doi:10.4149/neo_2011_01_65

The role of $p27^{Kip1}$ phosphorylation at Serine 10 in the migration of malignant glioma cells in vitro

D. WANG[1], F. HE[1], L. ZHANG[1], F. ZHANG[1], Q. WANG[1], X. QIAN[1], X. PAN[1], J. MENG[1], C. PENG[1], A. SHEN[2], J. CHEN[3]

[1]Department of pathology, Medical College, Nantong University, Nantong, Jiangsu, 226001, P.R. China; [2]The Jiangsu Province Key Laboratory of Neuroregeneration, Nantong University, Nantong, Jiangsu, 226001, P.R. China, e-mail: shen_aiguo@yahoo.com; [3]Department of Neurosurgery, Affiliated Hospital of Nantong University, Nantong, Jiangsu, P.R. China

Received May 15, 2010

Until recently, Cip/Kip members were almost solely viewed as nuclear proteins with a principal function of inhibiting cyclin/cyclin dependent kinase (CDK) activity and hence, inhibiting cell cycle progression. $P27^{Kip1}$ (hereafter p27) belongs to the Cip/Kip family that binds and inhibits all the cyclin/CDK complexes, thus often referred as a universal CDK inhibitor. However, emerging studies now suggest that Cip/Kip proteins play additional roles outside of the nucleus. Indeed, previous reports have linked p27 to the regulation of actin dynamics and cell migration. In this study, we constructed a model of migration-activated glioma cells by using the migration-stimulating substrate, a kind of ECM, laminin in vitro. Our results present evidence that laminin drives glioma cell migration without altering cell proliferation. Further, actively migrating cells which expressioned high phosphorylation of p27 at Ser10, and induced its cytoplasmic localization. In this process, Jab1 and CRM1 were also involved. Thus phosphorylation of p27 at Ser10 is necessary for both cytoplasmic localization and induction of cell migration. These observations solidified a genetic role of p27 in cell migration and this was independent of cyclin/CDK inhibition. Eventually, we transiently transfected p27S10A into T98G glioma cells, found that overexpression of p27S10A inhibited cell migration but not cell proliferation. These data linked phosphorylation of p27 at Ser10 and cell motility. Therefore, the major phosphorylation site at Ser10 of p27 played a pivotal role in the migration of malignant glioma cells.

Key words: $p27^{Kip1}$, Ser^{10}phosphorylation, glioma, migration

Glioblastoma multiforme (GBM) is the most common primary brain tumor, affecting 20,000 patients per year; the peak age of occurrence is between 50–60 years of age. Despite advances in diagnosis and treatment, life expectancy for patients suffering from this disease still remains at 18 months [1]. Currently, surgery and radiation therapy followed by treatment with temozolomide is the treatment protocol of choice for patients with glioblastoma multiforme [2; 3]. However, despite advanced multimodal treatment, the median life expectancy for patients with glioblastoma multiforme is approximately 14 months with less than 5% of patients alive at 5 years after diagnosis. Althoug systemic metastases of malignant gliomas are relatively rare, the highly infiltrative nature of glioma cells that deeply infiltrated in the normal surrounding brain parenchyma is a major cause of treatment failure and postoperative recurrence [4]. Glioma invasion is a multifactorial process consisting of glioma cell interactions with normal cellular constituents such as astrocytes, neurons, and endothelial cells, interactions with the extracellular matrix (ECM), proteolysis of ECM components, and migration. To migrate, glioma cells must modify their shape and rigidity to interact with the surrounding environment, which may act both as a mechanical barrier to the cells, and as a (permissive) substratum for traction for the migrating cells [5]. Recently, a report by AM. Joy et al. have showed that, when cells were plated onto the nonpermissive substrate BSA and the migration-stimulating substrate laminin, the migration rate were markedly different between these two groups, laminin effectively stimulated the migration of SF767 and T98G glioma cells approximately 8-12-fold more than BSA did [6].

Abbreviations: GBM – Glioblastoma multiforme, CDK – cyclin-dependent kinase, BSA – bovine serum albumin, LN – laminin, Jab1 – Jun activation domain-binding protein 1, EGFP – enhanced green fluorescent protein, PI3K – phosphatidylinositol 3-kinase, DMSO – dimethyl sulfoxide

$P27^{Kip1}$ (hereafter p27) is a member of the Cip/Kip family of cyclin/CDK inhibitor and plays a critical role in regulating the progression through the G1 and S phases of the cell cycle [7]. Recently, several research groups reached the conclusion that diverse biological functions of p27 are correlated to its cellular localization. Among these studies, the role of p27 on cell motility has become an interesting matter of scientific literature [8; 9; 10; 11; 12]. In the absence of p27, mouse embryonic fibroblasts have been shown to have increased focal adhesion and an increased amount of actin stress fibers [9], which are hallmarks of decrease in cell motility [13]. Further investigations revealed that the phosphorylation of p27 at Ser10 (p-p27-Ser10) and its subsequent relocalization to the cytoplasm would increase the capability of mouse embryonic fibroblasts to migrate [10]; this has been confirmed by p27-null fibroblasts which displayed a dramatic decrease in cell motility [9]. Moreover, overexpression of p27 efficiently promoted the migration of cortical neurons, while p27 downregulation largely inhibited such a migration [14].

Although p27 has proven to increase cell motility, its migration inhibitory effect on some cells was also supported by several studies [11; 12; 15]. Baldassarre et al. [8] demonstrated that p27 inhibits sarcoma cell migration through its interaction and inhibition on the microtubule destabilizing protein stathmin [16]. Their data supports the possibility that p27 interferes with the ability of stathmin to sequester the free tubulin heterodimers, resulting in decreased cell motility. Furthermore, a low p27/stathmin ratio in primary and metastatic sarcomas in vivo was shown to correlate with increased metastasis [8]. M. Schiappacassi et al. demonstrated that p27 expression inhibits glioblastoma growth, invasion, and tumor-induced neoangiogenesis [17].

