Genetic aberrations resulting in increased EGFR signaling are seen in about half of GBMs (13-15) and PTEN loss occurs with approximately the same frequency (40). Both of these events result in increased signaling through the PI3K pathway, underlining the importance of this pathway in GBM pathogenesis. PI3K signaling results in decreased p27 levels and increased proliferation in GBM cells (83, 84); however, the mechanism by which this occurs is not fully understood.

I have shown that siRNA-mediated depletion of the PI3K effector PKCι results in an increase in p27 levels (**figure 3.1**). However, experiments involving exogenous siRNAs must be interpreted carefully for several reasons. First, there is a possibility that that they could bind to the UTRs of other genes to cause off-target gene silencing. Microarray analysis has shown that this can occur at high concentrations of siRNAs, but that highly specific targeting could be achieved with concentrations in the 5-20 nM range (136). For this reason, siRNAs were not used at concentrations above 20 nM. Of course, the microarray study does not take into account translational silencing effects. However, because the effect of PKCι on p27 was observed with two different siRNAs, and because the control siRNA does not increase p27 protein levels above those observed in the mock-

transfected cells, the increase in p27 levels is not likely to be due to an off-target effect. Second, it was recently shown that the RNAi pathway is saturable. The expression of different short hairpin RNAs in the livers of mice resulted in dose-dependent liver injury which was associated with down-regulation of liver-derived miRNAs. As both shRNA- and miRNA- mediated silencing requires the same machinery, the effect was attributed to competition for limiting cellular factors required for small RNA processing (137). This is an important consideration as I have shown that p27 is regulated by miRNA (**figure 3.10**), and that the effect of PKCι depletion on p27 is at least in part dependent on the p27 3'UTR (**figure 3.14**). It is conceivable that the increase in p27 levels observed upon siRNA-mediated depletion of PKCι could be attributed to saturation of the RNAi pathway resulting in a global decrease in miRNA-mediated silencing and consequently relieving the miR-221-mediated inhibition of p27 translation. However, this possibility is ruled out by the demonstration that PKCι null MEFs also have high p27 levels compared to PKCι expressing cells (**figure 3.2**). Therefore, I have shown conclusively that PKCι is a regulator of p27.

The observation that the combined inhibition of PKCι and MEK results in a greater-than-additive increase in p27 levels (**figure 3.5**) is interesting, although interpretation of these results is certainly not straightforward. Knockout experiments are often used to determine if two proteins function in the same biochemical pathway. In such experiments, the deletion of the second gene will result either in an additive effect, meaning that the protein products of each gene function in different pathways, or have no effect, meaning that they are in the same pathway. However, siRNA-mediated depletion of PKCι and inhibition of MEK does not completely abolish the activity of either protein as deletion would; in this case if PKCι

and MEK functioned in the same pathway both treatments could have additive or greater-than-additive effects on p27 levels. Certainly, this could be the case as figure 3.5 shows that PKCι contributes to ERK activation. However, it is unlikely that this is the sole mechanism by which PKCι regulates p27. **Figure 3.5** also shows that ERK activity is virtually undetectable in the presence of the MEK inhibitor; thus it is unlikely that the additional decrease in ERK activity which would result from PKCι depletion could account for the greater-than-additive effect on p27 levels. Therefore although PKCι may control p27 levels by increasing ERK activity, there is probably an additional mechanism by which PKCι regulates p27.

The greater-than-additive effect observed could also be the result of two parallel pathways converging at p27. This type of synergy is often observed in signaling; for instance, the proapoptotic protein BAD is regulated by such a mechanism. BAD has two phosphorylation sites; one is MEK-dependent and the other is PI3K-dependent. BAD is released and apoptosis is induced only when both residues are dephosphorylated in response to inhibition of both pathways (138). Although the mechanism of synergy between PKCι and MEK with respect to p27 regulation is unknown, these results, along with those of She *et al*, provide a rationale for the combined inhibition of PI3K and MEK signaling in the treatment of GBM.

Figure 3.6 shows that dicer levels can be effectively reduced by siRNA transfection. This is an interesting result in itself, as evidence from *Drosphila melanogaster* indicates that the role of dicer-1 extends beyond the cleavage of RNAi intermediates. Of course, the exogenous siRNAs used in this work can be loaded into the RISC directly; they do not need

to be cleaved by dicer. However, in *Drosophila* dicer-1 was shown to be required for assembly of the siRNA-RISC complex and its ability to silence gene expression (139, 140). If this is true in humans as well, then one might expect that siRNA-mediated knockdown of dicer itself would be inefficient at best. However, *Drosophila* has two different dicer isoforms, Dicer-1 and Dicer-2, whereas in humans and mice dicer is encoded by a single locus (119). It was recently shown that dicer-deficient murine embryonic stem cells could induce siRNA-mediated gene silencing as well as their wild-type counterparts (119). Thus dicer function appears to be essential for silencing mediated by exogenous siRNAs in *drosophila* but not in mice. The results presented here suggest that dicer function is also dispensable for gene silencing mediated by exogenous siRNAs in human cells and that siRNA-mediated dicer depletion could be useful as a preliminary step in determining whether a gene is regulated by miRNA.

Figures 3.10 - 3.12 show that miR-221/222 regulates p27. This makes a considerable contribution to our knowledge of the regulation of cancer-associated genes by miRNA, as the only other cancer-associated miRNA targets that have been identified thus far are PTEN, Bcl2, E2F1 and ras (108, 112, 114, 115) and only 30 miRNA-target interactions in total have been validated experimentally (105). Thus there has been considerable speculation on the role of miRNAs in cancer. While it cannot be disputed that miRNA expression is vastly different in cancerous versus normal tissues, we have not yet determined whether these differences play a causative role or are merely innocent bystanders in the oncogenic transformation process. Pillai made the tentative hypothesis that "miRNAs might have a role in networking and fine-tuning gene expression in the cell" (104), while others have

asserted that "miRNAs play a crucial role in cancer" (116). Certainly, miRNAs play a crucial role in CLL (111), but it remains to be seen if miRNAs promote carcinogenesis in other cancers as well. I have shown that dicer depletion results in a robust increase in p27 levels (**figure 3.7**) and a delay in cell cycle progression (**figure 3.8**), suggesting that miRNAs play an important role in cancer by promoting cell cycle progression. While a similar increase in p27 levels was observed upon miR-221 inhibition, cell cycle analysis was not performed under these conditions, and as dicer is involved in several small RNA pathways (134), miRNAs cannot be directly implicated in promoting cell cycle progression. In the future it will be important to evaluate the functional consequence of miR-221 inhibition by analyzing its effect on the cell cycle. However, the strong increase observed in p27 levels upon miR-221 inhibition, coupled with the fact that this miRNA is strongly upregulated in glioblastoma (122), suggest an important role for miR-221 in this cancer type.

