Role of p27$^{\text{Kip1}}$ in Cell Proliferation and Motility during Oncogenic Transformation

Stefania Berton

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ROLE OF p27Kip1
IN CELL PROLIFERATION AND MOTILITY
DURING ONCOGENIC TRANSFORMATION
(Settore scientifico-disciplinare MED06)
| SUPERVISOR | Dr. Gustavo Baldassarre, MD  
Division of Experimental Oncology 2,  
National Cancer Institute (CRO)  
Via Franco Gallini, 2. 33081 Aviano, (PN), Italy  
Tel.: +39 0434 659759 (Office), +39 0434 659233 (Lab.)  
Fax: +39 0434 659428  
email: gbaldassarre@cro.it |
|---|---|
| EXTERNAL SUPERVISOR | Dr. Matthew Fero, Assistant Member,  
Clinical Research Division, D2-100  
Fred Hutchinson Cancer Research Center  
1100 Fairview Ave. N, Seattle, WA  98109  
Ph: (206) 667-5065 Lab: (206) 667-3333, Fax: (206) 667-1494  
email: mfero@fhcrc.org |
| PRESIDENTE EFFETTIVO | Prof.ssa Savoia Anna,  
Università degli Studi di Trieste,  
Dip. Scienze Riproduzione e Sviluppo  
IRCCS Burlo Garofano  
to dell’Istria 65/1, 34137 TRIESTE. |
| COMPONENTE EFFETTIVO | Prof.ssa Giordano Silvia  
Università degli Studi di Torino  
Dip. Scienze Oncologiche  
Strada Provinciale 142 di Plovesi, 10060 Candiolo (TO) |
| COMPONENTE EFFETTIVO | Prof. Brancolini Claudio  
Università degli Studi di Udine  
Dip. Scienze e Tecniche Biomediche  
Piazzale Kolbe 4, 33100 UDINE. |
| PRESIDENTE SUPPLENTE | Prof. Vittur Franco  
Università degli Studi di Trieste  
Dipartimento di Biochimica, Biofisica e chimica delle Macromolecole (BBCM),  
P.le Europa 1, 34127 TRIESTE. |
| COMPONENTE SUPPLENTE | Prof. Gustincich Stefano  
Scuola Superiore di Studi Avanzati di Trieste,  
Settore di Neurobiologia,  
Area Science Park  
SS 14, Km163.5 34012, Basovizza (TS) |
| COMPONENTE SUPPLENTE | Dott. Massimo Levrero  
Università degli Studi di Roma “La Sapienza”  
Dip. Medicina Interna, Regina Elena Cancer Institute,  
via delle Messi d’Oro 156, 00158 ROMA |
| COORDINATORE DEL CORSO DI DOTTORATO | Prof. Del Sal Giannino  
Università degli Studi di Trieste  
Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole (BBCM),  
LNCIB Area Science Park  
Padriciano 99 34012, Basovizza |
| DIRETTORE DEL DIPARTIMENTO DI RIFERIMENTO (BBCM) | Prof. Renato Gennaro  
Università degli Studi di Trieste  
Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole (BBCM),  
P.le Europa 1, 34127 TRIESTE |
A chi ogni giorno mi regala un sorriso...
A chi da una vita mi sostiene...
A chi mai mi fa mancare un abbraccio...
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abstract</strong></td>
<td>1</td>
</tr>
<tr>
<td>Papers included in the Thesis</td>
<td>2</td>
</tr>
<tr>
<td>Papers not directly included in the Thesis</td>
<td>3</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>4</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>5</td>
</tr>
<tr>
<td>1. Cell motility in 3D environment as a cue for metastatic process</td>
<td>5</td>
</tr>
<tr>
<td>2. The RAS-GTPases superfamily in actin and tubulin cytoskeleton</td>
<td>12</td>
</tr>
<tr>
<td>3. p27\textsuperscript{Kip1} and cell cycle: a finely designed picture</td>
<td>18</td>
</tr>
<tr>
<td>3.1 p27 and tumors: the utility of knock out model</td>
<td>23</td>
</tr>
<tr>
<td>3.2 p27 shows a role in cell migration</td>
<td>25</td>
</tr>
<tr>
<td>4. The oncogenic v-Src and its normal mammalian counterpart c-Src</td>
<td>28</td>
</tr>
<tr>
<td>4.1 Structure and regulation of Src</td>
<td>28</td>
</tr>
<tr>
<td>4.2 The v-Src transformed phenotype</td>
<td>30</td>
</tr>
<tr>
<td>4.3 Molecular mechanisms of Src functions</td>
<td>31</td>
</tr>
<tr>
<td>4.4 Src is altered in tumors</td>
<td>32</td>
</tr>
<tr>
<td><strong>AIM of the Study</strong></td>
<td>34</td>
</tr>
<tr>
<td><strong>Material and Methods</strong></td>
<td>35</td>
</tr>
<tr>
<td>1. Cell cultures and development of stable cell lines</td>
<td>35</td>
</tr>
<tr>
<td>2. Cell culture in 3D collagen lattices</td>
<td>35</td>
</tr>
<tr>
<td>3. Time-lapse microscopy and quantification of cell migration</td>
<td>35</td>
</tr>
<tr>
<td>4. Transwell-based migration assay</td>
<td>36</td>
</tr>
<tr>
<td>5. Matrigel\textsuperscript{TM} and Collagen I evasion assay</td>
<td>37</td>
</tr>
<tr>
<td>6. Cell adhesion assay</td>
<td>37</td>
</tr>
<tr>
<td>7. Preparation of cell lysates, immunoprecipitation and immunoblotting</td>
<td>38</td>
</tr>
<tr>
<td>8. Growth curve, MTT assay and FACS analysis</td>
<td>39</td>
</tr>
<tr>
<td>9. Kinase assay</td>
<td>39</td>
</tr>
<tr>
<td>10. Double thymidine block and nocodazole assay</td>
<td>40</td>
</tr>
</tbody>
</table>
11. Anchorage-independent cell growth ................................................. 40
12. In vivo analysis ............................................................................... 40
13. RNA extraction and RT-PCR from tissue samples .......................... 41
14. Immunofluorescence analysis ......................................................... 42
15. Statistical analysis ........................................................................ 42

RESULTS .............................................................................................. 43

1. p27⁻/⁻ v-src transformed cells exhibit higher proliferation potential than p27⁺/⁺ cells ................................................................. 43
2. p27 expression discriminates between mesenchymal and amoeboid morph-dynamics ................................................................. 49
3. The amoeboid motility of p27⁻/⁻ v-src cells is associated with higher cell speed and invasive ability ....................................................... 51
4. p27 expression in p27⁻/⁻ v-src cells reduced proliferation and tumor growth ................................................................. 57
5. p27¹¹⁸⁷A but not p27¹⁻¹⁷⁰ rescued cell shape and motility .................. 60
6. Migration rate and invasive potential were reduced after p27¹¹⁸⁷A expression ................................................................. 63
7. H-Ras transformation induces in p27⁻/⁻ the same proliferative advantage displayed after v-src over-expression ..................................... 67
8. p27⁻/⁻ fibroblasts displayed an increased cell motility also after H-Ras transformation ................................................................. 72
9. p27-rescue in p27⁻/⁻ H-Ras transformed cells: a more complicated scenario ................................................................. 73

DISCUSSION .......................................................................................... 77

1. p27 controls cell proliferation in transformed cell cultures and in vivo tumor growth ................................................................. 78
2. p27 influences 3D cellular morph-dynamics and in vivo tumor dissemination ................................................................. 79

Acknowledgements ............................................................................. 83

REFERENCES ....................................................................................... 84
ABSTRACT

In many human cancers, p27 down-regulation correlates to a worse prognosis suggesting that p27 levels could represent an important determinant in cell transformation and cancer development. Using a mouse model system based on \( v\text{-}src \)-induced transformation, we show that p27 absence is linked to a more aggressive phenotype, with an increased cell growth and motility. In 3D systems, transformed p27null fibroblasts shift from a mesenchymal spindle-like shape to a more rounded cell morphology, accompanied by amoeboid-like morphodynamics, membrane ruffling zones and loss of dendritic-like cell extensions. Importantly, the acquirement of amoeboid motility in p27\(^{-/-} \) transformed cells is associated with a higher ability to move in and colonize distant sites, \textit{in vivo}. The reintroduction of different p27 mutants in transformed p27null cells clearly demonstrates that the control of cell proliferation and motility by p27 represents two distinct functions, both necessary to fully act as a tumor suppressor. In fact, while the N-terminus region is necessary to block cell proliferation, p27 controls cell shape and motility through its C-terminus domain.

Interestingly, both the proliferative and motile advantage displayed by \( v\text{-}src \)-transformed p27\(^{-/-} \) cells highlighted also after transformation with the oncogene H-Ras, suggesting the existence and the involvement of common pathways responsible for p27 functions.

All together, our results demonstrate that p27 expression is an important determinant both in the first steps of cell transformation and tumor establishment as well as in the following progression to tumor dissemination and colonization of distant sites. Moreover, our study demonstrate for the first time that p27 can affect the motile and the invasive behavior by driving and regulating cell plasticity that, in turn, influences tumor cell morphology and movement.
During the Ph.D. course I worked on the role of the CKI p27\textsuperscript{kip1} in tumor cell growth and motility. In particular, I studied the role of p27 in cell proliferation, motility and invasion both \textit{in vitro} and in mouse models \textit{in vivo}. These studies led to the understanding that p27 was able to block not only cancer cell growth but also invasion and metastasis formation. In particular for the first time it was demonstrated that p27 can affect the motile and the invasive behavior by driving and regulating cell plasticity that, in turn, influences tumor cell morphology and movement. In this period, two papers describing most of the data presented in the PhD thesis are in preparation.

\textbf{Berton S}, Belletti B, Wolf K, Lovat F, Colombatti A, Friedl P, Baldassarre G. ”p27\textsuperscript{kip1} expression regulates the switch from mesenchymal to amoeboid motility in v-src transformed fibroblasts”. 2008. \textit{In preparation.}

Papers not directly included in the Thesis.


ABBREVIATIONS

ECM: extracellular matrix
2D: two-dimension/two-dimensional
3D: three-dimension/three-dimensional
MMPs: matrix metallo-proteinases
EMT: epithelial-mesenchymal transition
MAT: mesenchymal–amoeboid transition
GEFs: guanine nucleotide exchange factors
GAPs: GTPase-activating proteins
MTs: microtubules
MTOC: microtubule organizing centre
LOH: loss of heterozygosity
MEFs: mouse embryo fibroblasts
SFK: Src family kinase
RSV: Rous sarcoma virus
SH: SRC homology (domain)
*t: temperature sensitive
FATIMA: fluorescence-assisted transmigration assay
CAFCA: centrifugal assay for fluorescence-based cell adhesion
HRP: horseradish peroxidase
INTRODUCTION

1. Cell motility in 3D environment as a cue for metastatic process.

Tumor metastasis represents the most common cause of death in cancer patients. Metastasis is a multi-stage process involving cancer cell motility, intravasation, transit in the blood or lymphatic vessels, extravasation and growth at a new site (Fig1; Chambers et al., 2002; Sahai, 2007).

The acquisition of invasive behaviour is one of the first steps in the metastatic process and it is necessary to allow the cells to detach by primary tumor and enter the blood or lymphatic vasculature to spread to distant organs. Only a subset of tumour cells can overcome these diverse challenges, and therefore metastasis is generally an inefficient process (Wong et al., 2001). Tumor cells dissemination is strictly linked to their ability to attach to and move within the Extracellular Matrix (ECM).

Both in physiological and in pathological conditions cells migrate through three-dimensional (3D) environments, thus experimental systems that reproduce the 3D setting could well mimic the in vivo situation. Further, using 3D experimental model systems it has been revealed that
exists a higher complexity in the cell migration and in the adaptation responses in 3D compared to the classical 2D environment (Friedl and Wolf, 2003; Wolf and Friedl, 2006; Sahai and Marshall, 2003; Cukierman et al., 2001). For example, the adhesive mechanisms are quite different in cells that move on the ECM substrates (2D) or through ECM lattices (3D). In fact, fully mature focal contacts have only been observed in cells that are firmly attached to 2D substrates, whereas when cells are placed in 3D substrates, integrins tend to cluster in less assembled focal interactions (Friedl and Wolf, 2003). Moreover, the speed and character of cell motility in vivo is quite different from that normally observed on 2D substrates in vitro (Condeelis and Segall, 2003). In vivo in fact, cancer cells move at high speeds (up to 15µm a minute) and can change shape and direction rapidly, whereas, in 2D cultures, the more adhesive interactions reduce the migration speed and the possibility to switch between different migration mechanisms (Condeelis and Segall, 2003).

A five-step model of cell migration in 2D was well established over the past 30 years (Lauffenburger and Horwitz, 1996) but this model does not perfectly fit with 3D-motility. For example, when a cell moves in a 3D environments an additional important feature is the proteolytic remodelling of the ECM (Friedl and Wolf, 2003).

The first step in 3D migration is represented by the protrusion formation at the leading edge. For this process it is necessary that the actin polymerizes by coupling to the actin-nucleating ARP2/3 complex and the multifunctional adaptor protein WASP. The ARP2/3-WASP complex is linked to the inner leaflet of the plasma membrane via clustered phosphoinosites and here can interact with actin filaments and induce their branching. During protrusion formation, the integrin receptors come in contact with ECM ligands and cluster in plasma membrane. Clustered integrins recruit adaptor and signaling proteins via their intracellular domains, thereby inducing phosphorylation and dephosphorylation signals into the cell and stabilizing focal contacts.

The assembly of focal contacts is also directly or indirectly induced by several signaling pathways, such as PI3K, PKC and RhoGTPases. Before and while focal contacts develop, actin filaments locally elongated and assemble, through the action of crosslinking proteins, such as α-actinin and myosin II. The branched actin networks below the inner leaflet of plasma membrane are termed cortical actin, whereas cytoplasmic bundles and elongated cables of actin filaments are termed stress fibres. After the focal contact formation, surface proteases become concentrated near substrate binding sites. Here proteases cleave ECM components, such as collagen, fibronectin, laminins and pro-MMPs obtaining active soluble MMPs and inducing a focalized proteolysis of the matrix.
Introduction

The fourth step is represented by cell contraction induced by the actin-myosin II binding. Active myosin II binds to actin filaments and generates actomyosin contraction that, in turn, promotes the shortening of cell’s length axis and generates inward tension. By several and not completely understood mechanisms, cell substrate linkages resolve preferentially in the back of the cells, whereas the leading edge remains attached to the ECM. Myosin II is activated by myosin light chain (MCL) that in turn is phosphorylated by the myosin light chain kinase. On the contrary, the MLC phosphatase, that is inhibited by the Rho effector ROCK, dephosphorylates MLC thus inhibiting actomyosin contraction. Following focal contact disassembly, the trailing edge, together with the cell body and the nucleus, slowly glide forward (Friedl and Wolf, 2003).

*In vitro* and *in vivo* observations have shown that tumor cells possess a broad spectrum of migration mechanisms to infiltrate the surrounding tissue matrices (Friedl and Wolf, 2003; Friedl 2004). They can disseminate as single cells, referred to as “individual cell migration” or expand in solid cell strands, sheets, files or clusters, called “collective migration” (Friedl and Wolf, 2003).

In many tumors, both single cells and collectives are simultaneously present. Whereas leukemias, lymphomas and most solid stromal tumors, such as sarcomas, disseminate via single cells, epithelial tumors commonly use collective migration mechanisms (Friedl and Wolf, 2003). Between the so called “individual migration”, the mesenchymal and the amoeboid mechanisms are the most known. Mesenchymal migration, typical of fibroblasts, is mainly found in cells from connective tissue-tumors, such as fibrosarcomas, gliomas and de-differentiated epithelial cancers and it is characterized by a fibroblast-like spindle shape (Wolf et al., 2003; Friedl and Wolf, 2003). It is dependent on integrin-mediated adhesion dynamics and on proteases focalization and it is characterized by a relative low velocity (Friedl and Wolf, 2003).

The amoeboid motility is a less adhesive mechanism and it is integrin independent. The mechanism is propulsive and the cells, highly deformable, are able to circumnavigate, rather than to degrade, the ECM barriers, moving faster than cells that use mesenchymal strategy (Table1; Friedl and Wolf, 2003; Wyckoff et al., 2006).
Mesenchymal and amoeboid migration are also characterized by a different involvement of small GTPases of the Rho family, even if many aspects are still not completely elucidated. High RhoA and Rho kinase (ROCK) activity seems associated with amoeboid motility, while mesenchymal migration needs localized RhoA degradation at the leading edge and high Rac activity to promote the extension of cellular protrusions (Sahai and Marshall, 2003; Webb and Horwitz, 2003). Further, it was demonstrated that the activity of calpain2 and the Src family kinases (which normally act upstream of calpain2 to induce integrin adhesion turnover) are important determinants for an optimal mesenchymal tumor invasion through fibrillar collagen or Matrigel matrix. Conversely, amoeboid tumor invasion results relatively insensitive to inhibition of these two molecular enzymes (Carragher et al., 2006).

Under certain circumstances, cancer cells can switch from a migration mechanism to another. This phenomenon could compensate for the loss of a particular motile ability and allows the cells to develop migratory escape strategies. Such adaptation responses is known as “plasticity” or “transition” (Friedl and Wolf, 2003).