Contradictory findings have been reported on whether p27 promotes or inhibits cell migration [8; 9; 10; 11; 12; 14; 15]. As cell migration and glioma postoperative recurrence are closely related, it is worthwhile to further contributing data to the action of p27 on tumor cell migration. To extend these observations, in this study, we constructed a model of migration-activated glioma cells by using the migration-stimulating substrate, laminin, in vitro, aimed to investigate the role of p27 and its main phosphorylation site, at the Ser10, in tumor cell migration and have suggested that, in response to migration-stimulating signal, p27 was phosphorylationed at the Ser10 site and induced its cytoplasmic localization. These results suggested that p27 developed its migration-promoting effect was due to its phosphorylation of Ser10, so its potential use was as a therapeutic target for drugs to limit the migration of brain tumor cells or the migration and invasion of other cancer cell types.

Materials and methods

Cells, reagents, and antibodies. The human glioblastoma cell lines U87MG and T98G were purchased from China academy of science cell library. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GibCo BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin mixture (GibCo BRL, Grand Island, NY, USA) at 37°C and 5% CO_2 in a humidified chamber. Purified Laminin (LN), bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO), LY294002, were obtained from Sigma; anti-CRM1, anti-a-tublin, anti-LaminB were obtained from Santa Cruz Biotechnology, anti-phospho-p27(Ser^{10}) was obtained from Zymed Laboratories, anti-Jab1 was obtained from BD Biosciences PharMingen, anti-Akt, anti-phospho-Akt(Ser^{473}), anti-phospho-Akt (Thr^{308}), anti-β-actin were obtained from was from Sigma. Peroxidase-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Cell Signaling Technology.

Cell-migration assay. Migration assays were performed using the Monolayer wound healing assay as previously described [18]

Cell proliferation assay. Cell proliferation was measured using the CCK-8 assay following the manufacturer's instructions. Briefly, cells were seeded on a 96-well cell culture cluster (Corning Inc., Corning, NY) at a concentration of 2×10^4/well in a volume of 100 μL, and grown overnight. Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) reagents were added to a subset of wells under different treatments, were incubated for 2 hours at 37°C, and absorbance was quantified on an automated plate reader. The experiments were repeated at least three times.

Nuclear and cytoplasmic extraction, Western blot analysis. Subcellular fractionation was performed as described previously [19]. Briefly, cell pellets from a culture were incubated in a hypotonic buffer (10 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM NaF, 100 μM Na_3VO_4, and protease inhibitor mixture) for 30 min at 4°C on a rocking platform. Cells were homogenized (Dounce, 30 strokes), and their nuclei were pelleted by centrifugation (10 min ×14,000 rcf, 4°C). The supernatant was saved as the cytosolic fraction, and nuclear pellets were incubated in nuclear lysis buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100] for 1 h at 4°C on a rocking platform. The nuclear fraction was collected by centrifugation (10 min ×14,000 rcf, 4°C).

Prior to immunoblotting, cells were washed twice with ice-cold PBS, resuspensed in 2 ×lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM Na_3VO_4, and protease inhibitor mixture), and incubated for 20 min at 4°Cwhile rocking. Lysates were cleared by centrifugation (10 min×12,000 rpm, 4°C). Fifty μg of total protein were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane filter (Immbilon, Millipore). The membranes were first blocked and then incubated with anti-phospho-p27(Ser^{10}) (34–6,300, 1:500; Zymed Laboratories, Inc., San Francisco, CA, USA), anti-Jab1 (611618, 1:500; BD Biosciences PharMingen, San Diego, CA, USA), anti-CRM1

(sc-5595, 1:500; Santa Cruz Biotechnology, Inc., CA, USA), anti-β-actin (1:1000; SigmaQ1), anti-a-tubulin (sc-8035, 1:100; Santa Cruz Biotechnology, Inc., CA, USA), anti-LaminB (sc-6217, 1:100; Santa Cruz Biotechnology, Inc., CA, USA) , antibodies respectively for 2 h at room temperature. After three washes, the membranes were incubated with horseradish peroxidaseconjugated human anti-mouse or anti-rabbit antibodies (1:1,000; Pierce) for 2 h at room temperature. Detection of immunocomplexes was performed with an enhanced chemiluminescence system (NEN Life Science Products, Boston, MA).

Immunofluorescence. The cells grown on coverslips were fixed with 4% formaldehyde for 30 min, treated with 0.1% TritonX-100/PBS for 5 min, and incubated with phosphate buffered saline (PBS) containing 3% normal goat serum for 1 h. Then the cells were incubated overnight at 4°C with anti-p27 antibodies. After being rinsed with PBS, the cells were incubated with FITC labeled secondary antibodies. Immunopositive cells were observed using a fluorescent microscopy (Leica, Germany). All photomicrographs shown in this study are representative of multiple experiments.

Expression plasmid and transient transfection. The point mutant of p27 Ser[10], Ser-10-Ala cDNA was generated by PCR from a cDNA library of Jurkat cells. The PCR fragment was cloned into the pcDNA3.1-EGFP expression vector at the EcoRI/XhoI. The proper construction of the plasmid was confirmed by DNA sequencing and the plasmid for transfection was prepared using an EndoFree Plasmid Maxi Kit (Qiagen, Tokyo, Japan). Transfection was performed using lipofectamine 2,000 transfection reagent (Invitrogen) according to the manufacture's protocol with minor modifications. The transfection efficiency was assessed by fluorescence microscope, and the efficiency of transfection was found to be the same for all constructs. The experiments were repeated at least three times.

Statistical analysis. All the data were calculated as means ± SE. The statistical significance of the means was calculated using a Student's *t* test. One-way ANOVA followed by the Tukey's post-hocmultiple comparison tests was used for statistical analysis of the data of immunoblotting. *P* values <0.05 was considered statistically significant. All statistical analysis was conducted with a STATA 7.0 software package (Systat Software Inc., San Jose, CA, USA).