It could be argued that the results of the luciferase assays are more consistent with the hypothesis that miRNAs are fine-tuners of gene expression rather than crucial mediators of carcinogenesis. The siRNA-mediated depletion of dicer or PKCι, the inhibition of MEK and the mutation of the miR-221 binding sites all resulted in a similar increase (approximately 2-fold) in luciferase activity from the pRLp27 3' UTR construct. The magnitude of this effect is relatively small in comparison to the difference between the basal level of luciferase activity from the pRL and pRLp27 3' UTR constructs; pRL has 50-fold more luciferase activity than pRLp27 3' UTR (**figure 3.7**). However, in terms of p27 levels, a two-fold difference is sufficient to induce biological effects. This is well-illustrated by the

fact that p27 hemizygous mice, which have half the normal gene dosage of p27, are predisposed to tumour development (141-143). Additionally, luciferase assays may not provide an accurate representation of how endogenous p27 is regulated. This seems likely given that the 2-fold increase in luciferase activity is inconsistent with the changes observed in endogenous p27 protein levels. Inhibition of miR-221, for example, resulted in a 10-fold increase in p27 protein levels (**figure 3.5**). The reason for the discrepancy could be that the overexpression of the transfected constructs creates more miR-221 binding sites than there are miR-221 molecules in the cell, so that the effect of miR-221 inhibition would be under-represented. Nonetheless, the 10-fold change in endogenous p27 levels does not approach the 50-fold change in luciferase activity resulting from expression of the p27 3' UTR, and it is likely that there are other regulatory elements in the p27 3'UTR which are not dicer-dependent. It would be interesting to perform deletion experiments using the pRLp27 3'UTR construct to identify the region which is responsible for causing such a robust decrease in luciferase activity. Two studies thus far have found evidence for a regulatory element in the p27 3'UTR. One group showed that ERK decreased p27 mRNA stability in response to PDGF-BB *via* an element in its 3'UTR (87). The second group showed that the RNA binding protein quaking associates with the 3'UTR of p27 to increase mRNA stability (132). Additionally, the difference in luciferase activity between the pRL and pRLp27 3'UTR plasmids could simply be explained by the fact that a small 252-base pair stretch of DNA containing the SV40 late poly adenylation signal was removed to allow for the directional cloning of the p27 3' UTR. Although the p27 3'UTR contains a poly-A signal as well, some component of the excised DNA could be responsible for increasing the stability of the luciferase mRNA.

Figures 3.14 and **3.15** show that PKCι and MEK regulate p27 *via* its 3'UTR, although the mechanism by which this occurs was not determined. Future studies should determine whether the mechanism is miR-221-dependent. This is a plausible hypothesis for several reasons. First, the only regulatory elements in the p27 3'UTR that have been discovered regulate p27 mRNA stability. It is unlikely that PKCι would regulate p27 by this mechanism as **figure 3.3** shows that PKCι does not affect p27 mRNA levels. Additionally, the effect of dicer inhibition on luciferase activity is comparable to the effect of miR-221 inhibition. Although Targetscan predicted binding sites for 8 other miRNAs, these data suggest that miR-221 is the primary miRNA which regulates p27. To test the hypothesis that regulation of p27 by PKCι is miRNA-dependent, the activity of the luciferase reporter constructs containing the wild type and mutated p27 3'UTRs could be compared in response to PKCι depletion and MEK inhibition. If the hypothesis is correct, mutation of the miR-221 binding sites should abrogate the effect of PKCι depletion and MEK inhibition on luciferase activity.

If this proves to be true, there are several conceivable mechanisms by which PKCι and MEK could increase miR-221 activity. The most obvious mechanism is by transcriptional upregulation, given that regulation of miRNA expression is thought to be controlled primarily at the transcriptional level. This was demonstrated in *C. elegans* by linking a GFP reporter gene to the promoter that directs transcription of the let-7 pri-miRNA. The GFP expression pattern matched the expression pattern of the endogenous miRNA gene (144). Very little is known about the transcriptional regulation of specific miRNAs, although it was

recently shown that transcription of the oncogenic miR-17/92 cluster is directly upregulated by the oncogene c-myc (115).

However, there is some evidence for post-transcriptional regulation of miRNA expression. For instance, sea urchin embryos express the let-7 miRNA precursor, but it is not cleaved and activated until adulthood (145). In mice, increased processing of the Let-7 precursor was associated with neuronal differentiation (146). The post-transcriptional regulatory mechanism could be as simple as altering dicer function to result in global changes in miRNA expression. *Xenopus* oocytes, for example, lack dicer activity and cannot process siRNAs (147). It has been hypothesized that alteration of dicer expression could promote carcinogenesis by causing global changes in miRNA expression. However, the small amount of evidence for this is contradictory; dicer levels are shown to be increased in prostate carcinoma (148), but decreased in lung cancer where low dicer levels are associated with poor prognosis (117). Additionally, **figure 3.14** shows that PKCι does not alter dicer levels, although this is not a direct indication of dicer activity. This question could be resolved by comparing miRNA processing in cells depleted of PKCι. Northern blots could be used to accomplish this, as both the pre-miRNA and its processed form can be detected. Perhaps the strongest evidence arguing against global deregulation of miRNA expression in cancer comes from microarray analysis. The majority of miRNA microarrays showed both up-and down-regulation of miRNAs in cancer (113, 123, 125, 148, 149), a fact that is difficult to reconcile with the notion of global regulation of miRNA expression in cancer.