The most well known example of changes in cancer cell migration is the epithelial-mesenchymal transition (EMT). Following the carcinoma progression and de-differentiation, epithelial cells switch from a collective invasion pattern towards a detached and disseminated cell migration mechanism. In this meaning, EMT represents an example of phenotypic and functional plasticity that spontaneously occurs during the course of tumor progression (Friedl and Wolf, 2003).
Moreover, cancer cells can also undergo conversion from a mesenchymal towards an amoeboid cell type, which is termed the mesenchymal–amoeboid transition (MAT) (Fig2). This transition is not only accompanied by a change in cell morphology (from fibroblast-like spindle-shaped towards rounded and elliptoid), but also results in altered integrin distribution, organization of the actin cytoskeleton, and changes in molecular strategies to overcome tissue barriers (Friedl and Wolf, 2003). Several factors can lead to MAT, including abrogation of pericellular proteolysis by protease inhibitors, weakening of cell–ECM linkages, and alterations in the RHO signalling pathways (Fig2; Friedl and Wolf, 2003).

The MAT was first observed in HT-1080 fibrosarcoma and MDA-MB-231 mammary carcinoma cells after abrogation of pericellular proteolysis (Wolf et al., 2003). In 3D collagen matrices, in fact, highly invasive and metastatic HT1080 and MDA-MB-231 cancer cells use a mesenchymal migration strategy, but, after pharmacological inhibition of MMPs, they don’t cease to move neither become entrapped in the matrix. Conversely, cells acquire a protease-independent migration that is characterized by cell alignment along pre-existing fibre strands, shape change and the ability to squeeze through narrow matrix regions (Wolf et al., 2003).

More recently also an increased RhoA activity has been associated to the MAT both in vitro and in vivo (Sahai et al., 2007; Gadea et al., 2007). It was demonstrated that Smurf1 protein, through the regulation of peripheral RhoA–ROCK–MLC2 signaling, can regulate in vitro and in vivo tumor cell motility and invasion in BE colon carcinoma cells and MDA-MB-231 (Sahai et al., 2007). Smurf1, which is an E3-ubiquitin ligase, induces RhoA proteasome degradation at the leading edge, favouring the correct extension of cellular protrusions and the establishment of cell polarity (Wang et al., 2003; Sahai et al., 2007). Thus Smurf1 inhibition induces the transition from the “path generating” mesenchymal to the “path finding”
amoeboid migration that is associated to high activities of Rho, ROCK, and MLC2 necessary to generate cortical contractile forces used for matrix deformation (Sahai et al., 2007). A recent work has shown also how p53 deficiency in mouse embryonic fibroblasts cultured in 3D matrices induces a switch from an elongated spindle morphology to a markedly spherical and flexible one associated with highly dynamic membrane blebs (Gadea et al., 2007).

In a recent study, it was shown that the non-metastatic breast cancer cells had a much more elongated morphology than their metastatic counterparts and, interestingly, conversion of cells that normally move with an elongated morphology to a more rounded morphology increased both their ability to move and to enter tumor vessels (Wyckoff et al., 2006). The authors showed that amoeboid tumor cells generate sufficient actomyosin force to deform collagen fibers and are able to push through the ECM. This study suggests that tumor cells with an elongated morphology may need to adopt a more rounded shape, characterized by the increased cortical acto-myosin contraction, to efficiently intravasate within blood vessels and withstand greater mechanical stress (Wyckoff et al., 2006).

Following introduction into the circulation, tumor cells rapidly lodge in capillaries (Fig1). Several different mechanisms for this arrest have been described, although their respective relevance to human disease is unclear. For example, the arrest can simply be caused by the large physical size of tumor cells relative to the capillary lumen or alternatively, tumor cells can actively adhere to endothelia even when not restricted by the diameter of the vessel (Sahai, 2007). After the attachment of tumor cells to vessel walls, leukocytes may be recruited, and they are thought to be the first cells to extravasate and cancer cells following them. It was described that extravasation typically occurs a few hours after attachment to the vessel wall; cell protrusions and deformation of the nucleus has been observed crossing an endothelium, implying that it is an active process (Tsuji et al., 2006). It is likely that many of the same molecular mechanisms required for cell mobilization from the primary tumour will be used for extravasation, but the actin remodelling and protease function have not been investigated in detail in this step of metastatic process (Sahai, 2007).

An important factor that determines the cell attachment and extravasation is the shear stress – the physical force exerted on cells in the vessels as a result of blood flow. Non-metastatic cells are generally more susceptible to shear stress and they are rapidly sheared and detached. Of course, the entity of the shear stress can vary considerably between tissues; for example, blood pressure in liver capillaries is low, whereas it is much higher in muscle, and the morphologies of tumor cells arresting at these locations are correspondingly different (Sahai,
However, a causal link between local blood pressure in the different organs and metastatic efficiency remains to be clarified.

Once tumor cells arrived at the secondary sites, most of them undergo apoptosis within 24 hours (Fig1; Chambers et al., 2002). Non-metastatic cells are more prone to apoptosis than their metastatic counterparts, indicating that increased cell survival at this stage of the metastatic process correlates with overall metastatic capability (Kim et al., 2004). Some oncogenes that promote cell proliferation and survival in the primary tumor are also shown to be involved in promoting the survival of cancer cells at metastatic sites. For example, the overexpression of BCL2 or active HRAS increased the survival and proliferation of cells that had lodged within lungs and the liver, respectively (Wong et al, 2001). Moreover, various external factors contribute to reduced tumor cell survival at metastatic sites. For example, the lack of the normal pro-survival adhesion signals present in the tissue of origin of the cancer cells may lead to anoikis, the so called apoptosis induced by lack of attachment to a substrate, or tumor cells may be cleared by immune cells (Sahai, 2007). Not all the cells that survive at secondary sites will begin to proliferate because not all tumor cells have the same replicative potential. A new model suggests that only when the so called 'cancer stem cell' (described as a cell that is able to self-renew and to divide giving rise to another malignant cell) arrives at a secondary site, macroscopic metastasis are formed (Bjerkvig et al., 2005). In all other cases there will be limited or no proliferation of the disseminated cells.

Even if many aspects remain to understand, altogether the data collected and the advances in microscopy and imaging probes allowed us to dissect more in detail the metastatic process, till now considered a 'black box' research.
2. The RAS-GTPases superfamily in actin and tubulin cytoskeleton.

GTPases are molecular switches that control a wide variety of signal transduction pathways in all eukaryotic cells. They are known mainly for their pivotal role in regulating the actin cytoskeleton, but in the last decade many studies have revealed their ability to influence also cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity. Being GTPase enzymes, they use a simple biochemical strategy to control complex cellular processes. They cycle between two conformational states: one bound to GTP ('active' state), the other bound to GDP ('inactive' state) and they are able to hydrolyze GTP to GDP. In the 'on' (GTP) state, GTPases recognize target proteins and generate a response until GTP hydrolysis returns the switch to the 'off' state (Fig3). The RAS-GTPase superfamily is the most known family of these enzymes and mammalian cells contain several hundred GTPase switches. These small, monomeric GTPases fall into five major groups: Rho, Ras, Rab, Arf and Ran (Etienne-Manneville and Hall, 2002).

![Figure3: The RhoGTPases cycle.](image)

Rho activity is very carefully regulated and, for this family, in the human genome over 60 activators (GEFs) and over 70 inactivators (GAPs) are expressed. The Rho gene was identified in 1985, but only in 1992 some observations provided the first insights into the cellular function of Rho GTPases. Constitutively activated mutants of Rho and Rac (a member of the family) were found to induce respectively the assembly of contractile actin and
myosin filaments (stress fibers) and actin-rich surface protrusions (lamellipodia) when introduced into fibroblasts (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1999). Later, Cdc42, another member of this family, was shown to promote the formation of actin-rich, finger-like membrane extensions (filopodia) (Nobes and Hall, 1995; Nobes and Hall, 1999). The conclusion that Rho, Rac and Cdc42 regulate three separate signal transduction pathways linking plasma membrane receptors to the assembly of distinct actin structures has been confirmed in a wide variety of mammalian cell types as well as in yeast, flies and worms (Etienne-Manneville and Hall, 2002). But how these proteins work together to regulate cell migration is not so well defined and the picture appears more complicated.

It has become a dogma that Rac and Cdc42 drive protrusion at the front of the cell, whereas Rho drives contraction at the rear and that there is an antagonistic relationship between Rac and Rho, with the two proteins suppressing each other's activities and phenotypes (Burridge and Doughman, 2006). This, however, has turned out to be a too simple model in some cases. For example, although Rac and Cdc42 promote actin polymerization and extension of the leading edge of cells, Rho can also promote actin polymerization through its interaction with the protein mDia, and this can contribute to membrane extension (Higashida et al., 2004). In particular, a localized RhoA activation is required for the induction of membrane ruffling, the induction of which is a typical phenotype of activated Rac (Kurokawa and Matsuda, 2005). Nevertheless, the activity of Rho downstream effector ROCK has been shown to suppress cell protrusion in a variety of cells (Tsuji et al., 2002; Worthylake and Burridge, 2003; Xu et al., 2003). Altogether these effectors and their spatial and temporal activation are required for cell polarization during migration (Ridley et al., 2003). Although localized Rac-induced actin polymerization is considered the driving force, migration may be facilitated by other cellular activities. The microtubule cytoskeleton, for example, is highly polarized during migration. Microtubules are polymers composed of α/β tubulin heterodimers that continuously switch between phases of polymerization and depolymerization, a property known as dynamic instability (Rubin and Atweh, 2004). The transition from a phase of growth to a phase of shrinkage is called ‘catastrophe’, while the transition from a phase of shrinkage to a phase of growth is called ‘rescue’. In interphase, microtubules are long and relatively stable and their dynamics of growth and shrinkage are relatively slow. In contrast, at the onset of mitosis, when the interphase arrays of microtubules depolymerize and then repolymerize to assemble the mitotic spindle, microtubules are highly dynamic as a result of a marked increase in their rate of catastrophe (Rubin and Atweh, 2004).
The most striking polarization of the microtubule cytoskeleton in many migrating cells is the orientation of the centrosome, the organizing centre of the radial interphase microtubule network (MTOC), either in front of or behind the nucleus, with respect to the direction of migration and respect to the cell type (Fig4; Yvon et al., 2002; Etienne-Manneville and Hall, 2002). Partly as a result of centrosome position, microtubules themselves are polarized and tend to be aligned along the axis of cell migration; many of them, particularly stabilized, detyrosinated microtubules, are preferentially oriented with their plus ends facing the leading edge (Fig4; Wittmann and Waterman-Storer, 2001). Many observations have suggested that microtubules are required to establish cell polarity during motility. For example fibroblasts require an intact microtubule cytoskeleton to maintain their polarization and, in neurons, it is fundamental to axonal growth (Wittmann and Waterman-Storer, 2001).

There are three major hypotheses suggesting how microtubules contribute to cell polarity and migration. First, microtubules could serve as tracks for directed membrane and organelle transport towards the leading edge of the cell providing building material for the protruding lamellipodium. Second, growing microtubules could directly promote lamellipodial protrusion and stabilize the leading edge to maintain a directed movement of the cell. Third, microtubules do not regulate protrusion but instead locally regulate adhesion and contraction. But besides these hypothesis, direct evidence suggests that Rho GTPases might also influence the organization and dynamics of microtubules and, conversely, a microtubule-dependent regulation of Rho GTPases exists (Wittmann and Waterman-Storer, 2001).
depolymerization of microtubules induces formation of contractile actin bundles and focal adhesions and increases the level of GTP-bound RhoA, whereas the induction of microtubule polymerization leads to lamellipodia formation and activation of Rac1 (Wittmann and Waterman-Storer, 2001). Moreover, Rho (acting through p160Rho kinase and mDia) promotes the accumulation of detyrosinated microtubules, Rac (acting through p65Pak) inactivates the microtubule destabilizing protein, stathmin and Cdc42 regulate the orientation of the MTOC (Etienne-Manneville and Hall, 2002).

Besides the RhoGTPase family, with RhoA, Rac1 and Cdc42 as most studied members, RasGTPase proteins and their signaling pathways have been extensively studied. The RasGTPases were discovered as proteins encoded by retroviral oncogenes that had been hijacked from the host genome by the Kirsten (K-RAS) and Harvey (H-RAS) rat sarcoma viruses. The prototypical RAS GTPase proteins -H-RAS, N-RAS and K-RAS- were so identified as the products of active oncogenes in human tumours, even if they primary contribute to cell-cycle regulation in normal, non-transformed cells. H-RAS, N-RAS and K-RAS are highly related proteins, sharing approximately 84% identity, and their divergence being almost exclusively confined to the carboxyl terminus. Although there might be differences in the signalling pathways that they activate and in their subcellular distribution, these three proteins show similar functions. Ras GTPases are targeted to endosomes, Golgi stacks, the plasma membrane and mitochondria, where they are constitutively associated by means of farnesylation and palmitoylation (Ten Klooster and Hordijk, 2007).

Human tumours very frequently express RAS proteins that have been activated by point mutation: 30% of all tumours have undergone an activating mutation in one of the RAS genes, with K-Ras mutations almost ubiquitous in pancreatic tumors and common in lung and colorectal cancer, H-Ras mutations has been found in bladder and kidney cancer and N-Ras mutations is common in leukemias (Table2; Bos, 1989; Downward, 2003).

**Table2: Activation of RAS pathway in different tumors (Modified from Downward, 2003)**
In these tumours, the activated RAS protein contributes significantly to several aspects of the malignant phenotype, including the deregulation of tumour-cell growth, programmed cell death and invasiveness, and the ability to induce new blood-vessel formation (Shields et al., 2000).

Early studies established the central position of the prototypical RAS GTPases in cell-cycle regulation. Microinjection of RAS-neutralizing antibodies, or introduction of a dominant-negative form of RAS, blocked growth-factor-induced S-phase entry (Mulcahy et al., 1985; Stacey et al., 1991). Conversely, quiescent non-proliferating cells could be pushed back into the cell cycle by the microinjection of oncogenic H-Ras protein, independently of growth-factor stimulation (Feramisco et al., 1984). Downstream of RAS GTPases, the cell-cycle regulation is mediated by a three-component mitogen-activated protein kinase (MAPK) cascade, consisting of the RAF, MEK (MAPK and extracellular signal-regulated kinase (ERK) kinase) and ERK/MAPK proteins. In addition, the phosphatidylinositol 3-kinases (PI3Ks) and the RAL-activating RALGDS proteins also signal downstream of RAS.

The main function of RAS in G1–S-phase progression is to inactivate RB and relieve cells from its growth-inhibitory actions: both the ERK/MAPK and the PI3K pathways make key contributions to RB inactivation. Moreover, RAS signalling elevates cyclin-D1 levels by influencing the transcription, translation and protein stability of cyclin D1 and induced down-regulation of p27 CKI expression through transcriptional and post-transcriptional mechanisms in a mitogen-dependent way. Ras, via the PI3K pathway, regulates p27 expression by inactivating members of the forkhead transcription-factor family (Medema et al., 2000). PI3K pathway is also involved in the proteasome-mediated degradation of p27, possibly through the transcriptional induction of Skp2, the F-box protein that specifically recognizes p27 (Mamillapalli et al., 2001). RAS-mediated activation of the RAF-MEK-ERK/MAPK pathway, moreover, reduces p27 levels through enhanced proteolysis and decreased protein
synthesis both in a CDK2 dependent and independent way (Delmas et al., 2001; Rivard et al., 1999).

Ras signaling pathways are well known for their involvement in tumour initiation, but less is known about their contribution to invasion and metastasis. Most models of cell motility place Ras proteins as upstream regulators of Rho family proteins, controlling the activation states of RhoA, Rac1, and cdc42 in response to growth factor or cytokine stimulation (Bar-Sagi and Hall, 2000). For example, K-Ras, more efficiently than H-Ras, is able to stimulate both cell motility and Rac1 activation in fibroblasts (Walsh and Bar-Sagi, 2001). Activation of a Ras-Raf-MAPK cascade was also shown to stimulate motility in fibroblasts through calpain activation and subsequent rear detachment (Glading et al., 2000). In COS cells, Ras mediated downstream activation of MAP kinase cascade, was shown to be necessary for cell motility, at least in part through its effects on myosin light chain phosphorylation status (Klemke et al., 1997; Cheresh et al., 1999). In breast epithelial cells, activated R-Ras was shown to stimulate migration in a PI3K and PKC dependent way (Keely et al., 1999). Moreover, the Ras-activated Raf-MEK-ERK pathway can specifically control the expression of integrin receptors in a variety of human and murine cell lines, inducing changes in cell adhesion and migration (Woods et al., 2001). Ras can also control cell motility by regulating focal adhesions and stress fibres formation and it is seems to be involved in the epithelial-mesenchymal transition in a variety of cell lines (Nobes and Hall, 1999; Huber et al., 2005). Moreover, in some Ras-transformed cancer cells, including epithelial cancer and transformed fibroblasts, lack of stress fibre formation has been correlated to the Rac-dependent induction of an amoeboid-like behaviour, accompanied by high migration velocity both in vitro and in vivo (Friedl and Wolf, 2003).

As suggested by these data and given the growing number of Ras effectors, it is not surprising that Ras can regulate cell motility interfering with many downstream pathways and using a variety of mechanisms, that only in part are elucidated (Oxford and Theodorescu, 2003).
3. p27Kip1 and cell cycle: a finely designed picture.