Results

Laminin could stimulate glioma cell migration without altering cell proliferation. A monolayer wound healing assay (as previously reported) was used to assess the migration rate of cells plated onto the nonpermissive substrate BSA and the migration-stimulating substrate, laminin [6]. A typical migration assay for cells on laminin was shown in Fig.1A, after allowing cells to migrate for overnight, the next day, the wounds were observed using phase contrast microscopy on an inverted microscope. Images were taken

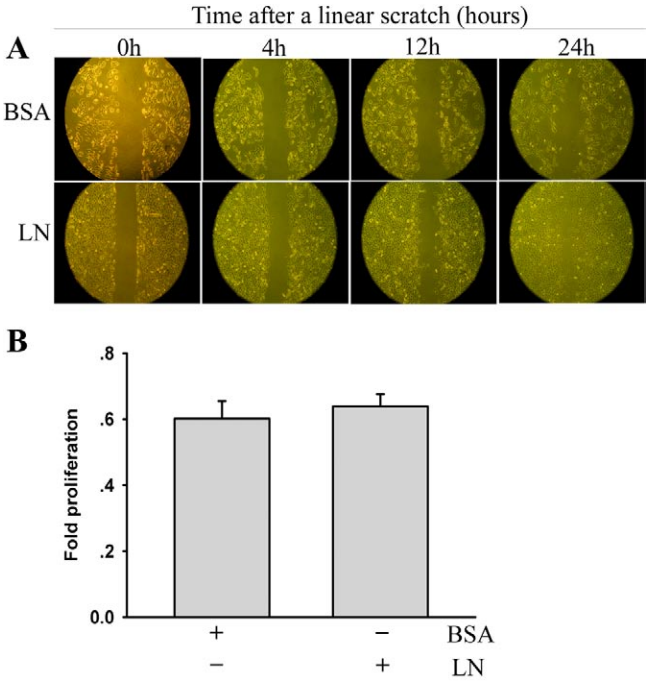

Figure 1. Laminin could stimulate glioma cell migration without altering cell proliferation
(A) A typical monolayer wound healing migration assay for cells on laminin at the initial migration measurement (2 hours following plating, t=0) and after allowing cells to migrate for 24 hours (t=24h). T98G glioma cells were plated onto 0.1% BSA or 10 μg/ml laminin-coated slides and migration rate obtained from 0 hours to 24 hours. (B) T98G glioma cells were subjected to CCK-8 analysis as described in Materials and Methods in order to examine cell proliferation. Experiments were performed in triplicate and representative results were shown.

at regular intervals over the course of 0-24 h of each area at the same place. Compared with BSA, laminin effectively stimulates the migration of T98G glioma cell at t=12h, and at t=24h reached the peak values, and even an apparent fusion (Fig.1A) could be seen.

To investigate whether engagement of the migratory phenotype affected the cells' susceptibility to proliferation, migration-restricted and migration-activated glioma cells were treated with CCK-8 then the percentage of proliferating cells present was determined (Fig.1B). Migration-activated cells are defined as those cells plated on laminin, and migration-restricted cells are operationally defined as those cells plated on the nonpermissive substrate BSA. Of note, this difference was not due to change in cell proliferation as the growth kinetics of T98G cells were unaffected by laminin over 24 hours compared with cells plated on BSA (Fig.1B). These results indicated that laminin could significantly stimulate T98G glioma cell migration in vitro without affecting cell proliferation.

Actively migrating cells which expressioned high phosphorylation of p27 at Ser10. P27, as a candidate tumor

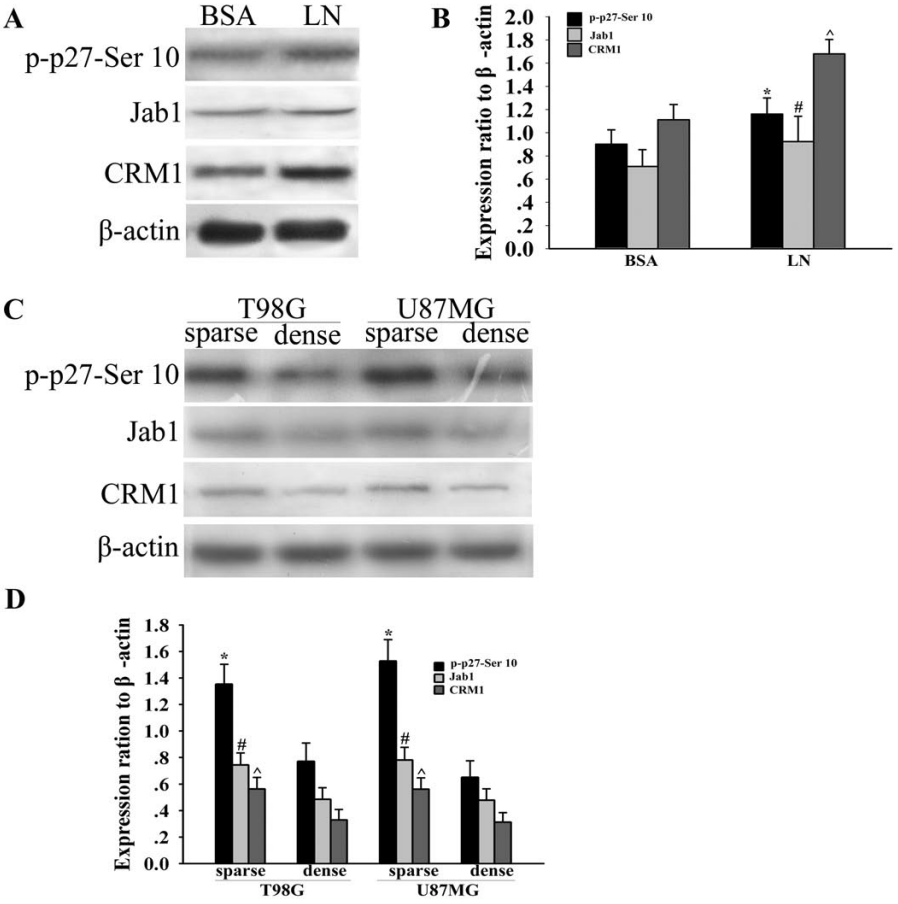

Fig.2. Actively migrating cells which expressioned high phosphorylation of p27 at Ser10
(A) T98G glioma cells were plated onto BSA or laminin-coated dishes and lysates collected. Western analysis for p-p27-Ser10, Jab1, CRM1 expressioned in cells and β-actin served as a loading control. (B) The bar chart beside demonstrated the ratio of p-p27-Ser10, Jab1 and CRM1 protein to β-actin by densitometry. (C) T98G and U87MG glioma cells were plated onto laminin-coated dishes at 7000 (sparse) or 55,000 (dense) cells/cm² and lysates collected. Sparse cells that acquired invasive phenotype were found to exhibit elevated levels of p-p27-Ser10, Jab1, CRM1 and β-actin was used as a loading control. (D) The bar chart below demonstrated the ratio of p-p27-Ser10, Jab1 and CRM1 protein to β-actin by densitometry. Experiments were performed in triplicate and representative results were shown. Columns, means of values from three independent experiments; bars, SE. (n=3, *, #, ^ p<0.05 glioma control: BSA or dense).