Therefore the hypothesis that regulation of dicer expression is responsible for global regulation of miRNAs may be too simplistic. A recent study provided evidence of post-transcriptional regulation of an individual miRNA. miR-138 pre-miRNA is ubiquitously expressed in the mouse, but the mature miRNA is only seen in brain tissue. The reason for the differential processing of this miRNA was attributed to an inhibitory factor present in the cells which did not express the mature RNA. This inhibitory factor binds the pre-miRNA and prevents its conversion into a mature RNA by dicer. (150)

Our knowledge of how miRNA expression is regulated is clearly very rudimentary. Theoretically, miRNA expression could be controlled at the level of transcription, processing, subcellular localization and stability. Understanding the regulation of miRNA expression should be a focus of future research in this field. I have shown that PKCι and MEK/ERK signaling regulate p27, at least in part via an element in the 3'UTR. My results are consistent with a model in which oncogenic signaling upregulates miR-221 expression, which then inhibits p27 translation to promote proliferation. Of course, further experiments will be required to link PKCι and MEK/ERK signaling to miR-221 regulation. Northern blotting is an excellent way to determine whether PKCι depletion and/or MEK inhibition elicits a change in miR-221 levels.

The idea of global deregulation of miRNA processing has also been invoked to explain the "de-differentiated" phenotype of many cancer cells. The first miRNAs discovered were involved in development (91, 92), and genes involved in development and morphogenesis represent a significant proportion of the miRNA target genes predicted by Targetscan. Thus

miRNAs probably play an important role in differentiation and specification of cell fate. One study analyzed miRNA expression profiles in a series of developmentally ordered samples and found that immature samples exhibited a less complex microRNA transcript profile than did mature samples (98). The idea that miRNAs control differentiation and cellular identity, and that this control is lost in cancer, is particularly relevant to GBM, as these tumours are composed of cells which resemble immature astrocytes and oligodendrocytes (2). Although the overexpression of miR-221 in GBM tissues argues against a global down-regulation of miRNA expression in this cancer type, changes in miRNA expression may well be responsible for the de-differentiation of GBM cells and thus play a role in gliomagenesis. In support of this notion, a microarray examining miRNA expression in response to TPA-induced differentiation of HL-60 cells found approximately equal numbers of up-regulated and down-regulated miRNAs (151).

In conclusion, I have shown that PKCι and MEK/ERK signaling regulate p27, at least in part via an element in the 3'UTR and identified miR-221 as a regulator of p27. The discovery that this important cell cycle regulator is regulated by miRNA helps to substantiate the hypothesis that microRNAs have a crucial function in cancer progression. The daunting task of experimentally validating the predicted targets of miRNAs is an important one, and will likely generate considerable insight into normal cellular processes, and how these processes are disrupted in diseases such as cancer.

Bibliography

1. Kleihues, P., and H. Ohgaki. 2000. Phenotype vs genotype in the evolution of astrocytic brain tumors. *Toxicol. Pathol.* 28:164-170.

2. Holland, E.C. 2001. Gliomagenesis: genetic alterations and mouse models. *Nat. Rev. Genet.* 2:120-129.

3. Smirniotopoulos, J.G. 1999. The new WHO classification of brain tumors. *Neuroimaging Clin. N. Am.* 9:595-613.

4. Halatsch, M.E., U. Schmidt, J. Behnke-Mursch, A. Unterberg, and C.R. Wirtz. 2006. Epidermal growth factor receptor inhibition for the treatment of glioblastoma multiforme and other malignant brain tumours. *Cancer Treat. Rev.* 32:74-89.

5. Shrieve, D.C., E. Alexander 3rd, P.M. Black, P.Y. Wen, H.A. Fine, H.M. Kooy, and J.S. Loeffler. 1999. Treatment of patients with primary glioblastoma multiforme with standard postoperative radiotherapy and radiosurgical boost: prognostic factors and long-term outcome. *J. Neurosurg.* 90:72-77.

6. Stupp, R., W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R.O. Mirimanoff, European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups., and National Cancer Institute of Canada Clinical Trials Group. 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* 352:987-996.

7. Friedman, H.S., and D.D. Bigner. 2005. Glioblastoma multiforme and the epidermal growth factor receptor. *N. Engl. J. Med.* 353:1997-1999.

8. Ohno, R. 2006. Treatment of chronic myeloid leukemia with imatinib mesylate. *Int. J. Clin. Oncol.* 11:176-183.

9. Braziel, R.M., T.M. Launder, B.J. Druker, S.B. Olson, R.E. Magenis, M.J. Mauro, C.L. Sawyers, R.L. Paquette, and M.E. O'Dwyer. 2002. Hematopathologic and cytogenetic findings in imatinib mesylate-treated chronic myelogenous leukemia patients: 14 months' experience. *Blood* 100:435-441.

10. Kantarjian, H.M., J.E. Cortes, S. O'Brien, F. Giles, G. Garcia-Manero, S. Faderl, D. Thomas, S. Jeha, M.B. Rios, L. Letvak, K. Bochinski, R. Arlinghaus, and M. Talpaz. 2003. Imatinib mesylate therapy in newly diagnosed patients with Philadelphia chromosome-positive chronic myelogenous leukemia: high incidence of early complete and major cytogenetic responses. *Blood* 101:97-100.

11. Kapoor, G.S., and D.M. O'Rourke. 2003. Receptor tyrosine kinase signaling in gliomagenesis: pathobiology and therapeutic approaches. *Cancer. Biol. Ther.* 2:330-342.

12. Gallagher, S.J., R.F. Kefford, and H. Rizos. 2006. The ARF tumour suppressor. *Int. J. Biochem. Cell Biol.* 38:1637-1641.

13. Ekstrand, A.J., C.D. James, W.K. Cavenee, B. Seliger, R.F. Pettersson, and V.P. Collins. 1991. Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. *Cancer Res.* 51:2164-2172.

14. Frederick, L., X.Y. Wang, G. Eley, and C.D. James. 2000. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res.* 60:1383-1387.

15. Fuller, G.N., and S.H. Bigner. 1992. Amplified cellular oncogenes in neoplasms of the human central nervous system. *Mutat. Res.* 276:299-306.