Besides cell migration and motility, ECM is essential also for cell survival and proliferation. The cell-ECM contact induces the activation of several signaling pathways that control cell cycle progression (Giancotti and Ruoslahti, 1999). The mitotic cell cycle is a tightly regulated universal process that ensures the correct division of one cell into two daughter cells and that underlies the growth and development of all living organisms. To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one (Hartwell and Weinert, 1989). The progression along the different phases of cell cycle is positively regulated by the sequential activation of the so called cyclin dependent kinases (CDKs), evolutionarily conserved serine-threonine kinases. CDKs are activated along the different phases by associating with their regulatory partner member of the cyclin family. Conversely, their activity is counteracted by small proteins known as CDK inhibitors (CKIs). Two different families of CKIs exist, the INK4 and the Cip/Kip proteins. The INK4 (Inhibitors of CDK4) family includes p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d}. All these members exert their inhibitory activity by binding to the CDK4 and CDK6 kinases and preventing their association with D-type cyclins. INK4 members are responsible for G1 cell cycle arrest and can block proliferation only through a functional pRB pathway. The Cip/Kip family includes p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}, all characterized by the presence of a conserved N-terminal region containing the cyclin-CDK binding domain, whereby they interact with the regulatory and catalytic subunit of every complex (Fig 5, Belletti et al., 2005).

Figure 5: Schematic representation of cell cycle regulation. (from Belletti et al., 2005)
Among these inhibitors, p27\textsuperscript{Kip1} (hereafter p27) is well known for its role in G1-S transition. It interacts with and inhibits cyclinE-CDK2 and cyclinA-CDK2 activity, blocking cell cycle progression. The crystal structure of the human p27 bound to the phosphorylated cyclinA-CDK2 complex revealed that p27 binds the complex as an extended structure interacting with both cyclin A and Cdk2 (Russo, 1996). On cyclin A, it binds in a groove formed by conserved cyclin box residues. On Cdk2, it binds and rearranges the amino-terminal lobe and also inserts into the catalytic cleft, mimicking the ATP (Russo, 1996).

The human p27 gene (\textit{CDKN1B}) resides in a region of chromosome 12p13 and contains two coding and one non coding exons. The murine \textit{cdkn1b} gene is located in a syntenic region on distal chromosome 6 and is similar to the human p27 gene. The cDNA sequence is more than 90% homologous to the human p27 cDNA (Philipp-Staheli et al., 2001). p27 sequence contains 198 aminoacids (197 in mouse) and presents some regulatory domains. From the residue 153 to 169 is present a nuclear localization signals, while a leucine rich nuclear export signal, responsible for p27 shuttling from nucleus to cytoplasm, is localized between aminoacids 32-45.

p27 expression in normal cultured cells is finely regulated and high levels of the protein induce the arrest in G1 phase and cells accumulation in G0. p27 levels increase in response to various stimuli that inhibit cell proliferation, such as cell-cell contact, loss of adhesion to extracellular matrix, induction of differentiation or TGFβ, INF-γ, c-AMP, rapamicin and lovastatin treatments (Belletti et al., 2005). The exit from the quiescent status requires the down-regulation of p27, which in turn results in CDKs activation. The enforced expression of p27 in tumor cells could also result in apoptotic cell death, probably due to the conflicting signals between proliferation and block of cell cycle progression.

p27 is mainly regulated at post-translational level. In contrast, p27 mRNA is usually constant through out the whole cell cycle. p27 could be regulated by sequestering into higher order complexes with cyclinD-CDK4 after activation of the MAPK pathway, that promotes cyclin D transcription (Cheng et al., 1998; Susaki et al., 2007). Also the proto-oncogene c-Myc, by increasing the expression of cyclin D and cyclin E, is responsible for p27 sequestration and this molecular event appears essential for Myc-induced cell cycle progression (Vlach et al., 1996).

Moreover, p27 can be displaced in the cytoplasm, with consequent progression in cell cycle, after activation of the Raf-Mek-Erk and PI3K-Akt pathways in a phosphorylation-dependent manner (described afterwards in detail. Philipp-Staheli et al., 2001).
But the most important way for p27 regulation seems to be its ubiquitin-proteasome dependent degradation (Fig6).

p27 proteolysis is cytoplasmatic and phosphorylation-independent in early G1, but it is nuclear and phosphorylation-dependent in late S-G2 phase (Fig7). The G0-G1 degradation is mediated by the KPC complex (Kip1 ubiquitination-promoting complex), consisting of KPC1 and KPC2 proteins, that interacts with and ubiquitinates p27 in the cytoplasm (Kamura et al., 2004; Kotoshiba et al., 2005). The nuclear export of p27 by CRM1 is necessary for KPC-mediated proteolysis and the recognition by CRM1 needs p27 phosphorylation on Ser10 (Kamura et al., 2004; Boehm et al., 2002; Ishida et al., 2002). Recently a role of cyclin D2 in p27 translocation from the nucleus into the cytoplasm for its KPC-dependent degradation was demonstrated (Susaki et al., 2007)

Instead, the S-G2 degradation is mediated by an SCF ubiquitin ligase, composed by Skp1, a cullin subunit called Cull, Rbx1/Roc1 and the F-box protein Skp2 that specifically recognizes p27 (Hara et al., 2001; Zeng et al., 2002). Skp2 binds to p27 and promotes its degradation.
only when p27 is phosphorylated on the conserved Thr187 by cyclinE-CDK2 or cyclinA-CDK2 complexes (Montagnoli et al., 1999; Carrano et al., 1999).

The kinases that determine p27 phosphorylation status are several and differently regulated (Fig8). The phosphorylation at S10 is mediated by the kinase interacting stathmin (hKIS), a nuclear protein that binds the C-terminal domain of p27 and phosphorylates it at S10 in vitro and in vivo, promoting its nuclear export to the cytoplasm. hKIS is activated by mitogens during G0/G1, and expression of hKIS overcomes growth arrest induced by p27 (Boehm et al., 2002). The involvement of mitogenic signals have suggested the hypothesis that also the MAPK pathway could promote p27 phosphorylation at S10 (Rodier et al., 2001). Moreover recently it was described also a role of Akt in this phosphorylation (Nacusi and Sheaff, 2006). The T187 of p27 is phosphorylated by the cyclinE-CDK2 and cyclinA-CDK2, thus a trimeric complex is necessary for p27 ubiquitination and degradation (Montagnoli et al., 1999). Other important phosphorylation sites of p27 are the T157 (not conserved in mouse) and the T198 (T197 in mouse), both regulated by Akt (Motti et al., 2004; Motti et al., 2005). The T157 residue maps within the nuclear localization signal of p27 and the Akt-induced T157

Figure7: Mechanisms regulating p27 ubiquitin-dependent degradation (From Belletti et al., 2005).
phosphorylation causes retention of p27 in the cytoplasm, precluding p27-induced G1 arrest (Viglietto et al., 2002; Shin et al., 2005). Also the PI3K/Akt dependent phosphorylation at T198 was initially described to contribute to p27 cytoplasmic mislocalization, since, once phosphorylated, it is recognized by the 14-3-3 proteins family and retained in the cytoplasm (Fujita et al., 2002; Motti et al., 2004). Moreover, it has been demonstrated that phosphorylation at T198 is able to regulate p27 stability (Kossatz et al., 2006). Phosphorylation of p27 at T198 prevents ubiquitin-dependent degradation of free p27 and controls progression through the G1 phase by regulating the association of p27 with cyclin-CDK complexes (Kossatz et al., 2006).

Recently, it has been shown that the growth-state-dependent tyrosine phosphorylation of p27 modulates its inhibitory activity in vivo (James et al., 2008). In fact, based on the observation that p27 bound to cyclinD-CDK4 can be both inhibitory and non-inhibitory, it was demonstrated that this behaviour is due to p27 growth-state-dependent tyrosine phosphorylation (James et al., 2008). In particular, two tyrosine residues (Y88 and Y89) in p27's CDK interaction domain are phosphorylated preferentially in proliferating cells, converting p27 to a non-inhibitor state (James et al., 2008). The residue Y88 can be phosphorylated by the Src-family kinase Lyn and the oncogene product BCR-ABL (Grimmler et al., 2007). It was reported that Y88-phosphorylated p27 is also efficiently phosphorylated on T187 by Cdk2 and in turn its SCF-Skp2-dependent degradation is promoted, suggesting an explanation for premature p27 elimination in cells transformed by activated tyrosine kinases (Grimmler et al., 2007).Besides the phosphorylation at Y88, the oncogenic kinase Src regulates p27 stability through its phosphorylation at tyrosine 74 and, in a less degree, at tyrosine 89: also in this case, the phosphorylation facilitates p27 proteolysis (Fig8; Chu et al., 2007).

![Diagram showing regulatory phosphorylation sites in p27 aminoacidic sequence.](image-url)

**Figure8**: Regulatory phosphorylation sites in p27 aminoacidic sequence.
3.1. p27 and tumors: the utility of knock out model.

Even though p27 has shown a new "dark side" displaying oncogenic functions in some less specialized cell types and in some circumstances (Sicinski et al., 2007; Besson et al., 2007; Besson et al., 2004; Denicourt et al., 2007), p27 is well known to be a tumor suppressor gene. But, while prototypic tumor suppressor genes follow the Knutson’s “two-hit” criterion, p27 is haplo-insufficient for tumor suppression (Fero et al., 1998). Animals lacking one copy of Cdkn1b gene develop tumors spontaneously late in life and are highly sensitive to tumor induction when challenged with carcinogens, displaying increased tumor frequency and decreased latency (Fero et al., 1998). Thus, a reduced dosage of p27, rather than its absolute absence, can contribute to cancer susceptibility. Nullizygous animals for p27 display a further increase in tumor rate and this observation lead many to defined p27 as a “dosage-dependent” tumor suppressor gene.

The use of mouse models resulted extremely useful to address the question of whether p27 is a causal or a secondary effect of tumor development. p27-/- mice display increased body size of about 20-30% respect to wild-type littermates, due to increased cellularity of tissues, demonstrating a role of p27 in the control of tissue growth also in vivo (Fig9. Fero et al., 1996; Nakayama et al., 1996; Kiyokawa et al., 1996).

Accordingly with the dosage-dependency of tumor suppressive potential of p27, heterozygous mice showed about 50% of p27 protein expression and an intermediate phenotype in terms of weight. This suggests that also in vivo the control of proliferation and body size is extremely sensitive to the levels of p27 protein. Further, p27-/- mice are prone to spontaneous pituitary adenomas and develop multiple organ hyperplasia. Challenging with radiation or chemical carcinogens, p27-/- mice are susceptible to developing tumors in multiple tissues, including
adenomas and adenocarcinomas of the intestine and lung, granulosa cell tumors of the ovary and uterine tumors. In addition to a reduced tumor free survival, p27-heterozygous and null mice show increased tumor multiplicity in different sites.

p27 deficiency enhances the malignancy and frequency of tumor formation in cooperation with several oncogenic stimuli, pointing to p27 as a major rate-limiting factor for tumor progression. For example, about 50% of PTEN+/− mice develop prostatic intraepithelial neoplasia after 9 months from birth, but concomitant inactivation of p27 (that normally is not associated to prostatic cancer) in PTEN+/− mice leads to prostate adenocarcinoma with 100% of penetrance within 3 months from birth (Di Cristofano et al., 2001). Similar results are obtained when p27 deficiency is combined with loss of eterozygosity (LOH) for pRB locus, resulting in developing of pituitary adenocarcinoma and high grade of thyroid C cell carcinoma (Park et al., 1999). Moreover, Myc over-expression results in decreased mice survival when associated to p27 deficiency and the absence of p27 in INK4a/ARF null background result in increased lethality, higher incidence of lymphomas and appearance of metastasis (Martin-Caballero et al., 2004). p27 results also as adjuvant and promoter of p53-driven tumorigenesis, since their concomitant absence in mice induces an accelerated tumor development, increased morbidity and the detection of tumor types not observable in p53 single mutants (Philipp-Staheli et al., 2004).

In colon, p27 null background combined with ApcMin/+ accelerate tumor development and increase tumor malignancy; loss of the CBP transcription factor in thymocytes, combined to p27+/− background, results in the development of T-cell lymphoma already after 13 weeks (compared to a 24 latency period in p27wt background) (Philipp-Staheli et al., 2002; Kang-Decker et al., 2004).

Together these data demonstrate that at least in mice, p27 functionally interacts and collaborates with several tumor suppressor genes and oncogenes to reduce the malignant phenotype, supporting the idea that p27 represents a nodal point in tumor suppression. This could, at least in part, explains why loss of p27 is so often associated with tumor development and progression in human cancers.

An increased body of literature reports frequent p27 functional inactivation in human cancers. LOH for p27 is not uncommon in human malignancies, but silencing or mutations of the remaining allele are very rare, which is consistent with the notion that p27 loss in tumors is mainly due to an accelerated proteolysis (Belletti et al., 2005). Accordingly, a plethora of studies shows the involvement of p27 protein reduction or loss in many tumors, such as carcinomas of the colon, breast, prostate, lung and ovary as well as brain tumors, lymphomas.
and soft tissue sarcomas (Belletti et al., 2005). Multivariate analyses show that reduced p27 levels are of independent prognostic significance for many of these tumors (Belletti et al., 2005). Other observations suggest that p27 in tumors can also be down-regulated by different mechanisms, as its cytoplasmic delocalization (Belletti et al., 2005).

3.2. p27 shows a role in cell migration.

Among the non-cell cycle dependent functions of p27, regulation of cell motility is one of the most controversial. This function requires the cytoplasmic localization of p27 so results dependent on all those mechanisms that rule p27 shuttling from nucleus to cytoplasm. Many authors have demonstrated a role of p27 in cell migration, but the conclusions appear at least apparently in contrast. It was shown that p27 stimulates the migration in cortical neurons, in hepatocellular carcinoma cells and in mouse embryonic fibroblasts where it induces rearrangements of the actin cytoskeleton (Kawauchi et al., 2006; Itoh et al., 2007; McAllister et al., 2003; Besson et al., 2004). Conversely p27 seems to reduces cell migration in endothelial cells, vascular smooth muscle cells, mesangial cells, sarcoma tumor cells and normal mouse fibroblasts (Daniel et al., 2004; Goukassian et al., 2001; Sun et al., 2001; Baldassarre et al., 2005).

In particular, we have demonstrated that p27 expression inhibits the migration of HT-1080 fibrosarcoma cells and murine fibroblasts and that the migration-inhibitory activity of p27 is localized to the C-terminal 28 amino acids of the protein (Baldassarre et al., 2005). Using a yeast two-hybrid assay, we identified stathmin as a partner protein that binds to p27 C-terminus and confirmed their in vivo interactions in HT-1080 sarcoma cells, pork brain, mouse fetal brain and normal mouse fibroblasts adherent to fibronectin.

Stathmin, also referred to as Op18, is a ubiquitous cytosolic phosphoprotein highly conserved among vertebrates: human and Xenopus stathmin are 79% identical (Maucuer et al., 1993). The 149 amino acid protein plays an important role in regulating microtubule dynamics in both interphase and mitosis and so it is crucial for maintenance of cell shape, intracellular transport, cell motility and cell division. As described previously, microtubules continuously switch between phases of polymerization and depolymerization, a property known as dynamic instability (Rubin and Atweh, 2004).

Microtubule dynamics are regulated by several families of proteins, including microtubule-associated proteins (MAPs) and microtubule-destabilizing proteins. Stathmin is a major microtubule-destabilizing protein that promotes microtubule depolymerization by two distinct mechanisms (Fig10; Howell et al., 1999; Rubin and Atweh, 2004). The first is a catastrophe-
promoting microtubule-depolymerization activity that requires the N-terminal region of the stathmin molecule and is necessary for the regulation of the mitotic spindle. The second is a tubulin-sequestering activity that requires the C-terminal region and is mainly important in the regulation of microtubule dynamics during interphase (Howell et al., 1999; Rubin and Atweh, 2004).

Figure 10: Stathmin regulation of microtubule dynamics Microtubules continuously switch between phases of polymerization and depolymerization. Stathmin can sequester un-polymerized tubulin by binding two αβ-tubulin heterodimers, thus diminishing the pool of tubulin available for polymerization. Stathmin can also bind to the end of polymerized microtubules and increase the rate of catastrophe by inducing a conformational change that promotes microtubule depolymerization. (From Rubin and Atweh, 2004)

Stathmin is negatively regulated by phosphorylation at four serine residues, Ser16, Ser25, Ser38 and Ser63 in response to a number of signals, including those necessary for cell proliferation, differentiation and progression through the cell cycle (Cassimeris, 2002). In particular, stathmin phosphorylation seems to be necessary for the cells to enter mitosis. Several studies have demonstrated that phosphorylation at Ser16 and Ser63 inhibit stathmin to a greater extent than phosphorylations at Ser25 and Ser38 and probably, multiple phosphorylations of stathmin serve to amplify the inhibition of this protein's activity (Cassimeris, 2002).

Besides its role in mitotic spindle formation, many literature data support the pro-migrative role of stathmin. For example, the RNA interference inactivation of *Drosophila* stathmin expression resulted in germ cell migration arrest and induced important anomalies in nervous system development (Ozon et al., 2002). Stathmin expression is also required in border cells of the *Drosophila* ovary for normal migration (Borghese et al., 2006). Moreover, reducing the expression of stathmin with an antisense oligonucleotide, results in the inhibition of migration
of new neurons from the sub-ventricular zone to the olfactory bulb via the rostral migratory stream, suggesting a role for stathmin in the migration of newborn neurons in the adult rodent brain (Jin et al., 2004).