suppressor gene, its expression is always reduced in many tumors, but in some malignant tumors, its expression is abnormaly increased. Recent studies have indicated that it relevant to its mislocation to the cytoplasm [20]. It was previously determined that Ser10 was the major phosphorylation site in p27, accounting for 70% of the total phosphorylation of this protein, due to cytoplasmic shuttling [21]. Western blot analysis showed that when the cells were plated on laminin, the level of phosphorylated $p27^{Kip1}$ (Ser10) was greatly increased and the levels of Jun activation domain-binding protein 1 (Jab1) and CRM1, carrier proteins for nuclear export [22] were also obviously increased (Fig.2A, B). Previous studies indicated that Akt is activated in migrating glioma cells, whereas p27 was a main target of Akt and its Ser10 could be phosphorylation by it, well how p27 was phosphorylated on Ser10 when cells were plated on laminin, also by PI3K/Akt pathway [23]? So we tested the expression of both p-Akt-Ser 473 and p-Akt-Thr 308, western analysis showed that the cells plated on laminin paralleled a greater increase in Akt phosphorylation at Ser 473 and Thr 308 than the cells on BSA (Supplementary Fig. S1). Further was investigated, a sparse/dense paradigm, as previously described [6; 24], p-p27-Ser10, Jab1 and CRM1 and were all found to be elevated in T98G and U87MG glioma cells propagated under sparse conditions relative to densely packed cognates (Fig.2C, D). Together, these findings indicated that phosphorylation at Ser10 of p27 played an important role in the migration of human glioma cells.

Critical role of p27 cytoplasmic localization. As known that since p27 bound to Jab1 and/or CRM1 after phosphorylation at Ser10, the extent of phosphorylation at that residue might regulate the nuclear-cytoplasmic shuttling

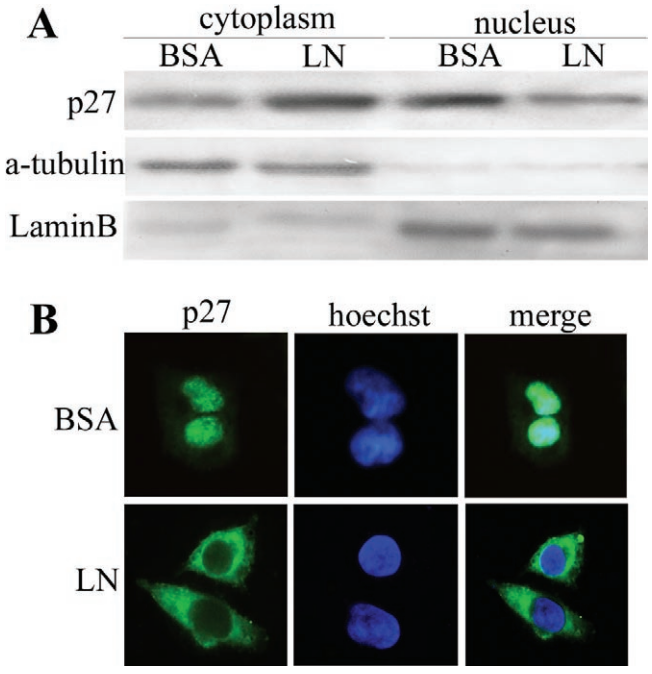

Fig.3. Critical role of p27 cytoplasmic localization
(A) Western analysis laminin induced migration-activated glioma cells which expressioned p27 levels predominant in the cytoplasmic fraction but fewer signals in the nucleus. Cytoplasmic and nuclear fractions were assayed for p27 protein (a-tubulin, cytoplasmic marker; LaminB, nuclear marker). (B) Immunocytofluorescence study of the subcellular localization of p27 in T98G glioma cells after acquired invasive phenotype, p27 signal was present largely in the cytoplasmic. Experiments were performed in triplicate and representative results were shown.

[22]. We then tested the expression of endogenous p27 and its subcellular localization under different growth substrates. Western blot analysis showed that migration-activated glioma cells which expressioned p27 levels predominantly in the cytoplasmic fraction but fewer signals even in the nucleus (Fig.3A). To corroborate these findings, we determined the subcellular localization of p27 in T98G glioma cells after plated on laminin, immunofluorescence analysis revealed that when the cells were plated on migration-stimulating substrate, p27 signal was present largely in the cytoplasmic fraction (Fig.3B). Together, these findings indicated that when p27 mislocalized from nucleus to the cytoplasm, it could show its another biological effect, which may promote the cell migration.