16. Chu, C.T., K.D. Everiss, C.J. Wikstrand, S.K. Batra, H.J. Kung, and D.D. Bigner. 1997. Receptor dimerization is not a factor in the signalling activity of a transforming variant epidermal growth factor receptor (EGFRvIII). *Biochem. J.* 324 (Pt 3):855-861.

17. Nagane, M., F. Coufal, H. Lin, O. Bogler, W.K. Cavenee, and H.J. Huang. 1996. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res.* 56:5079-5086.

18. Batra, S.K., S. Castelino-Prabhu, C.J. Wikstrand, X. Zhu, P.A. Humphrey, H.S. Friedman, and D.D. Bigner. 1995. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Differ.* 6:1251-1259.

19. Lund-Johansen, M., R. Bjerkvig, P.A. Humphrey, S.H. Bigner, D.D. Bigner, and O.D. Laerum. 1990. Effect of epidermal growth factor on glioma cell growth, migration, and invasion in vitro. *Cancer Res.* 50:6039-6044.

20. Nishikawa, R., X.D. Ji, R.C. Harmon, C.S. Lazar, G.N. Gill, W.K. Cavenee, and H.J. Huang. 1994. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc. Natl. Acad. Sci. U. S. A.* 91:7727-7731.

21. Li, B., M. Yuan, I.A. Kim, C.M. Chang, E.J. Bernhard, and H.K. Shu. 2004. Mutant epidermal growth factor receptor displays increased signaling through the phosphoinositide-3 kinase/AKT pathway and promotes radioresistance in cells of astrocytic origin. *Oncogene* 23:4594-4602.

22. Shinojima, N., K. Tada, S. Shiraishi, T. Kamiryo, M. Kochi, H. Nakamura, K. Makino, H. Saya, H. Hirano, J. Kuratsu, K. Oka, Y. Ishimaru, and Y. Ushio. 2003. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. *Cancer Res.* 63:6962-6970.

23. Shepherd, F.A., J. Rodrigues Pereira, T. Ciuleanu, E.H. Tan, V. Hirsh, S. Thongprasert, D. Campos, S. Maoleekoonpiroj, M. Smylie, R. Martins, M. van Kooten, M. Dediu, B. Findlay, D. Tu, D. Johnston, A. Bezjak, G. Clark, P. Santabarbara, L. Seymour, and National Cancer Institute of Canada Clinical Trials Group. 2005. Erlotinib in previously treated non-small-cell lung cancer. *N. Engl. J. Med.* 353:123-132.

24. Rich, J.N., D.A. Reardon, T. Peery, J.M. Dowell, J.A. Quinn, K.L. Penne, C.J. Wikstrand, L.B. Van Duyn, J.E. Dancey, R.E. McLendon, J.C. Kao, T.T. Stenzel, B.K. Ahmed Rasheed, S.E. Tourt-Uhlig, J.E. Herndon 2nd, J.J. Vredenburgh, J.H. Sampson, A.H. Friedman, D.D. Bigner, and H.S. Friedman. 2004. Phase II trial of gefitinib in recurrent glioblastoma. *J. Clin. Oncol.* 22:133-142.

25. Stragliotto, G., F. Vega, P. Stasiecki, P. Gropp, M. Poisson, and J.Y. Delattre. 1996. Multiple infusions of anti-epidermal growth factor receptor (EGFR) monoclonal antibody (EMD 55,900) in patients with recurrent malignant gliomas. *Eur. J. Cancer* 32A:636-640.

26. Scaltriti, M., and J. Baselga. 2006. The epidermal growth factor receptor pathway: a model for targeted therapy. *Clin. Cancer Res.* 12:5268-5272.

27. Klingler-Hoffmann, M., P. Bukczynska, and T. Tiganis. 2003. Inhibition of phosphoinositide 3-kinase signaling negates the growth advantage imparted by a mutant epidermal growth factor receptor on human glioblastoma cells. *Int. J. Cancer* 105:331-339.

28. Klingler-Hoffmann, M., M.T. Fodero-Tavoletti, K. Mishima, Y. Narita, W.K. Cavenee, F.B. Furnari, H.J. Huang, and T. Tiganis. 2001. The protein tyrosine phosphatase TCPTP suppresses the tumorigenicity of glioblastoma cells expressing a mutant epidermal growth factor receptor. *J. Biol. Chem.* 276:46313-46318.

29. Galabova-Kovacs, G., A. Kolbus, D. Matzen, K. Meissl, D. Piazzolla, C. Rubiolo, K. Steinitz, and M. Baccarini. 2006. ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. *Cell. Cycle* 5:1514-1518.

30. Cooper, G.M. 2000. Regulators of cell cycle progression. *In* The Cell: a Molecular Approach. ASM Press, Washington, DC USA.

31. Holgado-Madruga, M.; Emlet, D.R.; Moscatello, D.K.; Godwin, A.K.; Wong, A.J. 1996. A Grb2-Associated Docking Protein in EGF- and Insulin-Receptor Signaling. *Nature.* 379: 560-564.

32. Milburn, C.C., M. Deak, S.M. Kelly, N.C. Price, D.R. Alessi, and D.M. Van Aalten. 2003. Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. *Biochem. J.* 375:531-538.

33. Egawa, K., H. Maegawa, K. Shi, T. Nakamura, T. Obata, T. Yoshizaki, K. Morino, S. Shimizu, Y. Nishio, E. Suzuki, and A. Kashiwagi. 2002. Membrane localization of 3-phosphoinositide-dependent protein kinase-1 stimulates activities of Akt and atypical protein kinase C but does not stimulate glucose transport and glycogen synthesis in 3T3-L1 adipocytes. *J. Biol. Chem.* 277:38863-38869.

34. Nicholson, K.M., and N.G. Anderson. 2002. The protein kinase B/Akt signalling pathway in human malignancy. *Cell. Signal.* 14:381-395.

35. Baldwin, R.M., M. Garratt-Lalonde, D.A. Parolin, P.M. Krzyzanowski, M.A. Andrade, and I.A. Lorimer. 2006. Protection of glioblastoma cells from cisplatin cytotoxicity via protein kinase Ciota-mediated attenuation of p38 MAP kinase signaling. *Oncogene* 25:2909-2919.