Accordingly, we have demonstrated that p27, binding the C-terminus of stathmin, interferes with its ability to sequester tubulin, leading to increased microtubule stabilization that in turn impairs migration function. Also in our systems, in fact, stathmin has a pivotal role in cell migration regulation: stathmin-null mouse embryo fibroblasts (MEFs) showed migration defects rescued by transfection of stathmin cDNA and stathmin inhibition reduces cell motility, while its over-expression increases migration in HT-1080 cells (Baldassarre et al., 2005). But intriguingly, in our system the stathmin activity in cell migration results to be regulated by p27 expression and together these data represent the first indication that cytoplasmic p27 regulates migration by a direct effect on microtubule dynamic via-stathmin (Iancu-Rubin and Atweh, 2005).
4. The oncogenic v-Src and its normal mammalian counterpart c-Src.

Several oncogenes are able to induce mouse fibroblasts transformation. Among them, one of the most powerful is certainly v-Src, whose normal counterpart in the mammalian cell (c-Src,) was the first proto-oncogene to be discovered in the vertebrate genome (Takeya and Hanafusa, 1983). The viral src gene of Rous sarcoma virus (RSV) encodes the first recognized tyrosine kinase (Hunter and Sefton, 1980). Src is a member of a multigene family (the prototype Src family kinase (SFK)) of membrane-associated non-receptor tyrosine kinases that comprises nine members, some of which (Src, Fyn and Yes) are ubiquitous and some of which display more-restricted expression (Thomas and Brugge, 1997).

4.1. Structure and regulation of Src

Both the avian and human forms of c-Src protein are composed of a unique amino-terminal domain, four SRC homology (SH) domains and a C-terminal tail containing a negative-regulatory tyrosine residue (Tyr527, chicken; Tyr530, human) (Xu et al., 1997). The functions of the N-terminal domain are not well defined, but mutations in this region seems to reduce the transforming potential of v-Src. The SH domains consist of the SH1 kinase domain, containing the auto-phosphorylation site necessary for full Src activation (Tyr416, chicken; Tyr419, human); the SH2 domain, interacting with the negative-regulatory Tyr527/530; the SH3 domain, which interacts with the kinase domain in the inactive form of the protein; and the SH4 domain, which contains the myristoylation site, important for membrane localization (Fig11. Yeatman, 2004).

As mentioned before, the C-terminal tail and the SH2 and SH3 domains are involved in the negative regulation of c-Src. Crystallographic studies have shown that interactions between the C-terminus and the SH2 domain, and between the kinase domain and the SH3 domain, cause the c-Src molecule to assume a closed configuration that hides the kinase domain and reduces substrate interaction (Yamaguchi and Hendrickson, 1996). Mutational studies primarily involving the avian forms of Src, have clearly elucidated a closed, inactive conformation and an open, active state. The inhibitory residue (Tyr527/530) can bind to the SH2 domain when phosphorylated and Src results inactive. When dephosphorylated, Src is active and show the potential for autophosphorylation and for downstream interactions with Src substrates (Cooper et al., 1986). Thus, phosphatases that dephosphorylate human c-Src at Tyr530 can bring about activation, even when protein levels are normal. Instead, the C-terminal phosphate of c-Src can be removed by several protein phosphatases. Protein tyrosine phosphatase-α (PTPα) has been shown to dephosphorylate the terminal tyrosine residue in
**Introduction**

...vitro and in vivo, and PTP1, SH2-containing phosphatase 1 (SHP1) and SHP2 might also regulate c-Src (Yeatman, 2004). The most direct evidence for a role in c-Src activation in cancer among these phosphatases is for PTP1B, which is present at higher levels in breast cancer cell lines and can dephosphorylate c-Src (Bjorge et al., 2000). Conversely, inactivation of c-Src by phosphorylation of the terminal tyrosine residue is now known to be performed by CSK and its homologue CHK, resulting in the closed, inactive c-Src conformation described above (Frame et al., 2002; Yeatman, 2004). In addition to Thy527 dephosphorylation, c-Src is also activated by the direct binding to the SH2 and SH3 domains of focal-adhesion kinase (FAK) or its molecular partner CRK-associated substrate (CAS, also known as p130CAS) (Yeatman, 2004).

Moreover, c-Src can also be activated as a result of a variety of extracellular signals. For example, interactions with ligand-activated receptor tyrosine kinases, such as EGFR, PDGFR, ERBB2 (HER2/NEU), FGFR, CSF1 and HGF can result in augmented and even synergistic c-Src activation. In particular, the activation of growth-factor receptors leads to their association with the SH2 domain, which disrupts inhibitory intramolecular interactions and promote c-Src activation (Yeatman, 2004). Other modes of c-Src regulation include ubiquitination, with subsequent degradation by the proteasome. The CBL ubiquitin ligase, found deregulated in some cancer cells, has been shown to be important in suppressing v-Src transformation through ubiquitin-dependent degradation (Kim et al., 2004).

While c-Src activity is finely regulated, v-Src results constitutively active because it lacks this crucial C-terminal negative-regulatory region and consequently shows higher levels of activity and transforming ability. In addition, v-src gene contains point mutations throughout its coding region that contribute to the high level of intrinsic activity and transforming potential of the v-Src protein (Fig11. Takeya and Hanafusa 1982; Jove and Hanafusa 1987; Yeatman, 2004).

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**Figure11: Structural domains of c-Src and v-Src.** The c-Src protein is composed of an amino-terminal myristoylation sequence (M), a unique region (U), Src-homology-2 (SH2) and SH3 protein-interaction domains, a kinase domain that contains Tyr416 and a carboxy-terminal regulatory domain (R) that contains Tyr527. v-Src proteins differ from c-Src in several ways, but the most important difference is the substitution of the negative regulatory carboxy-terminal region (modified from Frame et al., 2002).
4.2. The v-Src transformed phenotype

The highly activated v-Src oncogene has been revealed very useful to understand the function of SRC proteins. The generation of RSV mutants that were non-conditionally defective or temperature sensitive (ts) for transformation provided the first demonstration that a protein, v-Src, can initiate and maintain cell transformation (Martin, 2001). v-Src transformation of normal fibroblasts is a visible event, leading to morphological changes in transfected cells. Transformed cells have a loss of bundled actin filaments and a reduction in the number and the size of cell–substrate adhesions (focal adhesions) into which actin filaments are tethered (Frame et al., 2002; Yeatman, 2004). This results in conversion from a well-spread morphology to a more refractile and fusiform cell shape, particularly in established cell lines. The lost of the intercellular, integrin-based cytoskeletal attachments could induce also the more extreme cell rounding and detachment from the substratum (Jove and Hanafusa, 1987).

v-Src-transformed cells are also more motile and more able to invade the basement-membrane matrix. Over the course of weeks, v-Src transformation can result in overgrown clumps of cells, known as foci, where cells lose their density inhibition - a hallmark of a cancer cell. These changes are consistent with the processes that are needed for a cancer cell to disaggregate from the primary tumor, invade the surrounding tissue and metastasize to distant organ sites. In addition to these effects, the v-Src oncoprotein has growth-promoting functions in fibroblasts. v-Src-transformed cells suppress the expression of p27, leading to more rapid transit through the G1 phase and a failure to enter the quiescent state when deprived of serum mitogens (Johnson et al., 1998; Riley et al., 2001). As well as suppression of p27, activation of ts v-Src in quiescent cells sequentially induces expression of cyclins D1, E and A and activity of cyclinD1/D3-CDK4/6, cyclinE-CDK2 and cyclinA-CDK2 (Riley et al., 2001). These combined effects on positive and negative regulators of the cell cycle require the activities of the PI3K and MEK pathways downstream of v-Src and drive cells through G1 and into S-phase (Riley et al., 2001). Recently, a direct phosphorylation of src on p27 Y74 has been also shown (see above). In vivo, transfected cells grow rapidly to form visible tumors within days of injection, and these tumors are capable of local invasion and metastasis to distant sites (Yeatman, 2004).

v-Src is transported to the cell periphery, where it has a crucial function in the regulation of the actin cytoskeleton and cellular adhesions, even if v-Src was also reported to be present in the perinuclear region of transformed cells (Rohrschneider, 1979). The effects of v-Src on the actin cytoskeleton and focal adhesion are responsible for the rounded morphology and
reduced adhesiveness of transformed cells. Many aspects have apparent parallels in the biology of c-Src, which is also transported to equivalent peripheral sites where its activity is required for cell migration. Although such parallels can be drawn, this is not to imply that v-Src simply reproduces an extreme and uncontrolled copy of the biological function of c-Src in normal homeostasis. Even when over-expressed, c-Src cannot transform cells because its kinase activity is subject to tight negative regulation (Thomas and Brugge, 1997). However, constitutively activated c-Src can induce oncogenic transformation, which implies that v-Src and c-Src share at least some effectors that determine the transformed phenotype (Frame et al., 2002).

4.3. Molecular mechanisms of Src functions

The complexity of Src phenotype is indicative of how wide its activity is. As mentioned before, v-src shows growth-promoting effects in fibroblasts, modulating the activity of both positive and negative cell cycle regulators. v-Src acts also as a survival factor affecting the fine balance between survival and death and promoting cell cycle progression in unfavorable conditions such as low serum concentration, via PI3K and AKT signaling. Moreover, v-Src is able to overcome the normal adhesion requirement for proliferation and induces anchorage independent growth (Frame, 2002).

But first of all, Src is well known to be a key regulator of cellular actin and adhesion network. Src is localized in the focal adhesions and assembled into supramolecular structures with over other 50 different cytoskeletal proteins. Focal adhesions are disassembled when the cell needs to move along or away from the ECM and Src contributes to this process leading to disruption of focal adhesions and actin stress fibers. This occurs both during normal cellular migration and mitosis, when cells round up and lose their matrix attachments and during transformation, when the integrity of these structures is disrupted, permitting increased motility (Hynes, 1992). v-Src, as c-Src, induces, a block downstream the RhoA signaling through activation of p190 RhoGAP, leading to focal-adhesion disruption. Src is also thought to affect focal adhesions by inducing tyrosine phosphorylation of R-Ras, by dephosphorylation and activation of the actin-severing protein cofilin and by phosphorylation and activation of FAK. Moreover, Src might affect migration and invasion by regulating matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs) and E-cadherin levels (Yeatman, 2004). In particular the E-cadherin down-regulation is a key event in the carcinoma progression and Src, in cooperation with EGF and HGF, promotes it by stimulating the ubiquitination of E-cadherin and leading to its endocytosis. In epithelial cancers the disruption or the weakening
of epithelial cell-cell adhesion facilitates invasion inducing the epithelial-mesenchymal transition (Fig12. Frame, 2002).

Figure12: Src activity promotes the epithelial-mesenchymal transition and cell migration. In carcinoma progression, Src and EGF/HGF cooperate to induce the weakening of cadherin-dependent cell-cell interactions and the switch to a mesenchymal-like motility (modified from Frame, 2002)

4.4. Src is altered in tumors

Despite the accumulation of information during decades of research, and the now refined molecular understanding of how Src is controlled, we still have an incomplete picture of whether, and how, misregulation of Src might contribute to the malignant phenotype. Among all the members of SFK family, it is primarily Src that has been implicated in the development of human cancer. Like oncogenic v-Src, activated mutants of c-Src can transform cells in culture and induce tumors in animal models (Frame, 2002). In addition, there are many reports showing how Src protein expression and/or activity is elevated in epithelial cancers, or cell lines derived from these, and there is often an association with stage of disease or with malignant potential (Frame, 2002). In fact, in many cancers, increased Src kinase activity is associated with advanced-stage tumors that readily metastasize and is thought to have an important role in the metastatic potential of these tumours, influencing directly tumour growth and cancer-cell adhesion, motility and invasion (Yeatmen, 2004).

In colon cancer, cellular Src kinase activity is frequently elevated compared to the adjacent normal mucosa and the activation is linked to malignant potential (Frame, 2002). Although an increased Src activity was also observed in premalignant lesions and in adenomas, it is generally highest in malignant polyps. The reduction of Src protein expression in HT29 colon
cancer cells using an antisense approach suppresses their growth both in vitro and in vivo. Further, higher Src activity is often observed in metastases compared to the corresponding primary tumors and activation of Src by receptor tyrosine kinases occurs in cells that have high metastatic potential (Frame, 2002). Taken together, current evidence implies that there is generally an early increase in Src activity accompanying tumour initiation and a stepwise increase during the progression of colon cancer.

There is also circumstantial evidence that Src activity might be playing a role in breast and other epithelial cancers. For example, Src is over-expressed or shows an elevated activity in breast carcinomas compared to normal tissue, in breast cancer cells lines and in mouse models of mammary tumours (Frame, 2002). Src is also frequently up-regulated in pancreatic cancers and cell lines derived from these, as well as in ovarian, esophageal, lung, head and neck and gastric cancers (Frame, 2002).

Src expression and activity are increased also in melanoma cell lines and in melanoma tumors. In melanoma, Src can activate STAT3, STAT5 and other downstream targets and their activated forms are present in both primary and metastatic melanoma in humans, although the expression level is variable (Frame, 2002).

All these observation strongly suggests that the elevated expression and/or activity of Src facilitate malignant progression. However, the way in which Src activation is stimulated and how this contributes to the cancer phenotype, are still not completely defined.
AIM OF THE STUDY

In cancer, the uncontrolled cell proliferation and the ability to invade tissues and form metastasis, are two of the most important features. Thus, the understanding of molecular mechanisms that regulate the cell growth and the motility behaviour becomes crucial for the development of new anticancer therapies.

Among all the molecular effectors that could have a role in tumor progression, particular interest has received the inhibitor of cyclin-CDK complexes p27\textsuperscript{kip1} (p27). p27 represents an ideal target displaying on one hand an inhibitory effect in cell cycle progression and on the other hand by regulating cell motility, as recently demonstrated by different authors.

In this PhD project we aimed to clarify whether and how p27 contributes to the proper coordination of proliferation and motility in the process of neoplastic transformation. In particular, we decided to evaluate if the presence or absence of p27 could be associated to different responses after oncogenic stimuli. We used murine immortalized fibroblasts, derived from embryos wild type or knock out for the \textit{cdkn1b} gene, encoding for the p27 protein, and then transformed by over-expression of v-src oncogene. The Harvey-Ras oncogene was used in the same manner to highlight common responses or different “oncogene-specific” effects.

The selected cell clones were then analysed with several \textit{in vitro} and \textit{in vivo} experimental approaches in order to characterize both their proliferative and motile behaviour and to gain new information about p27 functions in the first steps of neoplastic transformation as well as in the later stages of tumor progression.
MATERIALS AND METHODS

1. Cell cultures and development of stable cell lines.

3T3 p27 wt and null fibroblasts were kindly provided by Dr. M. Fero. All described cell lines were grown in DMEM (Dulbecco's Modified Eagle's Medium, Cambrex Bio Science) supplemented with 10% FBS (Sigma). To obtain stable cell clones, pM-vSrc (Johnson et al., 1985) was co-transfected with pTRE2pur (Clontech) or pMSCV-Hyg (Clontech), using the calcium phosphate procedure or FuGENE6® (3,5µl of FuGENE6® / µg of DNA). pMSCV-HRAS and all the p27 expressing vectors were retrovirally transduced. Retroviral transduction was performed by transfecting the pMSCV vectors together with the pHIT60 vector, containing the gag-pro-pol genes and the pHIT123, containing the env gene for mouse tropism, into the HEK-293 cells, using the calcium phosphate procedure. Three days later, conditioned medium harvested from 3 dishes (containing retroviruses) was used to transduce one dish of exponentially growing 3T3 p27wt or null fibroblasts. Clones and pools were selected in complete medium with 1,5µg/ml Puromycin and/or 0,4mg/ml Hygromycin. The stable expression of the different constructs was tested by western blot analysis.

2. Cell culture in 3D collagen lattices.

3D collagen matrix was prepared using both native dermal bovine (Vitrogen; PURE-COL/Nutacon) or rat tail (BD) collagen type I (Wolf et al., 2003). In this study both individual cells and multicellular spheroids were used. Spheroids were obtained seeding cells onto not-adhesive bacterial petri dish or in polyHEMA-coated dish, collecting the obtained cell clumps 24hours after plating. Then, spheroids or single cells from subconfluent cultures (detached with 2mM EDTA) were included into the collagen mix (1.7 mg/ml) at physiological pH. The suspension was allowed to polymerize for 45 minutes at 37°C in a 5% CO₂ atmosphere, in a self-constructed chamber (Friedl et al., 1993). After polymerization of the collagen lattice, FBS-containing medium (10% final FBS concentration) was added as supernatant and the chamber was sealed.

3. Time-lapse microscopy and quantification of cell migration.

Individual cells or multicellular spheroids incorporated within 3D collagen matrices were monitored by digital bright-field microscopy in order to discriminate cells along the different
axes. The pictures were collected every 4 or 5 min for 20 hours, using a CCD camera mounted onto the microscope. A 10X (for speed analysis) or 20X (for morphological analysis) objectives were used. The cells were maintained at 37°C during all the recording time using a remote temperature control system and a heating device. At the end of the recording, pictures were used to create a movie (10 images per second, Quicktime), and used for cell tracking analysis. After conversion of pixel values into micrometers, single cell migration was quantified by computer-assisted cell tracking of at least 40 randomly selected cells for each experiment, obtaining several locomotion parameters, such as velocity, speed and paths.

Velocity describes the actual translocation efficiency of cells in the process of migration, delineating the “true” speed without interference of stopping frequencies. Speed represents a more general parameter describing overall motility as a function of the fraction of locomoting cells and their locomotor duration, individual step lengths and step number. Using the $xy$ coordinates of cell paths, the speed and velocity were calculated as the length of each cell path divided by time (step interval, 12 minutes). Statistical analysis was obtained from the two-tailed unpaired Student's t-test or the two-tailed unpaired Mann-Whitney test.