Overexpression of p27S10A inhibited cell migration in the absence of significant effects on cell proliferation . To investigate a possible causal relationship between p27 phosphorylation at Ser10 and cell migration, T98G cells were transient transfected with mock, constructs encoding enhanced green fluorescent protein (EGFP) or EGFP-p27 Ser10 point mutants: Ser-10-Ala, which prevents p27 from being phosphorylated at position 10. To facilitate quantifica-

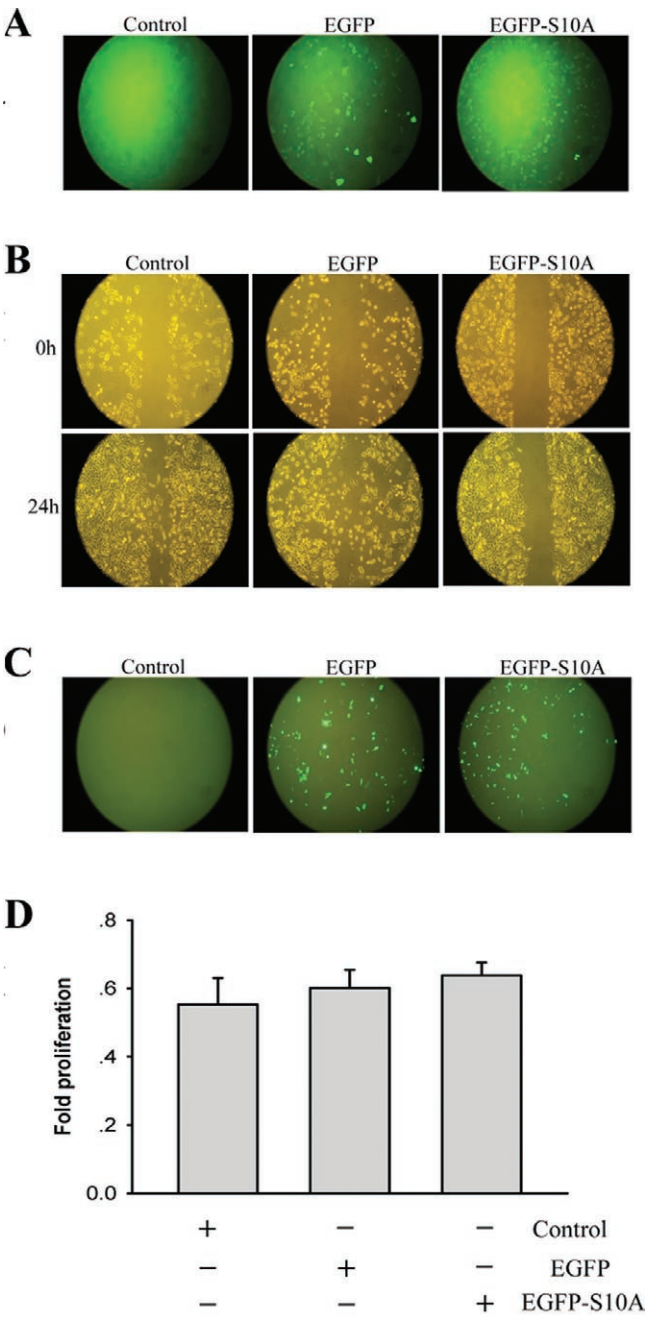

Fig.4. Overexpression of p27S10A inhibited cell migration in the absence of significant effects on cell proliferation
(A) T98G glioma cells were plated in separated 35 mm dishes and then transfected with mock, a vector encoding EGFP (enhanced green fluorescent protein) or EGFP-p27S10A; the transfection efficiency was assessed by fluorescence microscope. (B) Compared with the mock or EGFP control, a monolayer wound healing assay suggested that EGFP- p27S10A expressing cells which were showed a decreased ability to migrate. (C) T98G glioma cells were plated in 96-well cell culture cluster and then transfected with mock, a vector encoding EGFP or EGFP-p27S10A, the transfection efficiency was assessed by fluorescence microscope. (D) Cell proliferation was not affected by the introduction of the Ser10 mutant of p27 by CCK-8 test. Experiments were performed in triplicate and representative results were shown.

tion, T98G glioma cells were plated in separated dishes and then transfected with mock, a vector encoding EGFP or EGFP-p27S10A, then the transfection efficiency was assessed by fluorescence microscope, and the efficiency of transfection was found to be the same for all constructs (Fig.4A, C). Our control studies indicated that cells expressing EGFP alone did not alter the rate of migration. Compared with the EGFP control, EGFP-p27S10A expressing cells showed a decreased ability to migrate (Fig.4B). Of note, avoiding physiological relevant levels of p27, we also used CCK-8 to measure cell proliferation (Fig.4D). Among the cells which express mock, EGFP or EGFP-p27S10A, there is no significant difference in their proliferation as evidenced by cell counting.

Inhibition of PI3-K/Akt suppressed the migration of actively migrating glioma cells. Evidence for supporting the critical role of phosphorylation of p27 at Ser10 in the migration of glioma cells in vitro, we next investigated whether the PI3-K inhibitor, LY294002 had effect on the phosphorylation p27 at Ser10 and cell migration, between the cells on BSA and the cells on laminin. As shown in Fig. S2, compared with the cells on BSA, LY294002 could inhibit the expression of phosphorylation of p27 at Ser10 when cells on laminin (Supplementary Fig. S2). From the Monolayer wound healing assay, we found that PI3-K inhibitor, LY294002 could inhibit the migration of actively migrating T98G glioma cells on laminin, but have no effect on the cells on BSA (Supplementary Fig. S3). These data suggest that when cells were plated on laminin, p27 was phosphorylated at Ser10 and actively migrating, probably by activation the pathway of PI3K/Akt (Supplementary Fig.S1 and S2). Cell proliferations by itself could not have caused differences in migration content because plated cells on BSA did not have changes (Supplementary Fig. S3).

Discussion

In glioblastoma, the most aggressive form of brain tumors, tumor cells disperse so extensively that current treatment approaches such as surgical resection or radiation therapy have little effect in checking progression [5; 25]. This is a significant problem and it is recognized as a major priority to better understand glioma dispersal and to develop therapies that limit this process [25]. A first step in this development is the identification of proteins that function aberrantly in dispersal-relevant processes such as cell migration, which is frequently deregulated in cancer cells [13; 26; 27; 28]. The inhibition of cell migration and invasion is, in principle, likely to prevent the spread of tumors into eloquent brain regions and, in turn, preserve neurologic function. Clinical attempts to specifically inhibit cell migration and invasion have, however, been limited [29].

Our findings suggested that laminin could effectively stimulate the migration of T98G glioma cells (Fig.1A). The difference between migration-activated and migration-restricted cells was not due to change in cell proliferation as the growth kinetics of T98G cells were unaffected by laminin over 24 hours compared with BSA (Fig.1B). Up-regulation of p-p27-Ser10 expression may be one mechanism through which cancer cells can acquire a migration phenotype, and Jab1 and CRM1 were also involved in this process (Fig.2). These results showed that p27 could alter the motility of cells, resulting in the ability of cell migration increase, and suggested that this was achieved through modulation of its phosphorylation at Ser10 (Fig.2) maybe by PI3-K/Akt signaling pathways (Supplementary Fig.S1) and its cytoplasmic mislocalization (Fig. 3).