36. Regala, R.P., C. Weems, L. Jamieson, J.A. Copland, E.A. Thompson, and A.P. Fields. 2005. Atypical protein kinase Ciota plays a critical role in human lung cancer cell growth and tumorigenicity. *J. Biol. Chem.* 280:31109-31115.

37. Eder, A.M., X. Sui, D.G. Rosen, L.K. Nolden, K.W. Cheng, J.P. Lahad, M. Kango-Singh, K.H. Lu, C.L. Warneke, E.N. Atkinson, I. Bedrosian, K. Keyomarsi, W.L. Kuo, J.W. Gray, J.C. Yin, J. Liu, G. Halder, and G.B. Mills. 2005. Atypical PKCiota contributes to poor prognosis through loss of apical-basal polarity and cyclin E overexpression in ovarian cancer. *Proc. Natl. Acad. Sci. U. S. A.* 102:12519-12524.

38. Regala, R.P., C. Weems, L. Jamieson, A. Khoor, E.S. Edell, C.M. Lohse, and A.P. Fields. 2005. Atypical protein kinase C iota is an oncogene in human non-small cell lung cancer. *Cancer Res.* 65:8905-8911.

39. Cully, M., H. You, A.J. Levine, and T.W. Mak. 2006. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat. Rev. Cancer.* 6:184-192.

40. Wang, S.I., J. Puc, J. Li, J.N. Bruce, P. Cairns, D. Sidransky, and R. Parsons. 1997. Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res.* 57:4183-4186.

41. Chakravarti, A., G. Zhai, Y. Suzuki, S. Sarkesh, P.M. Black, A. Muzikansky, and J.S. Loeffler. 2004. The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. *J. Clin. Oncol.* 22:1926-1933.

42. Hickey, F.B., K. England, and T.G. Cotter. 2005. Bcr-Abl regulates osteopontin transcription via Ras, PI-3K, aPKC, Raf-1, and MEK. *J. Leukoc. Biol.* 78:289-300.

43. Castoria, G., A. Migliaccio, M. Di Domenico, M. Lombardi, A. de Falco, L. Varricchio, A. Bilancio, M.V. Barone, and F. Auricchio. 2004. Role of atypical protein kinase C in estradiol-triggered G1/S progression of MCF-7 cells. *Mol. Cell. Biol.* 24:7643-7653.

44. Gustafson, W.C., S. Ray, L. Jamieson, E.A. Thompson, A.R. Brasier, and A.P. Fields. 2004. Bcr-Abl regulates protein kinase Ciota (PKCiota) transcription via an Elk1 site in the PKCiota promoter. *J. Biol. Chem.* 279:9400-9408.

45. Shelton, J.G., L.S. Steelman, E.R. White, and J.A. McCubrey. 2004. Synergy between PI3K/Akt and Raf/MEK/ERK pathways in IGF-1R mediated cell cycle progression and prevention of apoptosis in hematopoietic cells. *Cell. Cycle* 3:372-379.

46. Holland, E.C., J. Celestino, C. Dai, L. Schaefer, R.E. Sawaya, and G.N. Fuller. 2000. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nat. Genet.* 25:55-57.

47. Zhang, W., D. Bergamaschi, B. Jin, and X. Lu. 2005. Posttranslational modifications of p27kip1 determine its binding specificity to different cyclins and cyclin-dependent kinases in vivo. *Blood* 105:3691-3698.

48. Blagosklonny, M.V., and A.B. Pardee. 2000-2005. The restriction point of the cell cycle. *In* Landes Bioscience,

49. Ali-Osman, F. 2005. Brain Tumours.

50. Alkarain, A., R. Jordan, and J. Slingerland. 2004. p27 deregulation in breast cancer: prognostic significance and implications for therapy. *J. Mammary Gland Biol. Neoplasia* 9:67-80.

51. Fero, M.L., E. Randel, K.E. Gurley, J.M. Roberts, and C.J. Kemp. 1998. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* 396:177-180.

52. Teixeira, L.T., H. Kiyokawa, X.D. Peng, K.T. Christov, L.A. Frohman, and R.D. Kineman. 2000. p27Kip1-deficient mice exhibit accelerated growth hormone-releasing hormone (GHRH)-induced somatotrope proliferation and adenoma formation. *Oncogene* 19:1875-1884.

53. Nakayama, K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, D.Y. Loh, and K. Nakayama. 1996. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85:707-720.

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

55. Sheaff, R.J., M. Groudine, M. Gordon, J.M. Roberts, and B.E. Clurman. 1997. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev.* 11:1464-1478.

56. Malek, N.P., H. Sundberg, S. McGrew, K. Nakayama, T.R. Kyriakides, and J.M. Roberts. 2001. A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. *Nature* 413:323-327.

57. Kamura, T., T. Hara, M. Matsumoto, N. Ishida, F. Okumura, S. Hatakeyama, M. Yoshida, K. Nakayama, and K.I. Nakayama. 2004. Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. *Nat. Cell Biol.* 6:1229-1235.

58. Coats, S., W.M. Flanagan, J. Nourse, and J.M. Roberts. 1996. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. *Science* 272:877-880.

59. Boehm, M., T. Yoshimoto, M.F. Crook, S. Nallamshetty, A. True, G.J. Nabel, and E.G. Nabel. 2002. A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. *EMBO J.* 21:3390-3401.

60. Tomoda, K., Y. Kubota, Y. Arata, S. Mori, M. Maeda, T. Tanaka, M. Yoshida, N. Yoneda-Kato, and J.Y. Kato. 2002. The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex. *J. Biol. Chem.* 277:2302-2310.

61. LaBaer, J., M.D. Garrett, L.F. Stevenson, J.M. Slingerland, C. Sandhu, H.S. Chou, A. Fattaey, and E. Harlow. 1997. New functional activities for the p21 family of CDK inhibitors. *Genes Dev.* 11:847-862.

62. Ishida, N., T. Hara, T. Kamura, M. Yoshida, K. Nakayama, and K.I. Nakayama. 2002. Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. *J. Biol. Chem.* 277:14355-14358.

63. Liang, J., J. Zubovitz, T. Petrocelli, R. Kotchetkov, M.K. Connor, K. Han, J.H. Lee, S. Ciarallo, C. Catzavelos, R. Beniston, E. Franssen, and J.M. Slingerland. 2002. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat. Med.* 8:1153-1160.