4. Transwell-based migration assay.

Cell lines were tested for invasion and motility by fluorescence-assisted transmigration assay (FATIMA, Spessotto et al., 2000), in response to haptotactic stimuli of ECM substrates. The procedure is based on the use of transwell-like inserts (HTS FluoroBlok™, Becton-Dickinson, Falcon, Milan, Italy) carrying fluorescence shielding porous polyethylene terephthalate (PET) membranes with 8μm pores.

For haptotaxis, bottom side of HTS Fluoroblok™ were coated with 20μg/ml fibronectin in carbonate buffer at 4°C overnight and then saturated with PBS 1% BSA for 2 hours at room temperature. Cells were labeled with DiI (Molecular Probes) for 20 minutes at 37°C before being seeded on the Fluoroblok™ upper chamber and then incubated at 37°C for the indicated times.

Migratory behavior of the cells was monitored at different time-points by detection of fluorescence from the top (corresponding to non-migrated cells) and the bottom (corresponding to migrated cells) sides of the membrane using the computer-interfaced GENios Plus (TECAN Italia Srl). Each experiment was performed at least 3 times, in
duplicate. As a further control, after the last measurement, Fluoroblok™ membranes were fixed in 4% PFA and mounted on a slide, to allow the manual count of migrated cells.


For 3D Matrigel™ and Collagen I evasion assay, cells (7,5x10^5/ml) were included in Matrigel™ (6 mg/ml, Becton Dickinson) or in Collagen I (1,7 mg/ml, Becton Dickinson) drops and maintained 1 hr up side down to polymerize at 37°C. Collagen I was diluted in a mix solution containing DMEM-10X (w/o phenol red), 7,5% sodium bicarbonate and complete medium. Then drops were incubated for the indicated times in complete medium.

Cell motility was observed by transmission microscopy using a Nikon TS100/F microscope and images collected using a CCD camera (Leica). Cells outside each drop (five drops/cell line/experiment) were counted to estimate the cell line evasion ability.

The evasion ability was also estimated by measuring the distance covered by the cells from the drop edges, after 5 days from inclusion. To perform this analysis, cells were stained with crystal violet and then pictures were taken. After conversion from pixel to millimeters, the actual distance covered was calculated.


The quantitative cell adhesion assay used in this study has been previously described (using centrifugal assay for fluorescence-based cell adhesion-CAFCA; Giacomello et al., 1999; Spessotto et al., 2000). Briefly, six-well strips miniplates (of flexible polyvinyl chloride), covered with double-sided tape (bottom units), were coated with the different substrates (20µg/ml Collagen I, 10 µg/ml Vitronectin and 10µg/ml Fibronectin. BSA was used as negative control). Cells were labeled with the vital fluorochrome Calcein AM (Molecular Probes) for 15 minutes at 37°C and then dispensed into the bottom CAFCA miniplates, which were then centrifuged to synchronize the contact of the cells with the substrate. The miniplates were then incubated for 20 minutes at 37°C and then mounted together with another CAFCA miniplate to create communicating chambers for subsequent reverse centrifugation. The relative number of cells bound to the substrate (i.e. remaining in the wells of the bottom miniplates) and cells that fail to bind to the substrate (i.e. remaining in the wells of the top miniplates) was estimated by top/bottom fluorescence detection in a computer-interfaced GENios Plus microplate fluorometer (TECAN Italia S.r.L., Milan, Italy).
The analysis of signal transduction pathways activated after cell adhesion was performed seeding about $1 \times 10^6$ cells in 100mm dishes, coated with 20µg/ml collagen I. At the indicated time points (15, 30, 60 minutes), cells were collected and total protein extracts were obtained as described below (point 7).

**7. Preparation of cell lysates, immunoprecipitation and immunoblotting.**

Total cell proteins were extracted using cold NP40 lysis buffer (0.5% NP40; 50mM HEPES pH7; 250mM NaCl; 5mM EDTA; 0.5mM EGTA pH8) containing a protease inhibitor cocktail (Complete™, Roche), 1mM sodium orthovanadate and 1mM DTT. After incubation on ice for 30 min, the lysates were centrifuged at max speed for 10 min at 4°C to recover the supernatant.

Immunoprecipitation experiments were performed using 0.5mg of total lysate, adding HNTG buffer (20mM HEPES, 150mM NaCl, 10%Glycerol, 0.1%Triton X-100, protease inhibitor cocktail, 1mM sodium orthovanadate and 1mM DTT) with the specific agarose-conjugated primary antibodies, gently rocking overnight at 4°C. When primary antibodies were not agarose-conjugated, Protein A or Protein G Sepharose 4 Fast Flow (Amersham Biosciences), was added for the last 2 hr of incubation. Immunoprecipitates were then washed six times in HNTG buffer and then nine parts were resuspended in 3X Laemli Sample Buffer (50mM TrisHCl pH6.8, 2% SDS, 10% glycerol, 0.5% Bromophenol Blue, with fresh 50mM DTT) for immunoblot analysis and one part was resuspended in kinase buffer (20mM TrisHCl pH6.8, 10mM MgCl$_2$) to perform a kinase assays (see the following chapter).

For immunoblot analysis, proteins were separated in 4-20% SDS-PAGE (Criterion Precast Gel, Biorad) and transferred to nitrocellulose membranes (Amersham). Membranes were blocked with 5% dried milk in TBS-0.1% Tween or in Odyssey Blocking Buffer (Licor, Biosciences) and incubated at 4°C overnight with primary antibodies.

Primary antibodies were from: Transduction Laboratories (p27, CDK1, CDK2, FAK, Cofilin), Santa Cruz (CDK1, CDK2-AC, Cyclin B1, Cyclin A, vinculin, c-Src, H-Ras, pY705-Stat3, Stat3, Akt), Cell Signaling (pS473-Akt, pT202/Y204-p42/44-MAPK, p42/44-MAPK, pS3-Cofilin), Biosource (pY397-Fak, pS473-Akt).

Then, membranes were washed, incubated 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies, for chemi-luminescent detection (ECL Kit, Amersham Inc.) or with Alexa-conjugated secondary antibodies for infrared detection.
(Odyssey Infrared Detection System, Licor). For immunoprecipitates, Rabbit IgG and Mouse IgG True Blot (eBioscience) secondary HRP-conjugated antibodies were used. The Restore™ Western Blot Stripping Solution (Pierce) was used to strip the membranes, when re-blotting was needed.

8. Growth curve, MTT assay and FACS analysis.

Cell proliferation was evaluated using growth curves, MTT assay and FACS analysis after Propidium Iodide labeling. For MTT assay (Sigma), 1000 cells/well were seeded in 96 well plates. At the indicated times, MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) in complete medium (0,28mg/ml final concentration) was added and incubated at 37°C for 4 hours. During this incubation, MTT is reduced to purple formazan in the mitochondria of living cells. The medium was then discard and the formazan salts were dissolved in DMSO. The colorimetric reaction was measured and quantified at 560 nm in Elisa plate reader.

For cell growth, 1x10^5 cells/well were seeded in 6 well plates in complete medium in duplicate. At the indicated time, cells were detached in trypsin-EDTA and counted.

Cell cycle distribution was analyzed by FACS analysis. Cells were collected and fixed in ice-cold 70% ethanol and maintained at -20°C until ready for the staining procedure. Cells were then washed and resuspended in propidium iodide staining solution (50µg/ml propidium iodide and 0,1mg/ml RNaseA, in PBS 1X). Stained cells were subjected to flow cytometry analysis (FACS) with a FACScan and a FACSCalibur instrument (BD Biosciences). The data were analysed using WinMDI2.8 software.


After immunoprecipitation with anti-CDK1, -CDK2, -CyclinA or -CyclinB1 antibodies, or with the control antibodies, the associated kinase activity was evaluated, as follows. Samples were mixed with a kinase reaction solution containing 50µM not labelled ATP + γATP-P32 and 2µg of H1-Histon as substrate in buffered solution (20mM TrisHCl pH6.8, 10mM MgCl₂). The reaction was carried out at 30°C for 30 min and then 2X Laemli Sample Buffer was added. After denaturation at 95°C for 5 min, proteins were loaded on a 4-20% SDS-PAGE (Criterion Precast Gel, Biorad). The gel was then dried and exposed on an
Materials and Methods

autoradiographic film (Amersham-Hyperfilm MP) at -80°C and developed after different time intervals.

10. Double thymidine block and nocodazole assay.

The double thymidine block was used to synchronize the cell cycle progression. Thymidine is a deoxynucleoside able to inhibit DNA replication if added in excess in the medium, inducing a reversible block at the G1/S transition. We performed a first treatment with 2mM thymidine for 15-16 hours in cells plated at about 20-30% of confluence. At the end of the first incubation, the cells were detached and seeded again at 30% of confluence and released in complete medium (DMEM-10%FBS) for 8 hours. Then a second thymidine block was performed (12 hours with 2mM thymidine in complete medium) at the end of which most of the cells resulted blocked at the beginning of S-phase. At this point, cells were released in complete medium for 6 hours and then incubated 5 hours with 50ng/ml nocodazole. Nocodazole is a MT destabilizing drug that inhibits the assembling of the mitotic spindle and blocks the cells at the beginning of M phase (in pro-metaphase). To analyse the progression along the mitosis, cells were collected at T0 (i.e. in pro-metaphase) and after 30, 60, 90, 120 and 180 minutes of release in complete medium and analyzed by FACS.

11. Anchorage-independent cell growth

To evaluate the cell ability to grow in an anchorage independent manner, soft agar assay was performed. Cells (1 × 10^3 and 5 × 10^3) were suspended in 2ml TOP agar medium (DMEM-10%FBS containing 0.4% Low Melting Agarose-SIGMA). The cell suspension was then layered on 2ml of jellified BOTTOM agar medium (DMEM-10%FBS containing 0.6% Low Melting Agarose) in six-well tissue culture plates in triplicate. DMEM-10%FBS with 1,5µg/ml Puromycin and/or 0,4mg/ml Hygromycin (depending from the selection needed) was added to the plates every 3 days, as a feeder layer. On day 8 for Src clones and day 12 for Ras clones, the number of colonies was counted in 15 randomly selected fields, at 10X magnification.

12. In vivo analysis

Primary tumors were established by s.c. injection of transformed cells into the flank of female athymic nude mice (Harlan, 8 weeks of age). 1x10^6 v-src transformed cells and 2 x10^6 v-ras transformed cells were inoculated in female athymic nude mice.
H-Ras transformed cells were injected. The tumor growth was followed every other day measuring the mass with a calibre. 15 days from injection in the case of v-src transformed cell and 22 days in the case of H-Ras cells, the animals were sacrificed and tumor analysis was performed. To analyze the invasive potential, spleen, lung and blood samples were also collected and rapidly frozen to preserve RNA integrity.

In a second type of experiment we tried to evaluate the ability of the transformed cells to settle and colonize distant sites. To this aim, 1x10⁶ cells have been injected into the tail vein. After about 20 days, the animals were sacrificed and lungs were fixed in Bouin’s solution to look for foci formation.

13. RNA extraction and RT-PCR from tissue samples

Isolation of RNA from spleen, lung and blood samples was performed using RNeasy-Mini Kit (QIAGEN), according to the manufacture’s instructions. Disruption of the tissue sample was achieved by grinding the frozen tissue thoroughly with lead blocks. The homogenization was performed passing the lysate at least 5 times through a blunt 23-gauge needle fitted to an RNase-free syringe.

RNA were then quantified and retro-transcribed with AMV Reverse transcriptase (according to provider’s instruction, Promega) and the obtained cDNAs were amplified with nested PCR, in order to evaluate the presence of ectopic injected cells in tissue samples. The following primers were used:

- pTREforward1 5’-CAGCAGGCAGAAGTATGCAA-3’
- pTREforward2 5’-TGCAAAGCATGCATCTCAAT-3’
- pTREreverse1 5’-CGTGAGGAAGAGTTCTTGCAG-3’
- pTREreverse2 5’-AGTTCTTGCAGCTCGGTGAC-3’
- pMSCVforward 5’-CCCTTGAACCTCCTCGTTCGACC-3’
- pMSCVreverse 5’-GAGACGTGCTACTTCCATTTGTC-3’
- H-RASforward 5’-GGATCCATGACGGAATATAAG-3’
- H-RASreverse 5’-GAATTCTCAGGAGAGCACACA-3’
- p27-170reverse 5’-GGATCCCTCAGAGTGTCTGTGGGTGCTTTT-3’

Samples were normalized by amplification of 18S ribosomal subunit. PCR products were then resolved on 1% agarose gels stained with ethidium bromide.
14. Immunofluorescence Analysis

For immunofluorescence staining, cells included in Matrigel™ or collagen I drops were fixed in PBS 4% paraformaldehyde (PFA) at room temperature (RT), permeabilized in PBS 0.2% Triton X-100 and blocked in PBS 1% BSA and 10% normal goat serum for at least 2 hours. Incubation with FITC-conjugated monoclonal anti-α-tubulin antibody (SIGMA) and Phallotoxin-AlexaFluor546 (Molecular probes-Invitrogen) for actin detection was performed at RT for two hours in PBS 1% BSA and 1% normal goat serum. Cover slips were mounted in Mowiol 4–88 (Calbiochem-Novabiochem) containing 2.5% (w/v) DABCO (Sigma). To visualize collagen fibers, laser light at a low density (the “reflection” parameter in confocal microscopy) was used and the light reflected by the sample was detected, allowing a sensitive recognition of fibers. Images were acquired with a Leica TCS SP2 confocal system (Leica Microsystems Heidelberg, Mannheim, Germany), using the Leica Confocal Software (LCS).

15. Statistical analysis

Statistical significance of the results was determined by using the paired and unpaired Student’s t-test. A p value <0.01 was considered significant.
RESULTS

1. p27<sup>−/−</sup> v-src transformed cells exhibit higher proliferation potential than p27<sup>+/+</sup> cells

To gain new information on the role of p27 in cell transformation, the oncogene v-src was used to transform mouse fibroblasts derived from p27<sup>+/+</sup> and p27<sup>−/−</sup> genetic background. Fibroblasts have been previously immortalized by 3T3 protocol (kindly provided by Dr. Fero). Two independent cell clones stably over-expressing comparable levels of v-src for each genotype (Fig. 1) were fully characterized.

The v-src transformation process is known to promote cell growth by altering the levels of both positive and negative cell cycle regulators and, among all, p27 results down-regulated after v-src over-expression (Johnson et al., 1998; Riley et al., 2001). In agreement with the literature data, we observed a decrease in the amount of endogenous p27 after transformation, but the relative low p27 levels were then retained throughout the time and confirmed after each experiments (Fig. 1).

Being p27 a well known tumor suppressor gene able to inhibit cell proliferation, the selected clones were initially analyzed for their proliferative behavior. First, cell distribution in the different phases of the cell cycle was evaluated by FACS analysis of DNA content. This analysis demonstrated that v-src p27<sup>−/−</sup> fibroblasts had a sensible higher S phase population respect to the wild type counterpart (38,4% for p27ko versus 25,4% for p27wt cells. Fig. 2A), suggesting that p27 absence increased v-src cell proliferation rate. Accordingly, both cell growth curve and MTT proliferation assays demonstrated that p27<sup>KO</sup> v-src cells grown significantly better than the p27<sup>WT</sup> transformed cell clone (Fig. 2B, C).
It is well accepted that p27 regulates cell cycle progression by interacting and inhibiting different cyclin-CDK complexes but with higher sensibility the CDK2 and CDK1 containing complexes (Belletti et al., 2005). To evaluate if the growth differences previously observed (Fig. 2) could be ascribed to a different CDKs activity, we performed a kinase assay in proliferating cells. As expected, the activity of CDK1 and CDK2 was significantly higher in p27\(^{KO}\) than in p27\(^{WT}\) v-src cells, although with some experimental variability (Fig. 3A, B). These results were then confirmed by assaying the cyclin A and B1 associated kinase activity (Fig. 3C, D), thus obtaining a molecular explanation for the different proliferation rate observed.

**Figure 2: Proliferative behaviour of v-src transformed cell clones.** A. Cell cycle distribution of p27WT and KO v-src cells was evaluated in exponentially growing cells by FACS analysis after PI staining of the DNA. The percentages of the cells in each phase is reported in the graph. A typical experiment is shown. B. Growth curve analysis of v-src transformed fibroblasts. 1x \(10^5\) cells/well were plated in triplicate and counted each day for 4 days. C. MTT assay in which 1x10\(^3\) cells/well were seeded in sixth-plate in 96well plates. Cells were harvested every 24 hours and cell proliferation quantified at 560nm in Elisa plate reader as described in materials and methods section.

**Figure 3: CDKs and cyclins-associated kinase activity in v-src transformed fibroblasts.** After immunoprecipitation for CDK1 (A), CDK2 (B), CyclinB1 (C) and CyclinA (D), the associated kinase activity was evaluated using a radioactive kinase assay in which the Histon H1 was used as substrate. A typical experiment is shown.
It is well known that p27 is a major regulator of the G1-S phase transition. To specifically analyze its contribution in this phase of the cell cycle in v-src transformed cells, we tried to synchronize the cells in the G1 phase by serum starvation and then re-stimulating cell cycle entry by release in complete medium. As control, normal p27+/+ and p27−/− 3T3 fibroblasts were used. After 24 hours of serum starvation, both p27WT and p27KO normal cells arrested in G1 phase and re-entered in S phase after about 15 hours of serum stimulation (Fig. 4A). This result was then confirmed by western blot analysis of typical markers of S (cyclin A) or G2-M (cyclin B1) phases of the cell cycle (Fig. 4B) in which we observed an increase in cyclin A levels after about 15 hours and of cyclin B1 after 21 hours from serum stimulation.