Cell migration is a highly regulated process that is required for many aspects of normal and disease biology [13; 26; 27; 28]. In normal cells, the continuous formation and disassembly of focal adhesions is a tightly controlled, dynamic process essential for migration [30], and the deregulation of this process has been linked to tumor invasion [31]. Migration plays a key role in the invasion and dispersal of tumor cells, and proteins that regulate migration are often up-regulated in these cells [28].

It has been proposed that p27 may contribute to oncogenic progression through an increase in cell motility. Nagahara et al. [32] first demonstrated that transduction of TAT-p27 in HepG2 hepatocellular carcinoma cells promotes cell migration. The C-terminal portion of cytoplasmic p27 was subsequently found to be required for activation of migration, but the mechanism thereof remained unclear [10]. Besson et al. [9] showed that p27 and RhoA interact both in vitro and in RhoA-transfected cells, and p27 interferes with RhoA activation by impairing the interaction between RhoA and its activator, the guanosine-nucleotide exchange factor (GEF).

Cytoplasmic mislocation of p27 has been reported in many human tumors and is most commonly associated with adverse patient outcome. In up to 40% of primary human breast cancers, cytoplasmic p27 is seen in association with PKB/AKT activation. In human breast cancers, p27pT198 was detected exclusively in the cytoplasm [33; 34]. Cytoplasmic p27 was associated with poor prognosis in acute myelogenous leukemia (AML) [35] and in pancreatic [36], ovarian [37], prostate [38], and breast carcinomas[33]. The notion that excess cytoplasmic p27 may actively contribute to malignant progression is supported by observations that expression of cytoplasmic p27 (NESp27) increased cell motility in vitro and increased metastatic capacity in a melanoma xenograft model [39]. Expression of p27 ΔNLS, a mutant localized in the cytoplasm, decreased RhoA activation and increased cell motility in MCF-7 cells [40]. In knock-in animals, $p27^{CK-}$ localized in cytoplasm and enhanced cell proliferation and promoted tumorigenesis [41].

Because phosphorylation of p27 at Ser10 is necessary for both cytoplasmic localization and induction of cell migration, while p27 Ser10 point mutants: Ser-10-Ala, which prevents p27 from being phosphorylated at position 10, so we transient transfected T98G glioma cells with mock, a vector encoding EGFP or EGFP-p27S10A, the transfection efficiency was assessed by fluorescence microscope (Fig.4A, C). Compared with the mock or EGFP control, EGFP- p27S10A expressing

cells which showed a decreased ability to migrate (Fig.4B), to avoid physiological irrelevant levels of p27; we also used CCK-8 to measure cell proliferation (Fig.4D). Among cells which expressing EGFP or EGFP-p27S10A, there is no significant difference in their proliferation as evidenced by cell counting. To investigate whether p27 phosphorylated on Ser10 when cells plated on laminin was by PI3-K/Akt signaling pathway, we used PI3-K inhibitor, LY294002, test its effect on the phosphorylation p27 at Ser10 and cell migration, between the cells on BSA and the cells on laminin. Compared with the cells plated on BSA, LY294002 greatly inhibited the expression of phosphorylation of p27 at Ser10 and the migration of actively migrating T98G glioma cells when cells on laminin (Supplementary Fig.S2 and S3). Cell proliferations by itself could not have caused differences in migration content because plated cells on BSA did not express changes.

In summary, a model of migration-activated glioma cells was constructed by using the migration-stimulating substrate, laminin, in vitro and a sparse/dense paradigm as models. Experimental results in the present study were in agreement with the promoting effect of p27 on cell migration through regulating phosphorylation of p27 at Ser10 and cytoplasmic localization, accordingly induction of cell migration. Thus, the cytoplasmic role of p27 in promoting cell migration and the absence of tumor selection for complete loss of p27 begins to look like a gene living a dual life in cancer, one as a nuclear tumor suppressor and the other as a cytoplasmic metastatic oncogene. The use of a transient transfected overexpression of p27S10A in vivo and the development of gene site-specific mutagenesis therapy will be instrumental in furthering our understanding of the role of p27 in human malignant gliomas. In conclusion, p27 and its major phosphorylation site at Ser10 played a pivotal role in the migration of malignant glioma cells.

Acknowledgement. This work was supported by the National Natural Science Foundation of China (No.30300099, No.30770488 and No.30870320); Natural Science Foundation of Jiangsu province (No.BK2009156, No.BK2009157 and No.BK2009161); Special Research Grant (XK200723) for the Key Laboratory from the Department of Health, Jiangsu Province; Specialized Research Fund for the College and University Natural Scientific Research Program of Jiangsu Province (No. 07KJD310172).

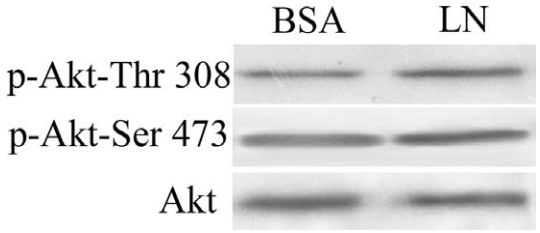

Fig.S1. Actively migrating cells which expressioned high phosphorylation of Akt. Phosphorylation of Akt relative to expression of total protein levels in BSA and laminin (LN), seeded T98G glioma cells assessed by immunoblotting (IB) with total or phospho-specific antibodies directed against Akt. Immunoblot shown is representative of three experiments with similar results.