64. Shin, I., F.M. Yakes, F. Rojo, N.Y. Shin, A.V. Bakin, J. Baselga, and C.L. Arteaga. 2002. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat. Med.* 8:1145-1152.

65. Viglietto, G., M.L. Motti, P. Bruni, R.M. Melillo, A. D'Alessio, D. Califano, F. Vinci, G. Chiappetta, P. Tsichlis, A. Bellacosa, A. Fusco, and M. Santoro. 2002. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat. Med.* 8:1136-1144.

66. Chiarle, R., L.M. Budel, J. Skolnik, G. Frizzera, M. Chilosi, A. Corato, G. Pizzolo, J. Magidson, A. Montagnoli, M. Pagano, B. Maes, C. De Wolf-Peeters, and G. Inghirami. 2000. Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. *Blood* 95:619-626.

67. Esposito, V., A. Baldi, A. De Luca, A.M. Groger, M. Loda, G.G. Giordano, M. Caputi, F. Baldi, M. Pagano, and A. Giordano. 1997. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. *Cancer Res.* 57:3381-3385.

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

69. Saigusa, K., N. Hashimoto, H. Tsuda, S. Yokoi, M. Maruno, T. Yoshimine, M. Aoyagi, K. Ohno, I. Imoto, and J. Inazawa. 2005. Overexpressed Skp2 within 5p amplification detected by array-based comparative genomic hybridization is associated with poor prognosis of glioblastomas. *Cancer. Sci.* 96:676-683.

70. Traub, F., M. Mengel, H.J. Luck, H.H. Kreipe, and R. von Wasielewski. 2006. Prognostic impact of Skp2 and p27 in human breast cancer. *Breast Cancer Res. Treat.* 99:185-191.

71. Yang, G., G. Ayala, A. De Marzo, W. Tian, A. Frolov, T.M. Wheeler, T.C. Thompson, and J.W. Harper. 2002. Elevated Skp2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival. *Clin. Cancer Res.* 8:3419-3426.

72. Sui, L., Y. Dong, M. Ohno, Y. Watanabe, K. Sugimoto, Y. Tai, and M. Tokuda. 2001. Jab1 expression is associated with inverse expression of p27(kip1) and poor prognosis in epithelial ovarian tumors. *Clin. Cancer Res.* 7:4130-4135.

73. Kouvaraki, M.A., A.L. Korapati, G.Z. Rassidakis, L. Tian, Q. Zhang, P. Chiao, L. Ho, D.B. Evans, and F.X. Claret. 2006. Potential Role of Jun Activation Domain-Binding Protein 1 as a Negative Regulator of p27kip1 in Pancreatic Adenocarcinoma. *Cancer Res.* 66:8581-8589.

74. Kouvaraki, M.A., G.Z. Rassidakis, L. Tian, R. Kumar, C. Kittas, and F.X. Claret. 2003. Jun activation domain-binding protein 1 expression in breast cancer inversely correlates with the cell cycle inhibitor p27(Kip1). *Cancer Res.* 63:2977-2981.

75. Gartel, A.L., and K. Shchors. 2003. Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. *Exp. Cell Res.* 283:17-21.

76. Matsuda, Y., T. Ichida, T. Genda, S. Yamagiwa, Y. Aoyagi, and H. Asakura. 2003. Loss of p16 contributes to p27 sequestration by cyclin D(1)-cyclin-dependent kinase 4 complexes and poor prognosis in hepatocellular carcinoma. *Clin. Cancer Res.* 9:3389-3396.

77. Le, X.F., F.X. Claret, A. Lammayot, L. Tian, D. Deshpande, R. LaPushin, A.M. Tari, and R.C. Bast Jr. 2003. The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. *J. Biol. Chem.* 278:23441-23450.

78. Kortylewski, M., P.C. Heinrich, M.E. Kauffmann, M. Bohm, A. MacKiewicz, and I. Behrmann. 2001. Mitogen-activated protein kinases control p27/Kip1 expression and growth of human melanoma cells. *Biochem. J.* 357:297-303.

79. Delmas, C., S. Manenti, A. Boudjelal, C. Peyssonnaux, A. Eychene, and J.M. Darbon. 2001. The p42/p44 mitogen-activated protein kinase activation triggers p27Kip1 degradation independently of CDK2/cyclin E in NIH 3T3 cells. *J. Biol. Chem.* 276:34958-34965.

80. Lenferink, A.E., D. Busse, W.M. Flanagan, F.M. Yakes, and C.L. Arteaga. 2001. ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1 through multiple signaling pathways. *Cancer Res.* 61:6583-6591.

81. Foster, J.S., R.I. Fernando, N. Ishida, K.I. Nakayama, and J. Wimalasena. 2003. Estrogens down-regulate p27Kip1 in breast cancer cells through Skp2 and through nuclear export mediated by the ERK pathway. *J. Biol. Chem.* 278:41355-41366.

82. Zhu, X., C.H. Kwon, P.W. Schlosshauer, L.H. Ellenson, and S.J. Baker. 2001. PTEN induces G(1) cell cycle arrest and decreases cyclin D3 levels in endometrial carcinoma cells. *Cancer Res.* 61:4569-4575.

83. Brandts, C.H., B. Bilanges, G. Hare, F. McCormick, and D. Stokoe. 2005. Phosphorylation-independent stabilization of p27kip1 by the phosphoinositide 3-kinase pathway in glioblastoma cells. *J. Biol. Chem.* 280:2012-2019.

84. Gottschalk, A.R., D. Basila, M. Wong, N.M. Dean, C.H. Brandts, D. Stokoe, and D.A. Haas-Kogan. 2001. p27Kip1 is required for PTEN-induced G1 growth arrest. *Cancer Res.* 61:2105-2111.

85. Kfir, S., M. Ehrlich, A. Goldshmid, X. Liu, Y. Kloog, and Y.I. Henis. 2005. Pathway- and expression level-dependent effects of oncogenic N-Ras: p27(Kip1) mislocalization by the Ral-GEF pathway and Erk-mediated interference with Smad signaling. *Mol. Cell. Biol.* 25:8239-8250.