![Figure 4: Serum dependent cell cycle entry of normal p27wt and null fibroblasts. A. FACS analysis of 3T3 fibroblasts serum starved for 24 hours (T0) and then released in complete medium for 9, 15 and 21 hours. B. The cells treated as in A were analyzed by western blot for the expression of the indicated proteins. Vinculin expression was used as loading control. A typical experiment is shown.](image)

Differently by normal cells, transformed v-src fibroblasts were less sensitive to growth factor deprivation (Fig. 5). However both FACS analysis of DNA content (Fig. 5A) and western blot (Fig. 5B), demonstrated that p27+/+ v-src cells are more sensitive to serum deprivation than
p27 null cells. In fact, a transient reduction of S phase population and of cyclin A and B1 expression was seen in normal but not in p27 null v-src cells.

Interestingly, FACS analysis of transformed cells grown to confluence (Fig. 6) demonstrated that p27 expression is important to induce cell cycle arrest after cell-cell contact in transformed but not in normal fibroblasts. In fact while normal fibroblasts, independently on their genotype arrest in G1 after cell-cell contact, v-src transformed cells respond to contact inhibition only when p27 is present.
Results

Overall these data demonstrated that p27 plays an important role in the control of cell cycle progression of v-src transformed fibroblasts and that, in these cells, it has a major function in the transition between G1 and S phase.

We next analyzed whether v-src fibroblasts were effectively transformed and tumorigenic and if p27 had any effects on the process of transformation. A typical hallmark of transformed fibroblasts is the ability to grow in an anchorage independent way and in several model systems v-src has been proved to be able to confer this ability (Pawlak and Helfman, 2001). Thus, as a first approach, p27+/+ and p27-/- v-src cells were assayed in a soft agar assay and, as expected, both resulted able to grow in an anchorage independent way. However, p27 null cells displayed a significant increase in the number and the size of formed colonies (Fig. 7A,B), a result that is in line with the 2D proliferation assays.

Figure 6: Proliferative behaviour of cells grown to confluence. Normal and v-src transformed cells were grown to confluence and then analysed by FACS. A typical experiment in which two different cell clones/genotype have been analyzed is shown. The distribution of the cells in the different phases of the cell cycle is reported in each histogram.

Figure 7: Characterization of anchorage-independent growth. v-src transformed cells were tested in soft agar assay in triplicate (0.4% TOP, 0.6% BOTTOM of agar concentration). After 8 days, colonies were counted (A) and representative pictures (B) were collected. 10X objective was used and for each cell lines 15 fields were counted. (Student’s t test: p27wt vs p27ko: p<0.001).
Results

Next, two different clones of p27<sup>+/+</sup> and p27<sup>-/-</sup> v-src fibroblasts were subcutaneously injected in nude mice (n=19 for p27<sup>+/+</sup> and n=15 for p27<sup>-/-</sup> v-src cells). Xenografts were followed for about 15 days and then the mice were sacrificed and the tumor masses analyzed. Data showed that tumors formed from p27<sup>-/-</sup> transformed cells were significantly bigger in size (Fig. 8A, B) and weight (Fig. 8C) than the wild type counterpart, indicating that also in vivo p27 expression could influence the proliferative behavior of transformed cells. Importantly, no significant variations were observed between the two wild type and the two knock out cell clones utilized (Fig. 8D), demonstrating that the differences observed between p27<sup>+/+</sup> and p27<sup>-/-</sup> v-src fibroblasts were not due to clonal selection.

**Figure 8: In vivo growth of v-src transformed cells.** 1x10<sup>6</sup> cells were injected subcutaneously in the flank of nude mice and mice have been sacrificed 15 days after. In A and B representative picture of the injected mice (A) and explanted tumors (B) are shown. C. The graph displays the tumor weight. The differences between p27wt and p27ko v-src cells are highly significant (p<1x10<sup>-5</sup>). D. The average tumor weight for each cell clones is reported. Whereas the differences among the two p27wt or the two p27ko clones are not statistically significant, T test results significant (p<0.006) for all the other compared couples (p27wt#A4 vs p27ko#3, p27wt#A4 vs p27ko#11, p27wt#A6 vs p27ko#3, p27wt#A6 vs p27ko#11).
2. p27 expression discriminates between mesenchymal and amoeboid morph-dynamics.

Recently, the role of p27 in cell motility has become a debated issue in the scientific literature. We previously demonstrated a reduction in 3D cell migration due to the p27 cytoplasmic expression (Baldassarre et al., 2005). Moreover, we also observed different morphological features in cells expressing or not expressing p27 included in a 3D environment (Belletti et al. in preparation and personal observations). We thus speculated that p27 could be involved in the regulation of cell morph-dynamics during oncogenic transformation. To test this hypothesis, p27<sup>+/+</sup> and p27<sup>-/-</sup> v-src fibroblasts were included in 3D collagen matrices and studied by time-lapse microscopy and immunofluorescence analyses. Their morphology was first evaluated at different time points by transmission microscopy in bright field to better discriminate the cell distribution along the three axes (Fig. 9A). v-src transformed fibroblasts included in 3D matrices acquired typically two different shapes: an elongated fibroblast-like spindle shape, with finger-like protrusion at the cellular edges or a rounded amoeboid-like morphology, characterized by numerous peripheral membrane ruffles. Counting the rounded versus the elongated cells after 10 hours from inclusion, we verified that while p27<sup>WT</sup> fibroblasts displayed a similar percentage of elongated and rounded cells (47.8% and 52.2±7.01% of elongated and rounded, respectively), p27<sup>KO</sup> v-src fibroblasts acquired an almost exclusively rounded morphology (14% and 86±3.3% elongated and rounded, respectively. Fig 9B).

![Figure 9: Morphologic characterization of v-src transformed cells. A. Representative pictures of typical morphology acquired by p27wt and p27ko transformed fibroblasts after 10 hours of inclusion in 3D collagen I matrix. The pictures were collected in bright field using the 10X objective. B. Percentage of cells characterized by the two different morphology (the elongated spindle shape and the rounded amoeboid-like morphology). The results were obtained counting 5 fields for each independent experiment. The dead cells were excluded by the count.]
Results

We also evaluated the cytoskeletal structures of transformed cells in 3D using immunofluorescence and confocal microscopy analysis. p27\textsuperscript{WT} and p27\textsuperscript{KO} v-src fibroblasts were cultured for 10 hours in collagen matrix and then fixed and stained for tubulin (green) and actin (red) (Fig. 10). The analysis highlighted the structural differences between cell expressing or not expressing p27. Most of transformed p27\textsuperscript{+/+} cells retained a fibroblast-like elongated morphology with cellular protrusions supported both by the microtubule network and actin stress fibers. Conversely, p27\textsuperscript{-/-} v-src cells mainly displayed a rounded shape, lose the dendritic-like extensions and showed an actin cortical distribution and a lower content of microtubule (Fig. 10).

The same morphological differences were observed also performing time lapse video microscopy of spheroids included in 3D collagen matrix.

Spheroids are cell clusters obtained by seeding cells in not-adhesive Petri dish or in polyHEMA-coated plates, thus favouring the cell-cell contacts and in turn the formation of cellular clumps. After 24 hours spheroids are collected and included in a 3D matrices to evaluate the 3D cell morphology and motility in a different condition than individual cell inclusion.

As shown in the photogram at 3, 6 and 9 hours from inclusion and in the movies resulting from the collection of pictures taken every 4 minutes for at least 12 hours, p27\textsuperscript{-/-} v-src cells
Results

detached by the cell cluster retaining a rounded cell shape and showing a higher deformability (Fig. 11 and supplementary movie). They were able to squeeze through the collagen lattices, moving in a propulsive way all around with a reduced directional persistence. Conversely, p27\(^{+/+}\) transformed fibroblasts moved outside the cluster using long protrusions to generate the traction forces needed for the cell body advancing (Fig. 11 and supplementary movie). All these features well fit with the described model for the amoeboid and the mesenchymal morph-dynamics, respectively (Friedl and Wolf, 2003). Thus, our results demonstrate that p27 control the shape and the type of motility during v-src transformation process, since its absence favors the acquirement of an amoeboid motility.

3. The amoeboid motility of p27\(^{-/-}\) v-src cells is associated with higher cell speed and invasive ability.

To evaluate if the two diverse cell shapes observed in p27 wt and null cells could be associated to a different migration rate in vitro, we performed a time lapse video microscopy and cell tracking analysis of individual cells included in 3D collagen matrices (Fig. 12 and supplementary movies). We analysed the tracking parameters of 40 individual cells in two independent experiments. Moreover, we evaluated both the cell speed (that describes overall motility as a function of the fraction of locomoting cells and their locomotors duration, individual step lengths and step number) and cell velocity (that describes the actual translocation efficiency of cells in the process of migration, without considering the stopping frequencies). Analysing both these parameters, p27\(^{-/-}\) v-src cells displayed a higher migration rate than p27\(^{+/+}\) transformed cells, with a speed value of 0.227\(\mu\text{m/min}\) versus 0.118 \(\mu\text{m/min}\)

Figure 11: Spheroid assay of transformed cells in 3D collagen matrix. Spheroids obtained by seeding cells in polyHEMA-coated plates were included in 3D collagen matrix and evaluated in bright field collecting one picture every 4 minutes for at least 12 hours. Here, photogram collected after 3, 6 and 9 hours are reported.
and a velocity of 0,33\(\mu\)m/min \textit{versus} 0,22\(\mu\)m/min. (Fig. 12A, B, C). In both cases the differences between the two cell lines resulted highly significant \((p<0,0001)\), demonstrating that p27 expression is an important determinant in the control of 3D locomotion.

\textbf{A}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cell_speed.png}
\caption{Cell speed}
\end{figure}

\textbf{B}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cell_velocity.png}
\caption{Cell velocity}
\end{figure}

\textbf{C}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cell_analysis.png}
\caption{Cell analysis}
\end{figure}

\textbf{Figure 12: Locomotion parameters of 3D motility.} After time lapse video microscopy of p27wt and p27ko v-src fibroblasts included in 3D collagen matrix, the analysis of cell speed (A) and velocity (B) was performed using a semi-automatic cell tracking software. Two independent experiments were performed and 40 cells for each cell line were tracked in each experiment. The obtained data result highly significant \((p<0,0001)\) both in cell speed and in cell velocity analysis (C). The cells were followed for 20 hours collecting one picture every 5 minutes. The step interval for tracking analysis corresponds to 12 minutes.
The different mobility is well represented also by the assumed cell trajectories that described how p27\(^{\text{KO}}\) transformed cells were able to cover longer paths compared to wild type in the same time frame (Fig. 13).

![Cell paths in 3D environment](image)

**Figure 13: Cell paths in 3D environment.** Using the cell tracking software, the trajectories assumed by the cells in 3D collagen matrix were collected.

Moreover, cell migration was assessed by an evasion assay using both Matrigel and collagen I matrices. In agreement with the tracking data, the obtained results showed that p27\(^{-/-}\) v-src cells displayed a higher ability than p27\(^{+/+}\) to migrate outside the drops, confirming again that p27 null cells had a higher 3D mobility (Fig 14).

![Evasion ability of p27wt and p27ko v-src transformed fibroblasts](image)

**Figure 14: Evasion ability of p27wt and p27ko v-src transformed fibroblasts.** Individual cells (7.5x10^3 cells/ml) were included in Matrigel or collagen I drops to evaluate their ability to exit from the matrices in response to chemotactic stimuli. The pictures shown a typical field of cells exited from the matrices drops 24 hours after the inclusion. The yellow dashed line indicates the drop edge.
Results

In order to evaluate if the differences in cell migration observed in 3D systems could be ascribed to an impaired adhesive function during cell-ECM interactions, we tested p27<sup>+/+</sup> and p27<sup>-/-</sup> v-src cells for their ability to attach to different ECM substrates. We performed a CAFCA assay (Centrifugal Assay for Fluorescence-based Cell Adhesion; Spessotto et al., 2000) by seeding fluorescent labelled cells in flexible polyvinyl chloride miniplates (well’s surface comparable to the 96well plates) coated with vitronectin, fibronectin and collagen I (BSA as control) and then assaying their adhesion to substrates with reverse centrifugation. The relative number of bound versus the unbound cells demonstrated that no significant differences exist in the adhesive properties between p27<sup>+/+</sup> and p27<sup>-/-</sup> v-src cells (Fig. 15).

Being v-src involved in focal adhesion turnover and in many signal transduction pathways activated after cell-ECM contact, we evaluated the activation of the main signalling pathways after adhesion of p27<sup>+/+</sup> and p27<sup>-/-</sup> v-src cells. Since most of motility experiments were performed in collagen I matrices, we analysed the cell signaling after 15, 30 and 60 minutes of adhesion to collagen coated plates (20µg/ml). The results showed no massive differences in the total amount of phosphorylated proteins between p27wt and null cell clones, even if p27ko v-src cells tended to have a different activation kinetics of some signal transduction pathways. They in fact displayed a faster activation in particular of FAK, Stat3 and RhoA (evaluated using as read-out the phosphorylation of cofilin on S3, an accepted marker of the activation of Rho-ROCK signals) pathways at 15’of adhesion, whereas p27wt cells showed a more gradual activation during the different time points (Fig. 16).

![Figure 15: Adhesive behaviour of p27wt and p27ko v-src transformed fibroblasts towards ECM substrates.](image)
Figure 16: Activation of signaling pathways after adhesion to collagen substrate. A. p27wt and ko cells were adhered for 15, 30 and 60 minutes to collagen coated dishes and then lysated and analyzed by western blot using phospho-antibodies specific to detect the phosphorylated residues of ERK1/2, Cofilin, FAK, Stat3 and AKT proteins. The total levels of the same proteins are also shown. B. Quantification of the blots shown in A (as indicated) expressed as the ratio of the phosphorylated/total protein levels. Quantification was performed using the Odyssey software.
We next evaluated the ability of p27\(^{+/+}\) and p27\(^{-/-}\) v-src cells to move and metastasize \textit{in vivo}. To this aim the mice injected subcutaneously with p27\(^{+/+}\) and p27\(^{-/-}\) v-src cells (\(n=9\) for genotype) were analyzed for the presence of transformed cells in the spleen by RT-PCR with primers able to recognize the exogenous circulating cells as described in the methods section. Interestingly, while none of the mice injected with p27\(^{WT}\) v-src cells (0/9) showed the presence of circulating transformed cells, 4/9 mice injected with p27 null cells resulted positive (Fig. 17A,B). Moreover, the ability to settle and colonize distant sites was evaluated. In a pilot experiment we injected transformed fibroblasts in the tail veins of nude mice (\(n=4\) for genotype). The mice were sacrificed when they displayed breathing failure and fatigue (1 month later), and just by macroscopic analysis of the lungs, we found a huge difference, with many tumor foci induced by p27\(^{-/-}\) v-src cells and only few foci induced by p27\(^{+/+}\) v-src cells (Fig. 17C).

\begin{figure}
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\includegraphics[width=\textwidth]{figure17.png}
\caption{\textit{In vivo} motility of p27\textit{wt} and p27\textit{null} transformed fibroblasts. A. The mice injected s.c. with v-src cell clones as indicated (\(n=9\) for genotype), were analysed for the presence of circulating cells performing an RT-PCR on RNAs extracts from mouse spleens and amplifying the resulted cDNA with primers specific for transformed cells. In the diagram the positive \textit{versus} the negative spleens are displayed. B. A representative RT-PCR is shown. The ribosomal 18S subunit was used as control of RNA state and amount. As positive control (+), the cDNA derived by one primary tumor was used. C. Representative picture of lung metastasis formed 1 month after the injection of v-src transformed fibroblasts in the tail veins of nude mice (\(n=4\) for genotype).}
\end{figure}
All the *in vitro* and *in vivo* results demonstrated that the absence of p27 in v-src transformed fibroblasts induces an increased proliferation coupled with a motile advantage that could be ascribed to the switch from a mesenchymal to an amoeboid-like motility.

4. **p27 expression in p27<sup>-/-</sup> v-src cells reduced proliferation and tumor growth.**

To definitively prove that the observed effects on v-src cell growth and motility were due to p27 itself, we reintroduced the p27 cDNA in p27<sup>KO</sup> null cells obtaining several clones and mass transfections. The only one strategy that allowed us to create clones stably expressing p27 at levels comparable to the endogenous protein, consisted in first transducing p27 in 3T3-p27<sup>KO</sup> cells and then transforming them with v-src. Conversely, when we tried to re-express p27 wild type (p27<sup>WT</sup>) protein in v-src transformed p27 null cells we never obtained stable p27 independently on the strategies used (data not shown). However, in all the clones obtained and characterized, the v-src protein was expressed at comparable levels as by western blot evaluation (Fig. 18).