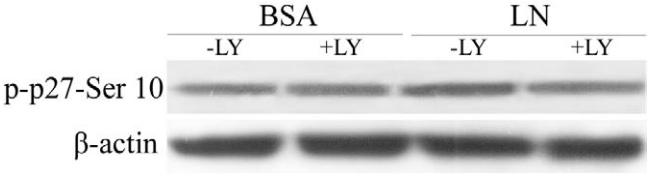

Fig.S2. Inhibition of PI3-K/Akt suppressed the expression of phosphorylation of p27 at Ser10 in actively migrating glioma cells. T98G glioma cells were grown to 70% confluence, serum starved overnight, then serum containing media with (+) or without (-) 50 μg/ml LY294002 was incubated for 24 h and cells were collected at the times indicated for western blot. Experiments were performed in triplicate and representative results were shown.

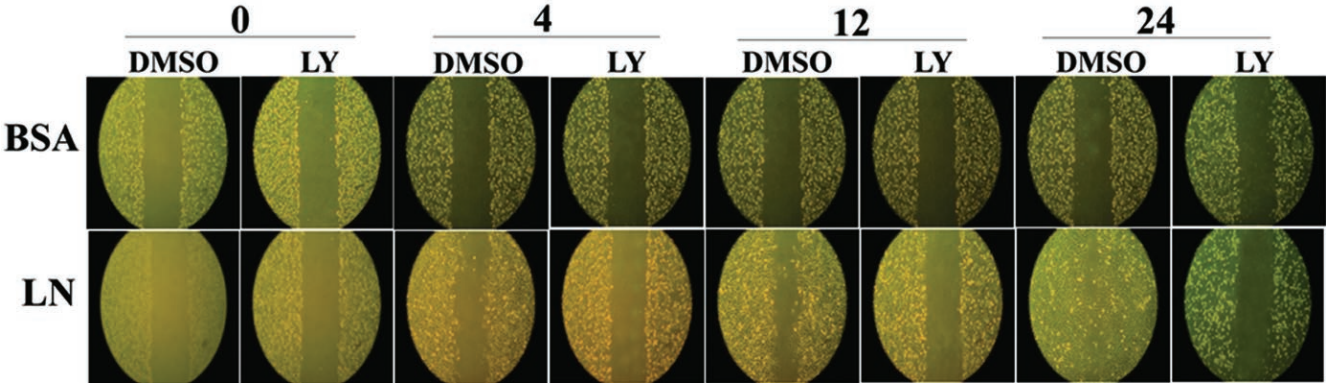

Fig.S3. Inhibition of PI3-K/Akt suppressed the migration of actively migrating glioma cells. T98G glioma cells were treated with DMSO or 50 μg/ml LY294002 and migration rate were obtained from 0 hours to 24 hours.

References

[1] DEMUTH T, RENNERT J, HOELZINGER D, REAVIE L, NAKADA M et al. Glioma cells on the run – the migratory transcriptome of 10 human glioma cell lines. BMC Genomics 2008; 9; 54–69. doi:10.1186/1471-2164-9-54

[2] STUPP R, HEGI M, GILBERT M, CHAKRAVARITY A Chemoradiotherapy in malignant glioma: standard of care and future directions. J Clin Oncol 2007; 25; 4127–4136. doi:10.1200/JCO.2007.11.8554

[3] SOFFIETTI R, LEONCINI B, RUDA R New developments in the treatment of malignant gliomas. Expert Rev Neurother 2007; 7; 1313–1326. doi:10.1586/14737175.7.10.1313

[4] HWANG J, SMITH C, SALHIA B, RUTKA J The role of fascin in the migration and invasiveness of malignant glioma cells. Neoplasia 2008; 10; 149–159. doi:10.1593/neo.07909

[5] DEMUTH T, BERENS M Molecular mechanisms of glioma cell migration and invasion. J Neurooncol 2004; 70; 217–228. doi:10.1007/s11060-004-2751-6

[6] JOY A, BEAUDRY C, TRAN N, PONCE F, HOLZ D et al. Migrating glioma cells activate the PI3-K pathway and display decreased susceptibility to apoptosis. J Cell Sci 2003; 116; 4409–4417. doi:10.1242/jcs.00712

[7] WANG X, LUI E, CAI Q, CHING W, LIU K et al. p27Kip1 promotes migration of metastatic hepatocellular carcinoma cells. Tumour Biol 2008; 29; 217–223. doi:10.1159/000152939

[8] BALDASSARRE G, BELLETTI B, NICOLOSO M, SCHIAPPACASSI M, VECCHOONE A et al p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. Cancer Cell 2005; 7; 51–63. doi:10.1016/j.ccr.2004.11.025

[9] BeESSON A, GURIAN-WEST M, SCHMIDT A, HALL A, ROBERTS J p27Kip1 modulates cell migration through the regulation of RhoA activation. Genes Dev 2004; 18; 862–876.

[10] MCALLISTER S, BECKER-HAPAK M, PINTUCCI G, PAGANO M, DOWDY S Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions. Mol Cell Biol 2003; 23; 216–228. doi:10.1128/MCB.23.1.216-228.2003

[11] GOUKASSIAN D, DIEZ-JUAN A, ASAHARA T, SCHRATZBERGER P, SILVER M et al. Overexpression of p27(Kip1) by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis. FASEB J 2001; 15; 1877–1885. doi:10.1096/fj.01-0065com

[12] J. SUN, S. O. MARX, H. J. CHEN, M. POON, A. R. Marks ey al. Role for p27(Kip1) in Vascular Smooth Muscle Cell Migration. Circulation 2001; 103; 2967–2972.

[13] RIDLEY A, SCHWARTZ M, BURRIDGE K, FIRTEL R, GINSBERG M et al. Cell migration: integrating signals from front to back. Science 2003; 302; 1704–1709. doi:10.1126/science.1092053

[14] NGUYEN L, BESSON A, HENG J, SCHUURMANS C, TEBOUL L et al. p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev 2006; 20; 1511–1524. doi:10.1101/gad.377106

[15] DANIEL C, PIPPIN J, SHANKLAND S, HUGO C The rapamycin derivative RAD inhibits mesangial cell migration through the CDK-inhibitor p27KIP1. Lab Invest 2004; 84; 588–596. doi:10.1038/labinvest.3700078

[16] CASSIMERIS L The oncoprotein 18/stathmin family of microtubule destabilizers. Curr Opin Cell Biol 2002; 14; 18–24. doi:10.1016/S0955-0674(01)00289-7

[17] SCHIAPPACASSI M, LOVAT F, CANZONIERI V, BELLETTI B, BERTON S et al. p27Kip1 expression inhibits glioblastoma growth, invasion, and tumor-induced neoangiogenesis. Mol Cancer Ther 2008; 7; 1164–1175. doi:10.1158/1535-7163.MCT-07-2154

[18] VALSTER A, TRAN N, NAKADA M, BERENS M, CHAN A etal. Cell migration and invasion assays. Methods 2005; 37; 208–215. doi:10.1016/j.ymeth.2005.08.001

[19] LENFERINK A, BUSSE D, FLANAGAN W, YAKES F, ARTEAGA C et al. ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1 through multiple signaling pathways. Cancer Res 2001; 61; 6583–6591.