86. Greulich, H., and R.L. Erikson. 1998. An analysis of Mek1 signaling in cell proliferation and transformation. *J. Biol. Chem.* 273:13280-13288.

87. Sakakibara, K., K. Kubota, B. Worku, E.J. Ryer, J.P. Miller, A. Koff, K.C. Kent, and B. Liu. 2005. PDGF-BB regulates p27 expression through ERK-dependent RNA turn-over in vascular smooth muscle cells. *J. Biol. Chem.* 280:25470-25477.

88. van Duijn, P.W., and J. Trapman. 2006. PI3K/Akt signaling regulates p27(kip1) expression via Skp2 in PC3 and DU145 prostate cancer cells, but is not a major factor in p27(kip1) regulation in LNCaP and PC346 cells. *Prostate* 66:749-760.

89. Brunet, A., A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, and M.E. Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96:857-868.

90. Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-297.

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

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

93. Pasquinelli, A.E., B.J. Reinhart, F. Slack, M.Q. Martindale, M.I. Kuroda, B. Maller, D.C. Hayward, E.E. Ball, B. Degnan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J.

Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson, and G. Ruvkun. 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408:86-89.

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

95. Bentwich, I., A. Avniel, Y. Karov, R. Aharonov, S. Gilad, O. Barad, A. Barzilai, P. Einat, U. Einav, E. Meiri, E. Sharon, Y. Spector, and Z. Bentwich. 2005. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat. Genet.* 37:766-770.

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

97. Lewis, B.P., C.B. Burge, and D.P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15-20.

98. Strauss, W.M., C. Chen, C.T. Lee, and D. Ridzon. 2006. Nonrestrictive developmental regulation of microRNA gene expression. *Mamm. Genome* 17:833-840.

99. Wienholds, E., W.P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H.R. Horvitz, S. Kauppinen, and R.H. Plasterk. 2005. MicroRNA expression in zebrafish embryonic development. *Science* 309:310-311.

100. Ason, B., D.K. Darnell, B. Wittbrodt, E. Berezikov, W.P. Kloosterman, J. Wittbrodt, P.B. Antin, and R.H. Plasterk. 2006. Differences in vertebrate microRNA expression. *Proc. Natl. Acad. Sci. U. S. A.* 103:14385-14389.

101. Ambros, V., B. Bartel, D.P. Bartel, C.B. Burge, J.C. Carrington, X. Chen, G. Dreyfuss, S.R. Eddy, S. Griffiths-Jones, M. Marshall, M. Matzke, G. Ruvkun, and T. Tuschl. 2003. A uniform system for microRNA annotation. *RNA* 9:277-279.

102. Nottrott, S., M.J. Simard, and J.D. Richter. 2006. Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat. Struct. Mol. Biol.* 13:1108-1114.

103. Pillai, R.S., S.N. Bhattacharyya, C.G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand, and W. Filipowicz. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* 309:1573-1576.

104. Pillai, R.S. 2005. MicroRNA function: multiple mechanisms for a tiny RNA? *RNA* 11:1753-1761.

105. Bentwich, I. 2005. Prediction and validation of microRNAs and their targets. *FEBS Lett.* 579:5904-5910.

106. Kloosterman, W.P., E. Wienholds, R.F. Ketting, and R.H. Plasterk. 2004. Substrate requirements for let-7 function in the developing zebrafish embryo. *Nucleic Acids Res.* 32:6284-6291.

107. Vella, M.C., K. Reinert, and F.J. Slack. 2004. Architecture of a validated microRNA::target interaction. *Chem. Biol.* 11:1619-1623.

108. Lewis, B.P., I.H. Shih, M.W. Jones-Rhoades, D.P. Bartel, and C.B. Burge. 2003. Prediction of mammalian microRNA targets. *Cell* 115:787-798.

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

110. Calin, G.A., and C.M. Croce. 2006. Genomics of chronic lymphocytic leukemia microRNAs as new players with clinical significance. *Semin. Oncol.* 33:167-173.

111. Calin, G.A., C.D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich, and C.M. Croce. 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U. S. A.* 99:15524-15529.

112. Cimmino, A., G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, S.E. Wojcik, R.I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C.G. Liu, T.J. Kipps, M. Negrini, and C.M. Croce. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. U. S. A.* 102:13944-13949.

113. Volinia, S., G.A. Calin, C.G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R.L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C.C. Harris, and C.M. Croce. 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. U. S. A.* 103:2257-2261.

114. Johnson, S.M., H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K.L. Reinert, D. Brown, and F.J. Slack. 2005. RAS is regulated by the let-7 microRNA family. *Cell* 120:635-647.

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

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

117. Karube, Y., H. Tanaka, H. Osada, S. Tomida, Y. Tatematsu, K. Yanagisawa, Y. Yatabe, J. Takamizawa, S. Miyoshi, T. Mitsudomi, and T. Takahashi. 2005. Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer. Sci.* 96:111-115.

118. Carmell, M.A., Z. Xuan, M.Q. Zhang, and G.J. Hannon. 2002. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev.* 16:2733-2742.

119. Murchison, E.P., J.F. Partridge, O.H. Tam, S. Cheloufi, and G.J. Hannon. 2005. Characterization of Dicer-deficient murine embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 102:12135-12140.

120. Bernstein, E., S.Y. Kim, M.A. Carmell, E.P. Murchison, H. Alcorn, M.Z. Li, A.A. Mills, S.J. Elledge, K.V. Anderson, and G.J. Hannon. 2003. Dicer is essential for mouse development. *Nat. Genet.* 35:215-217.

121. Hatfield, S.D., H.R. Shcherbata, K.A. Fischer, K. Nakahara, R.W. Carthew, and H. Ruohola-Baker. 2005. Stem cell division is regulated by the microRNA pathway. *Nature* 435:974-978.

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

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

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

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

126. Griffiths-Jones, S., R.J. Grocock, S. van Dongen, A. Bateman, and A.J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* 34:D140-4.

127. Felli, N., L. Fontana, E. Pelosi, R. Botta, D. Bonci, F. Facchiano, F. Liuzzi, V. Lulli, O. Morsilli, S. Santoro, M. Valtieri, G.A. Calin, C.G. Liu, A. Sorrentino, C.M. Croce, and C. Peschle. 2005. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc. Natl. Acad. Sci. U. S. A.* 102:18081-18086.