To reduce the v-src induced degradation of p27 wild type protein, we used the cDNA encoding for two less degradable form of p27, namely p27<sup>T187A</sup> and p27<sup>1-170</sup> (Fig 18). p27<sup>T187A</sup> carries a point mutation that results in the substitution of the threonine 187 with an alanine, thus impairing p27 degradation via the ubiquitin-dependent proteasome pathway (Montagnoli et al., 1999; Vlach et al., 1997). The deletion mutant p27<sup>1-170</sup> also lacks this residue, resulting in turn less degradable, and, as we previously demonstrated, retained the ability to block cell proliferation while loose the migratory inhibitory properties of the wild type protein (Baldassarre et al., 2005). It is to note that we obtained some cell clones expressing the p27<sup>1-170</sup> mutant also by transducing the v-src p27<sup>KO</sup> clones 3 and 11. The biological behaviour of these clones was identical to the one observed in the p27<sup>1-170</sup> expressing cells derived from first transducing p27 in 3T3-p27<sup>KO</sup> cells and then transforming them with v-src. These observations demonstrate that is the expression of p27 and not the way by which it is obtained to influence the biological behaviour of v-src transformed cells. For simplicity we will present in the next part of this thesis only the results obtained by the characterization of cell clones expressing p27<sup>T187A</sup> and p27<sup>1-170</sup> derived from first transducing p27 in 3T3-p27<sup>KO</sup> cells and then transforming them with v-src since this represents the only way to co-express p27<sup>T187A</sup> and v-src in p27 null cells.
Results

Proliferation assays of v-src transformed fibroblasts demonstrated that both p27 mutants were able to reduce cell proliferation to the levels observed in the p27<sup>WT</sup> v-src cells, as shown by growth curve obtained seeding 1x10<sup>5</sup> cells and counting them for other four days (Fig. 19A, B).

Western blot analysis of cell cycle regulating proteins demonstrated that all the analyzed cell clones expressed similar amount of cyclin A, cyclin B1, CDK1 and CDK2 (Fig. 20A). However, kinase assays demonstrated that, although with little clonal variability, reintroduction of both p27<sup>T187A</sup> and p27<sup>1-170</sup> strongly reduced the CDK activity associated to both cyclin A and cyclin B1 (Fig. 20B) confirming that the expression of p27 is able to...
negatively regulate cell proliferation through an inhibitory effect on the cyclin/CDKs complexes activity.

We next evaluated whether p27 could rescue also the transformed and tumorigenic potential in vitro and in vivo. First using soft agar assay, we showed that both p27\textsuperscript{T187A} and p27\textsuperscript{1-170} were able to reduce anchorage independent cell growth in term of both size and number of the colonies (Fig. 21A, B) demonstrating that during v-src transformation p27 is an important determinant for cell growth in semisolid media.

![Figure 20: Cyclin-CDK expression and activity in v-src p27-rescued cell lines. A. The expression of cyclin A, cyclin B1, CDK1 and CDK2 in exponentially growing cells was evaluated by western blot. Vinculin was used to normalize the protein lysates. B. A representative panel of kinase assay performed after immunoprecipitation for cyclin A (marker for S phase) and cyclin B1 (marker for G2-M phases). The Histon H1 was used as substrate to evaluate the rate of phosphorylation (with γATP-P\textsuperscript{32} radioactive labelling).](image)

![Figure 21: Analysis of the anchorage-independent growth in p27-rescued clones. A. A soft agar assay was performed using two different clones for each cell lines. The diagram was obtained counting, with 10x objective, the colonies in 15 fields for each cell lines, p27\textsuperscript{T187A} and p27\textsuperscript{1-170} significantly reduced colonies number compared to p27ko v-src clones (*: p=0.007 and §: p<0.0001). B. A representative picture of colonies in 3D agar is reported to show the different colony sizes obtained by each cell line.](image)
This effect was paralleled by \textit{in vivo} analysis of tumor growth in nude mice after subcutaneously injection ($n=6$ for p27$^{+/+}$ and p27$^{-/-}$ v-src cells, $n=8$ for p27$^{T187A}$ rescued mass transfections and $n=9$ for p27$^{1-170}$ rescued clones) showing that p27 expression in p27$^{-/-}$ transformed cells significantly reduced the weight of tumors formed by v-src transformed fibroblasts (Fig. 22).

![Figure 22: \textit{In vivo} tumor growth of p27-rescued fibroblasts. 1x10$^6$ cells were injected s.c. in nude mice and after 15 days mice were sacrificed and the tumor masses evaluated. In the diagram the tumor weight is reported. p27$^{T187A}$ and p27$^{1-170}$ significantly reduced tumor growth compared to p27ko v-src clones ($\ast$ and $\S$: $p<0.0001$)](image)

5. p27$^{T187A}$ but not p27$^{1-170}$ rescued cell shape and motility.

We next analysed the 3D-cell shape and the motile behaviour of p27-rescued cell lines in order to verify whether p27$^{T187A}$ and p27$^{1-170}$ had any effects in the control of cell shape and motility. First, we included cells in 3D collagen matrices and analysed their morphology after 10 hours from the inclusion. As described above (Fig. 9), also in p27-recued clones we can discern two diverse 3D cell shapes, namely elongated and rounded morphology. Interestingly, most p27$^{T187A}$ transformed cells clearly appeared to have the elongated and bipolar shape, in a proportion similar to the one observed in p27$^{+/+}$ v-src cells (Fig. 23A). On the contrary, almost all p27$^{1-170}$ cells resulted rounded, as described for p27$^{1-170}$ v-src cells (Fig. 23A).

This result was then confirmed by counting the rounded versus the elongated cells in five different fields for each experiment (Fig. 23A, B). p27$^{T187A}$ cells displayed about 50\% of rounded and 50\% of the elongated shape (50,4±16\% versus 49,6\%), comparable to p27$^{+/+}$ transformed cells, having the 52,2±7,01\% of elongated and 47,8\% of rounded cells. On the contrary, 96±2,9\% of the p27$^{1-170}$ population showed a rounded shape, resulting more similar to the p27$^{-/-}$ v-src cells (86±3,3\% of rounded shape) (Fig. 23B).
The structural differences were evaluated by confocal microscopy analysis after staining of α-tubulin (green) and polymerized actin (red). p27T187A cells, as the p27WT, appeared to have an elongated spindle shape based both on microtubules and actin stress fibers content, while p271-170 expression was not able to rescue the morphological phenotype of p27 null v-src fibroblasts and these cells still displayed a typical rounded amoeboid-like shape with cortical actin distribution (Fig 24).

Figure 23: Morphologic characterization of p27-rescued transformed cells. A. Representative pictures of the morphology acquired by p27T187A and p271-170 p27wt and p27ko v-src transformed fibroblasts after 10 hours of inclusion in 3D collagen I matrix. The pictures were collected in bright field using a 10X objective. B. Quantification of the elongated and rounded cell population in the indicated cell lines shown as percentage of the total cells analyzed. The results represent the mean (± SD) of cells counted in 5 different fields for each independent experiment (n=3). The dead cells were excluded by the count.
Interestingly also the behaviour of cell clusters support the data coming from the individual cells analysis. The spheroids obtained maintaining the cells in suspension for 24 hours, were included in 3D collagen matrix and a time lapse video microscopy was performed (Fig. 25 and supplementary movies). The expression of p27<sup>T187A</sup> reverted the p27<sup>KO</sup> phenotype and cells in 3D displayed long cellular protrusion (Fig. 25) that allowed the cells to detach from the cell cluster using the mesenchymal mechanism. On the contrary, p27<sup>1-170</sup>-rescued cells showed a different clustering ability and detached from the spheroid in an amoeboid manner, showing a markedly spherical shape (Fig. 25), highly dynamic membrane blebs and squeezing through the collagen lattices in a very flexible way (supplementary movies).

Figure 24: Cytoskeletal structures in p27-rescued transformed cells in 3D matrix. Cells included in 3D collagen I matrix were evaluated 10 hours after the inclusion for their actin and tubulin content. The actin network was stained with Phalloidin-AlexaFluor546 (RED), whereas for tubulin detection FITC-conjugated monoclonal anti-α-tubulin antibody (GREEN) was used.
Results

6. Migration rate and invasive potential were reduced after p27<sup>T187A</sup> expression.

The different motility acquired by p27-rescued clones in 3D matrices was coupled by a higher ability of p27<sup>1-170</sup> cells to evade from 3D matrices, as demonstrated by evasion assays of individual cells included in Collagen I and Matrigel drops. As shown in Fig. 26A, 24 hours after inclusion only few p27<sup>+/+</sup> v-src and p27<sup>T187A</sup> rescued cells were present outside the matrix. Conversely, p27<sup>1-170</sup> expression was not able to reduce cell migration of p27 null v-src cells and many cells were present outside the drops in the same time frame (Fig. 26A).

Counting all the evaded cells (five drops for each cell lines) we confirmed the result in both the collagen and the Matrigel matrix, suggesting a similar motile behaviour in both the matrices (Fig. 26B).

Figure 25: Spheroid assay of p27<sup>T187A</sup> and p27<sup>1-170</sup> v-src cells in 3D collagen matrix. Spheroids obtained by seeding cells in Petri dish were included in 3D collagen matrix and evaluated in bright field by collecting one picture every 5 minutes for at least 12 hours. Here, photogram collected with a 20X objective after 3, 6 and 9 hours are reported.
To analyse the in vivo invasive potential, we evaluated the presence of circulating transformed cells by analyzing the spleen of the mice injected subcutaneously with p27-rescued clones. After RT-PCR of RNA extracted from spleen, we performed a nested PCR with two different couples of primers in order to amplify the exogenous cDNAs and specifically detect only the injected transformed cells. This analysis showed that only 1/7 mouse injected with p27\(^{T187A}\)-rescued cells displayed a positive spleen (corresponding to the 14.2%), whereas 3/5 mice injected with p27\(^{1-170}\)-rescued cells had circulating cancer cells (corresponding to the 60%) (Fig. 27).

**Figure 26: Evasion ability of p27-rescued transformed fibroblasts.** A. Individual cells (7.5x10^3 cells/ml) were included in Matrigel and collagen I drops to evaluate their ability to exit from the matrices. Typical images of matrix drops incubated for 24 hours from inclusion at 37°C are shown. B. Quantification of the experiment shown in A. The number of cells evaded from matrix drops within 24 hours is reported. p27\(^{T187A}\), but not p27\(^{1-170}\) (p=ns), significantly reduces matrix cell evasion compared to p27ko cells (p<0.001).
Moreover, in order to assess the ability to colonize distant organs \textit{in vivo} and based on the observation obtained by our pilot experiment (Fig. 17C), we injected in the tail veins 4 mice with p27\textsuperscript{+/+} and p27\textsuperscript{-/-} v-src cells as control, 7 mice with two different p27\textsuperscript{T187A} rescued cell lines and 6 mice with two different p27\textsuperscript{1-170} rescued clones. The mice were sacrificed after 20 days from injection. Lungs were fixed in Bouin’s solution and first macroscopically analyzed. In agreement with the results above reported, p27\textsuperscript{-/-} v-src cells showed a higher ability to settle and create tumor foci in the lungs (an average of 10.5 macroscopic foci for mouse), while p27\textsuperscript{+/+} cells were much less aggressive (with none macroscopically evident focus) (Fig. 28A, B). Interestingly, mice injected with p27\textsuperscript{T187A}-rescued cells displayed on average 1.95 foci/animal, while the p27\textsuperscript{1-170} counterpart showed 6.41 foci (Fig. 28B).

Together these results demonstrate that p27 expression at least in v-src transformed fibroblasts is able to reduce both cell migration rate \textit{in vitro} and invasion ability \textit{in vivo}. Importantly, p27 controls these functions by regulating cell plasticity and these effects are independent on its ability to block cell cycle progression.

\textbf{Figure 27:} \textit{In vivo} metastatization of p27-rescued transformed fibroblasts. A. Nude mice injected s.c. with the different cell clones were analysed for the presence of circulating cells by performing an RT-PCR analysis on spleen samples as reported in figure 18. In the diagram the positive (black) \textit{versus} the negative (grey) spleens are displayed. B. The same results are shown as percentage of positive spleen versus total animals analysed.

Moreover, in order to assess the ability to colonize distant organs \textit{in vivo} and based on the observation obtained by our pilot experiment (Fig. 17C), we injected in the tail veins 4 mice with p27\textsuperscript{+/+} and p27\textsuperscript{-/-} v-src cells as control, 7 mice with two different p27\textsuperscript{T187A} rescued cell lines and 6 mice with two different p27\textsuperscript{1-170} rescued clones. The mice were sacrificed after 20 days from injection. Lungs were fixed in Bouin’s solution and first macroscopically analyzed. In agreement with the results above reported, p27\textsuperscript{-/-} v-src cells showed a higher ability to settle and create tumor foci in the lungs (an average of 10.5 macroscopic foci for mouse), while p27\textsuperscript{+/+} cells were much less aggressive (with none macroscopically evident focus) (Fig. 28A, B). Interestingly, mice injected with p27\textsuperscript{T187A}-rescued cells displayed on average 1.95 foci/animal, while the p27\textsuperscript{1-170} counterpart showed 6.41 foci (Fig. 28B).

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Together these results demonstrate that p27 expression at least in v-src transformed fibroblasts is able to reduce both cell migration rate \textit{in vitro} and invasion ability \textit{in vivo}. Importantly, p27 controls these functions by regulating cell plasticity and these effects are independent on its ability to block cell cycle progression.
Results

Figure 28: In vivo lung colonization of p27-rescued transformed fibroblasts. A. Representative pictures of the ribcages of mice injected in the tail veins with p27wt and p27ko v-src cells and sacrificed after 20 days. While it appears “macroscopically” tumors-free in the case of p27wt cells, in the case of p27ko injection many tumor foci and colonies are present on both lungs and on the ribcage wall, suggesting a severe respiratory dysfunction. B. Representative pictures of explanted lungs of nude mice injected in vein with p27T187A and p271-170-rescued compared to p27wt and p27ko v-src transformed fibroblasts. Lungs were fixed in Bouin’s solution. The red arrows pinpoint the presence of tumor foci.
7. **H-Ras transformation induces in p27<sup>-/-</sup> the same proliferative advantage displayed after v-src over-expression.**

Based on the interesting features differentially displayed by p27<sup>+/+</sup> and p27<sup>-/-</sup> cells after v-src transformation, we asked whether the proliferative and motile behaviour, as well as the morphologic plasticity, linked to p27 expression could be oncogene-specific or could be reproduced by the activation of other oncogenetic signals. To this aim, we transduced 3T3-p27<sup>+/+</sup> and 3T3-p27<sup>-/-</sup> murine fibroblasts with Harvey-Ras oncogene (H-Ras) and different cell clones were selected. Similar levels of H-Ras were observed in the different cell clones and in both p27<sup>+/+</sup> and p27<sup>-/-</sup> clones the activation of two downstream Ras kinases, ERK1 and 2, was readily observed (Fig. 29). In p27<sup>WT</sup> cells the endogenous p27 levels were not reduced by Ras transduction (Fig. 29 last lane).

The selected clones were characterized for their growth ability by FACS analysis, growth curve and MTT proliferation assay. As in the case of v-src transformation, the absence of p27 was linked to a higher proliferation rate also in H-Ras transformed cells, as shown by the percentage of cells progressing along the S-phase in exponentially growing conditions (38.3% of p27<sup>-/-</sup> versus 17% of p27<sup>+/+</sup> H-Ras cells (Fig. 30A) and by growth curve experiments (Fig. 30B, C).
Next we evaluated the effects of serum starvation and cell-cell contact inhibition (typical anti-proliferative signals for normal cells) in p27<sup>+/+</sup> and p27<sup>−/−</sup> H-Ras transformed fibroblasts (Fig. 31). H-Ras clones were serum starved for 24 hours and then re-stimulated with medium containing 10% Fetal Bovine Serum (FBS) and harvested at the indicated time points (9, 15 and 21 hours). The FACS (Fig. 32A) and western blot (Fig. 31B) analyses demonstrated that H-Ras transformed cell lines still retained a certain sensitivity to growth factors depletion and both genotype accumulate in G1 after serum deprivation. Even though both cell lines re-entered the cell cycle with similar kinetics, p27 null cells progressed through the S and G2 phase faster than the wt counterpart (Fig. 31A). In fact, already after 15 hours of release in complete medium, p27<sup>−/−</sup> transformed cells were consistently accumulated in G2-M phases, as confirmed by the high expression of cyclinB1 (Fig. 31B), and after 21 hours resulted

![Figure 30: Proliferative behaviour of H-Ras transformed cells. A. Cell distribution in the cell cycle phases was evaluated in exponentially growing cells by FACS analysis after PI staining. The numbers indicate the cell percentages in the different phases. B. Growth curve of H-Ras transformed fibroblasts is shown. 1x 10<sup>5</sup> cells/well were plated in triplicate day 0 and counted each day for 5 days. C. 1x10<sup>3</sup> cells/well were seeded in sixth-plicate in 96well plates to perform the MTT assay. Two different clones were used for each genotype. The results were obtained measuring and quantifying the colorimetric reaction at 560nm in Elisa plate reader each day for 4 days.](image-url)
already accumulate in the G1 phase of a new cell cycle, whereas p27wt cells were still progressing through the G2-M phases (Fig. 31A,B).

Then we asked whether the response to contact inhibition was similar in p27wt and ko Ha-Ras fibroblasts. Interestingly, while p27wt cells retained a certain sensitivity to growth inhibition after achievement of confluence, p27ko cells still proliferate (Fig. 31C) confirming the importance of p27 in the control of growth arrest induced by cell-cell contact.

**Figure 31:** Proliferation after serum starvation and achievement of confluence in H-Ras transformed fibroblasts. **A.** FACS analysis of transformed cells after 24 hours of serum starvation (T0) and then released in complete medium for 9, 15 and 21 hours. **B.** Western blot analysis of cells treated as in A and evaluated for the expression of cyclinA, cyclinB1 and p27. **C.** FACS analysis of H-ras transformed cells after the achievement of confluence. The percentage of the cells in the different cell cycle phases is reported.