[20] BESSON A, GURIAN-WEST M, CHEN X, KELLY-SPARTT K, KEMP C et al. A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression. Genes Dev 2006; 20; 47–64. doi:10.1101/gad.1384406

[21] ISHIDA N, HARA T, KAMURA T, YOSHIDA M, NAKAYAMA K et al. Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. J Biol Chem 2002; 277; 14355–14358. doi:10.1074/jbc.C100762200

[22] WANG Y, CHENG C, JI Y, ZHAO Y, ZOU L et al. Expression of Jun activation domain-binding protein 1 and Ser10 phosphorylated p27 protein in human epithelial ovarian carcinoma. J Cancer Res Clin Oncol 2009; 135; 951–959. doi:10.1007/s00432-008-0531-y

[23] KELLY-SPRATT KS, PHILIPP-STAHELI J, GURLEY KE, HOON-KIM K, KNOBLAUGH S et al. Inhibition of PI-3K restores nuclear p27Kip1 expression in a mouse model of K-ras driven lung cancer. Oncogene 2009; 28; 3652–3662 doi:10.1038/onc.2009.226

[24] GAVERT N, CONACCI-SORRELl M, GAST D, SCHNEIDER A, ALTEVOGT P et al. L1, a novel target of beta-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. J Cell Biol 2005; 168; 633–642. doi:10.1083/jcb.200408051

[25] LOUIS D, POSNER J, JACOBS T, KAPALN R Report of the Brain Tumor Progress Review Group; 2000.

[26] GUO W, GIANCOTTI F Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 2004; 5; 816–826. doi:10.1038/nrm1490

[27] BOGENRIEDER T, HERLYN M Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 2003; 22; 6524–6536. doi:10.1038/sj.onc.1206757

[28] HSU M, MEIER F, HERLYN M Melanoma development and progression: a conspiracy between tumor and host. Differentiation 2002; 70; 522–536. doi:10.1046/j.1432-0436.2002.700906.x

[29] LEVIN V, PHUPHANICH S, YUNG W, FORSYTH P, MAESTRO R et al. Randomized, double-blind, placebo-controlled trial of marimastat in glioblastoma multiforme patients follow-

ing surgery and irradiation. J Neurooncol 2006; 78; 295–302. doi:10.1007/s11060-005-9098-5

[30] WEBB D, DONAIS K, WHITMORE L, THOMAS S, TURNER C et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 2004; 6; 154–161. doi:10.1038/ncb1094

[31] CARRAGHER N, FRAME M Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. Trends Cell Biol 2004; 14; 241–249. doi:10.1016/j.tcb.2004.03.011

[32] NAGAHARA H, VOCERO-AKBANI A, SNYDER E, HO A, LATHAM D et al. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Med 1998; 4; 1449–1452. doi:10.1038/4042

[33] LARREA M, HONG F, WANDER S, SILVA T, HELFMAN D et al. RSK1 drives p27Kip1 phosphorylation at T198 to promote RhoA inhibition and increase cell motility. Proc Natl Acad Sci U S A 2009; 106; 9268–9273. doi:10.1073/pnas.0805057106

[34] KOSSATZ U, VERVOORTS J, NICKELEIT I, SUNDBERG H, ARTHUR J et al. C-terminal phosphorylation controls the stability and function of p27kip1. EMBO J 2006; 25; 5159–5170. doi:10.1038/sj.emboj.7601388

[35] MIN Y, CHEONG J, KIM J, EOM J, LEE S et al. Cytoplasmic mislocalization of p27Kip1 protein is associated with constitutive phosphorylation of Akt or protein kinase B and poor prognosis in acute myelogenous leukemia. Cancer Res 2004; 64; 5225–5231. doi:10.1158/0008-5472.CAN-04-0174

[36] FUKUMOTO A, IKEDA N, SHO M, TOMODA K, KANEHIRO H et al. Prognostic significance of localized p27Kip1 and potential role of Jab1/CSN5 in pancreatic cancer. Oncol Rep 2004; 11; 277–284.

[37] ROSEN D, YANG G, CAI K, BAST R, Jr. et al. Subcellular localization of p27kip1 expression predicts poor prognosis in human ovarian cancer. Clin Cancer Res 2005; 11; 632–637.

[38] LI R, WHEELER T, DAI H, SAYEEDUDDIN M, SCARDINO P et al. Biological correlates of p27 compartmental expression in prostate cancer. J Urol 2006; 175; 528–532. doi:10.1016/S0022-5347(05)00151-5

[39] DENICOURT C, SAENZ C, DATNOW B, X CUI, DOWDY S et al. Relocalized p27Kip1 tumor suppressor functions as a cytoplasmic metastatic oncogene in melanoma. Cancer Res 2007; 67; 9238–9243. doi:10.1158/0008-5472.CAN-07-1375

[40] WU F, WANG S, SANDERS M, SHIN I, ROJO F et al. Reduction of cytosolic p27(Kip1) inhibits cancer cell motility, survival, and tumorigenicity. Cancer Res 2006; 66; 2162–2172. doi:10.1158/0008-5472.CAN-05-3304

[41] BESSON A, HWANG H, CICERO S, DONOVAN S, GURIAN-WEST M et al. Discovery of an oncogenic activity in p27Kip1 that causes stem cell expansion and a multiple tumor phenotype. Genes Dev 2007; 21; 1731–1746. doi:10.1101/gad.1556607