128. Mellinghoff, I.K., M.Y. Wang, I. Vivanco, D.A. Haas-Kogan, S. Zhu, E.Q. Dia, K.V. Lu, K. Yoshimoto, J.H. Huang, D.J. Chute, B.L. Riggs, S. Horvath, L.M. Liau, W.K. Cavenee, P.N. Rao, R. Beroukhim, T.C. Peck, J.C. Lee, W.R. Sellers, D. Stokoe, M. Prados, T.F. Cloughesy, C.L. Sawyers, and P.S. Mischel. 2005. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N. Engl. J. Med.* 353:2012-2024.

129. Garratt-Lalonde, M. 2003. The Role of Atypical PKC iota in Glioblastoma Multiforme. 53.

130. Jackson, A.L., J. Burchard, J. Schelter, B.N. Chau, M. Cleary, L. Lim, and P.S. Linsley. 2006. Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA* 12:1179-1187.

131. Dijkers, P.F., R.H. Medema, C. Pals, L. Banerji, N.S. Thomas, E.W. Lam, B.M. Burgering, J.A. Raaijmakers, J.W. Lammers, L. Koenderman, and P.J. Coffer. 2000. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol. Cell. Biol.* 20:9138-9148.

132. Larocque, D., A. Galarneau, H.N. Liu, M. Scott, G. Almazan, and S. Richard. 2005. Protection of p27(Kip1) mRNA by quaking RNA binding proteins promotes oligodendrocyte differentiation. *Nat. Neurosci.* 8:27-33.

133. Bhatt, K.V., L.S. Spofford, G. Aram, M. McMullen, K. Pumiglia, and A.E. Aplin. 2005. Adhesion control of cyclin D1 and p27Kip1 levels is deregulated in melanoma cells through BRAF-MEK-ERK signaling. *Oncogene* 24:3459-3471.

134. Duchaine, T.F., J.A. Wohlschlegel, S. Kennedy, Y. Bei, D. Conte Jr, K. Pang, D.R. Brownell, S. Harding, S. Mitani, G. Ruvkun, J.R. Yates 3rd, and C.C. Mello. 2006. Functional proteomics reveals the biochemical niche of C. elegans DCR-1 in multiple small-RNA-mediated pathways. *Cell* 124:343-354.

135. Di Gennaro, E., M. Barbarino, F. Bruzzese, S. De Lorenzo, M. Caraglia, A. Abbruzzese, A. Avallone, P. Comella, F. Caponigro, S. Pepe, and A. Budillon. 2003. Critical role of both p27KIP1 and p21CIP1/WAF1 in the antiproliferative effect of ZD1839 ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor, in head and neck squamous carcinoma cells. *J. Cell. Physiol.* 195:139-150.

136. Semizarov, D., L. Frost, A. Sarthy, P. Kroeger, D.N. Halbert, and S.W. Fesik. 2003. Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. U. S. A.* 100:6347-6352.

137. Grimm, D., K.L. Streetz, C.L. Jopling, T.A. Storm, K. Pandey, C.R. Davis, P. Marion, F. Salazar, and M.A. Kay. 2006. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441:537-541.

138. She, Q.B., D.B. Solit, Q. Ye, K.E. O'Reilly, J. Lobo, and N. Rosen. 2005. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. *Cancer. Cell.* 8:287-297.

139. Lee, Y.S., K. Nakahara, J.W. Pham, K. Kim, Z. He, E.J. Sontheimer, and R.W. Carthew. 2004. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117:69-81.

140. Pham, J.W., J.L. Pellino, Y.S. Lee, R.W. Carthew, and E.J. Sontheimer. 2004. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. *Cell* 117:83-94.

141. Fero, M.L., E. Randel, K.E. Gurley, J.M. Roberts, and C.J. Kemp. 1998. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* 396:177-180.

142. Teixeira, L.T., H. Kiyokawa, X.D. Peng, K.T. Christov, L.A. Frohman, and R.D. Kineman. 2000. p27Kip1-deficient mice exhibit accelerated growth hormone-releasing hormone (GHRH)-induced somatotrope proliferation and adenoma formation. *Oncogene* 19:1875-1884.

143. Nakayama, K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, D.Y. Loh, and K. Nakayama. 1996. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85:707-720.

144. Johnson, S.M., S.Y. Lin, and F.J. Slack. 2003. The time of appearance of the C. elegans let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. *Dev. Biol.* 259:364-379.

145. Pasquinelli, A.E., B.J. Reinhart, F. Slack, M.Q. Martindale, M.I. Kuroda, B. Maller, D.C. Hayward, E.E. Ball, B. Degnan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J. Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson, and G. Ruvkun. 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408:86-89.

146. Wulczyn, F.G., L. Smirnova, A. Rybak, C. Brandt, E. Kwidzinski, O. Ninnemann, M. Strehle, A. Seiler, S. Schumacher, and R. Nitsch. 2006. Post-transcriptional regulation of the let-7 microRNA during neural cell specification. *FASEB J.*

147. Lund, E., S. Guttinger, A. Calado, J.E. Dahlberg, and U. Kutay. 2004. Nuclear export of microRNA precursors. *Science* 303:95-98.

148. Chiosea, S., E. Jelezcova, U. Chandran, M. Acquafondata, T. McHale, R.W. Sobol, and R. Dhir. 2006. Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma. *Am. J. Pathol.* 169:1812-1820.

149. Bottoni, A., M.C. Zatelli, M. Ferracin, F. Tagliati, D. Piccin, C. Vignali, G.A. Calin, M. Negrini, C.M. Croce, and E.C. Degli Uberti. 2007. Identification of differentially expressed microRNAs by microarray: A possible role for microRNA genes in pituitary adenomas. *J. Cell. Physiol.* 210:370-377.

150. Obernosterer, G., P.J. Leuschner, M. Alenius, and J. Martinez. 2006. Post-transcriptional regulation of microRNA expression. *RNA* 12:1161-1167.

151. Kasashima, K., Y. Nakamura, and T. Kozu. 2004. Altered expression profiles of microRNAs during TPA-induced differentiation of HL-60 cells. *Biochem. Biophys. Res. Commun.* 322:403-410.

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