Based on the results demonstrating a faster progression along the cell cycle of p27KO transformed cells, we asked whether they properly progressed along the M-phase. To this aim, we synchronize cells through a double thymidine block followed by nocodazole treatment as
Results

reported in the methods section to block the cells in pro-metaphase. Cells were then released in complete medium from nocodazole block and analyzed by FACS after 30, 60, 90, 120 and 180 minutes to follow their progression along mitosis (Fig. 32).

Our data suggested that p27 absence did not alter the time to complete M phase in mouse fibroblasts transformed with Ras but conversely it rendered the cells more sensitive to the M phase block induced by nocodazole (Fig. 32). In fact the biggest difference between p27 wt and null cells was observed in nocodazole arrested cells in which the percentage of cells in the G2/M phase was significantly higher in p27ko cells (71%) respect to wt cells (65%). This effect is in line with our previous studies that demonstrated the lower MTs stability of p27ko cells due to the higher stathmin activity (Baldassarre et al., 2005).

![Figure 32: Analysis of progression along M phase.](image)

To test the effective tumorigenic potential of H-Ras transformed cells and to evaluate the effects of p27 expression in this context, we first analysed the ability to grow in an anchorage independent way using the soft agar assay. The obtained results demonstrated that p27-/- H-Ras cells formed significantly more colonies in comparison to the wt counterpart (Fig. 33).
We next analysed the *in vivo* ability of H-Ras transformed cells to grow and induce tumors. We injected nude mice with two different clones derived from p27wt and p27ko fibroblasts transformed with H-Ras.

In a preliminary experiment conducted on 5 mice/genotype we demonstrated that 1x10^6 of p27ko-H-Ras cells formed in all mice a visible tumor after 12 days from the initial subcutaneous injection of the cells while the same number of p27wt-H-Ras cells did not form tumors.

For this reason in the next experiment we injected 2x10^6 p27wt or ko-H-Ras cells (Fig. 34). Under these conditions, tumors from p27ko-H-Ras cells became visible at day 10, while at the same time point the p27wt-H-Ras formed only very small tumors often not even appreciable at the palpation. Mice were sacrificed for humanitarian reasons when the biggest tumor reached the volume of approximately 800 mm^3, 22 days after cells injection. At this time point all animals of the experiment were sacrificed and tumors excised and analyzed. As shown in figure 35, p27ko-H-Ras cells formed tumors bigger in size (Fig. 34A, B) and weight (0.96g *versus* 0.047g of p27wt cells. Fig. 34C) compared to the wt counterpart. No significant difference was observed between the two different clones used for each genotype (data not shown).
8. p27\textsuperscript{−/−} fibroblasts displayed an increased cell motility also after H-Ras transformation.

To evaluate whether H-Ras transformed cells were differentially able to migrate or invade the ECM components as observed in v-src model, p27\textsuperscript{+/+} and p27\textsuperscript{−/−} fibroblasts were analysed in Matrigel evasion assay and in haptotactic-like migration assay toward fibronectin (Fig. 35). The results demonstrated that the absence of p27 conferred higher ability to exit from 3D matrices, as shown in Figure 35A, since p27\textsuperscript{+/−} H-Ras cells are consistently present outside the matrigel drops after 2 days from inclusion, whereas p27\textsuperscript{+/+} cells are still entrapped in the matrix (Fig. 35A). Moreover, p27\textsuperscript{+/−} H-Ras cells displayed also an increase in the migration rate in haptotactic-like experiments, compared to p27 wt counterpart (Fig. 35B).
Further, we tested in vivo motility by analysing the metastatization potential of p27^{+/+} and p27^{-/-} H-Ras cells after injection in nude mice. Thus all the animals injected s.c. were analysed for the presence of circulating transformed cells in the blood and for the presence of distant metastasis in lungs by RT-PCR. Data shown that p27^{WT} Ras cells were not able to invade the blood or the lungs while p27 null tumour cells were present both in the blood (3/5) and in the lungs (4/5) in p27ko of nude mice (part of these results are reported in Fig. 39), demonstrating that p27 absence increased the metastatic potential also of H-ras transformed fibroblasts.


In order to understand whether the expression of p27 protein was able to revert the proliferative and motile phenotype shown in vitro and the oncogenic potential shown in vivo, we de novo expressed p27 cDNA into p27^{-/-} H-Ras cells. We used a p27 wild type form and the deletion mutant lacking the last 28 aminoacids (p27^{1-170}) described above to generate stable mass transfections (Fig. 36).
Results

As in the case of v-src-induced transformation model, we first evaluate the proliferative behaviour of p27-rescued cell lines. Surprisingly, the expression of both p27 forms in cells already transformed by H-Ras, did not significantly reduce the *in vitro* proliferation rate, as demonstrated by growth curve and MTT assay (Fig. 37A, B).

![Figure 36: p27-rescued clones selection.](image)

**Figure 36: p27-rescued clones selection.** p27ko H-Ras fibroblasts were transduced with p27wt and p271-170 (170), in order to obtain stable p27-rescued mass transfections. In the western blot analysis the expression of p27 and Ras is shown. Vinculin was used to normalize the blot.

![Figure 37: Proliferative behaviour of p27-rescued clones.](image)

**Figure 37: Proliferative behaviour of p27-rescued clones.** The proliferation rate of p27wt and p271-170-rescued H-Ras fibroblasts was tested performing growth curve (A) and MTT assay (B) following the previously described procedures.

Although in soft agar assay p27 rescue induced a significant reduction in the numbers of colonies compared to p27ko cells, the decrease was not sufficiently to completely revert the phenotype to the levels of p27wt cells. In fact, the colonies number induced by p27rescued cells resulted significantly higher compared to p27wt cells (Fig. 38A). Moreover, after
injection in nude mice, the expression of p27 in both forms was not sufficient to reduce tumor growth (Fig. 38B).

All the results collected about the proliferative behaviour suggest that additional genetic alterations probably came out after H-Ras transformation and made the phenotype not more reversible with the expression of p27.

Interestingly, when motility of p27-rescued cells was analysed, we noticed that the reintroduction of p27wt cDNA but not p27<sup>1-170</sup> in H-Ras transformed cells strongly affected cell motility both in vitro (in evasion assay. Fig. 39A) and in vivo, as analysed by RT-PCR looking for circulating cells in the blood and for distant metastasis in lungs (Fig. 39B).

All together, the results collected using the H-Ras model well confirm those obtained after v-src-transformation, suggesting that the proliferative and motile advantage, displayed after oncogene transformation in cells lacking p27, could be, at least in part, ascribed to common “oncogene-independent” pathways.
Figure 39: Characterization of motile behaviour in p27-rescued H-Ras transformed fibroblasts. A. The *in vitro* motility was assayed by evasion assay. The exit of the cells from Matrigel drops was evaluated as the migration distance covered by the cells in 5 days. To measure this parameter, a crystal violet staining was previously performed and representative pictures of stained drops are shown below the diagram. B. Representative nested PCRs performed on lungs and blood samples to detect exogenous cells are reported. Tumors were used as positive control. The results were confirmed performing PCR three times. C. The results obtained by nested PCR are described as diagram. Each column represent the number of the obtained positive samples. 5 mice for genotype were analyzed.
DISCUSSION

Uncontrolled cell proliferation as well as tissue invasion and metastasis represent two of the most important hallmarks in cancer (Hanahan and Weinberg, 2000). Metastasis is one of the main cause of death in cancer patients affected by several types of neoplasia and the progression to a metastatic tumor phenotype often is associated to a worse prognosis (Condeelis and Segall, 2003). Thus, one of the most successful strategy in cancer treatment could be represented by the combination of drugs that minimize the growth of existing tumors and, at the same time, limit their spread and diffusion to new sites. But, while treatments for primary tumor growth have clear and measurable end points, the development of anti-metastasis treatments is more difficult and will depend on a detailed understanding of the basic steps in metastasis formation, beginning at the primary tumor and ending with the establishment of new tumors in distant organs (Condeelis and Segall, 2003).

The recent advances in techniques of in vivo imaging and in the use and development of animal models have clearly demonstrated the complexity of motile strategies and molecular adaptive responses displayed by the cells when placed in a more physiological context than classical experimental 2D environment (Friedl and Wolf, 2003; Wolf and Friedl, 2006; Sahai and Marshall, 2003; Condeelis and Segall, 2003). Moreover, emerging evidences have suggested that the several described migration mechanisms could be associated to a different ability of tumor cells to locally invade and/or to disseminate at distance, resulting differently able to induce metastasis in vivo.

Among all the molecular effectors that could display a role in tumor progression, particular interest has received the inhibitor of cyclin-CDK complexes p27kip1 (p27). p27 represents an ideal target displaying on one hand an inhibitory effect in cell cycle progression (Belletti et al., 2005) and on the other hand by reducing cell migration both in vitro and in vivo (Baldassarre et al., 2005; Schiappacassi et al., submitted 2008; Yuan et al., 2007). Moreover, many human cancers express decreased amounts of p27 compared to normal tissues. The reduced p27 expression often correlates with increased tumor aggressiveness and a poor clinical outcome, and p27 protein levels result an independent prognostic factor for many of these tumors (Bloom and Pagano, 2003; Slingerland and Pagano, 2000; Philipp-Staheli et al., 2001).

Thus, in this PhD thesis we have focused our attention on the role of p27 in the process of neoplastic transformation, pinpointing the effects strictly associated to p27 expression or to its absence. To this aim, we have used murine fibroblasts, previously immortalized with 3T3...
Discussion

protocol, derived from embryos wild type or knock out for the cdkn1b gene, encoding for the p27 protein. 3T3p27+/+ and p27−/− were then transformed by over-expression of v-src oncogene and different cell clones were analysed and fully characterized. H-Ras oncogene was used in the same manner to highlight common responses or different “oncogene-specific” effects.

1. p27 controls cell proliferation in transformed cell cultures and in vivo tumor growth.

The characterization of in vitro proliferative behaviour demonstrated that p27 absence in v-src transformed cells correlated with an increased cell growth, as demonstrated by the S-phase cell fraction and growth curves. The same result was also observed after H-Ras transformation, indicating that the proliferative advantage could be an “oncogene-independent” effect.

Whether this observation agrees with the role of p27 as tumor suppressor gene, it differs from the behaviour described in normal fibroblasts, in which the absence of p27 is not associated, at least apparently, to major differences in the proliferation rate (our observations and Nakayama et al., 1996). These observations suggest that the correct cell cycle progression in normal p27−/− cells is ensured by compensatory and redundant mechanisms that, after challenging with oncogene stimuli, result no more sufficient to exert a full inhibition of cell cycle progression and, consequently, to control the cell proliferation. These effects of p27 seem to be mediated by a higher CDK1 and CDK2 activity that could be readily observed in p27−/− v-src fibroblasts. Moreover, although p27+/+ and p27−/− embryonal fibroblasts are characterized by similar proliferative response to contact inhibition (Nakayama et al., 1996), we were able to highlight some differences after oncogenes transformation between the two genotypes. In particular, p27 expression resulted important to retain at least a partial activity in cell cycle inhibition after cell-cell contact in both v-src and H-Ras transformed cells. This observation is of extreme importance in the understanding of cell transformation process since loss of contact inhibition of cell growth is a hallmark of cancer cells. Our observation, although not completely confirmed in normal p27 null mouse fibroblasts, was not completely unexpected since previous work linked the contact-dependent block of cell proliferation to p27 expression (Polyak et al., 1994; Coats et al., 1996). Moreover, the observation that p27ko mice are bigger in size respect to the wild type littermate (Nakayama et al., 1996; Fero et al., 1996; Kiokawa et al., 1996) suggests that also in vivo the cell-cell contact inhibition could not properly works. In accord with our in vitro and in vivo observations it has been demonstrated that p27 null animals are not able to guarantee the same response after oncogenetic challenges. In fact, even if p27 KO mice are relatively free of malignancies, they show a
decreased tumor-free survival following γ-irradiation and an increased tumor-related mortality following treatment with chemical carcinogens, with a dose dependent effect (Fero et al., 1998).

The *in vitro* proliferative advantage of transformed p27 null cells was confirmed also *in vivo*, as demonstrated by the growth of xenograft sarcomas after s.c. injection in nude mice. In both v-src and H-Ras models, p27 absence is strictly associated to the induction of significantly bigger masses. But while in v-src transformation p27-rescued expression impairs tumor growth and completely reverts the phenotype, in H-Ras model the picture appears more complicated. In fact the expression of p27 is not sufficient to decrease *in vivo* growth, suggesting that other genetic alterations could be induced during Ras transformation.

Moreover, the tumor suppressive activity of p27 is well supported by the data collected using an adenoviral approach for target gene therapy in glioblastomas (Schiappacassi et al., accepted for publication 2008). In this tumor model, the over-expression of p27 strongly impairs the *in vivo* tumor growth, suggesting that p27 expression could be an important determinant in progression of several tumor types (Schiappacassi et al., accepted for publication 2008).

2. **p27 influences 3D cellular morph-dynamics and *in vivo* tumor dissemination.**

The more interesting feature demonstrated in this study is represented by the description of a new p27 function in the control of cellular morph-dynamics.

Tumor cells possess a broad spectrum of migration mechanisms to infiltrate surrounding matrices and to overcome tissue barriers, but they haven’t been clearly elucidated yet (Friedl and Wolf, 2003; Friedl, 2004). In our work, we have observed that after v-src transformation, 3T3 fibroblasts can assume mainly two different cell morphologies in 3D collagen context. They can display an elongated spindle-like cell shape, typical of mesenchymal cells, characterized by finger-like protrusions at the cellular edges and actin stress fibers and, moreover, they can assume a rounded amoeboid-like cell shape, with several peripheral membrane ruffles, lost of the dendritic-like extensions and an actin cortical distribution.

Interestingly, the percentage of cells that display the two different morphologies significantly changes according to p27 expression. The absence of p27 increases the number of rounded cells from the 52.2% to the 86%, whereas only the 14% of p27 null cells appear elongated. Till now, no one study has demonstrated a correlation between the expression of any cell cycle inhibitors and cell shape, even if it is known that cell proliferation requires important
Discussion

rarrangements in cell morphology to allow a proper cell division (Théry and Bornens, 2006; Meyers et al., 2006). The use of different p27 cDNAs allowed us to map the function of cell shape regulation in the C-terminus region of p27 protein. In fact, although p27 full length protein completely reverts the morphologic traits of p27null v-src cells, the deletion mutant lacking the last 28 aminoacids (p271-170) is not able to rescue the morphology and, consequently, cells result almost all rounded and amoeboid.

Importantly the different cell morphologies assumed by p27+/+ and p27−/− v-src cells in 3D matrices strongly influence the motile behavior and the migration rate. With the aid of time lapse video microscopy and cell tracking software to analyze migration parameters, we had the opportunity to study and directly visualized motility of individual and clustered cells in 3D environment, mimicking in a fascinating way a “physiologic” context. Using these experimental approaches, p27−/− v-src cells clearly display an amoeboid motility characterized by a very flexible cell shape associated with highly dynamic membrane blebs and by the ability to rapidly squeeze through the matrix lattices overcoming the ECM barriers. Conversely, p27+/+ transformed cells mainly display a mesenchymal migration mechanism, establishing long cellular protrusions to generate the traction forces necessary for cell body advancing. Further, the amoeboid migration mechanism assumed by p27null cells induces a higher cells speed compared to the mesenchymal strategy and cells move faster than the wt counterpart. Importantly the in vitro behavior is associated in vivo to a high ability to settle at distant sites and induce metastasis formation, suggesting that, besides the role in primary tumor growth, p27 absence induces a more aggressive phenotype also by increasing the invasion capability and metastasis induction.

Our previous observations have highlighted similar morphological and migration differences also in normal cells expressing or not p27 (Belletti et al., in preparation 2008). The absence of p27 or its down-regulation in fibroblasts results both in a more rounded cell shape compared to the typical elongated mesenchymal-like morphology of p27-expressing cells and in higher migration rates (Belletti et al., in preparation 2008).

We have previously demonstrated that p27 binds and inhibits the MTs destabilizing protein Stathmin and, through this interaction, p27 could play a pivotal role in in vitro motility (Baldassarre et al., 2005). In fact by inhibiting Stathmin, p27 induces stabilization of the MT network thus impairing cell migration. Recently we have demonstrated that p27/stathmin interaction controls also tumor cell invasion in in vitro models, confirming our previous in vivo results (Schiappacassi et al., accepted for publication 2008).
Whether p27/stathmin interaction could be involved also in the motile phenotype observed during v-src transformation is still to be determined, although some of the collected data strongly suggest this possibility. In fact, our preliminary results, collected using a small interference adenoviral system able to strongly reduce stathmin levels, suggest that the migration rate of p27<sup>−/−</sup> v-src cells is significantly down-regulated, whereas the same siRNA does not significantly change the motility of p27-expressing cells. Moreover, we have demonstrated that the expression of p27<sup>1-170</sup> in p27null cells does not revert neither the morphologic traits nor the migration advantage and we previously demonstrated that this deletion mutant is not able to bind and inhibit stathmin (Baldassarre et al., 2005). Conversely a full length p27 cDNA completely rescues the phenotype both <i>in vitro</i> and <i>in vivo</i>. Thus, at least in part, the motility inhibition exerted by p27 seems to be mediated by its interaction with stathmin.

Besides the p27 function in the control of MTs stability, we can not excluded a p27-mediated effect in the regulation of the actin cytoskeleton too. In fact, during cell migration both the tubulin and actin cytoskeleton result to play crucial functions (Etienne-Manneville, 2004). Besson et al., demonstrated that in over-expression conditions, p27 directly interacts with RhoA and inhibits its activity (Besson et al., 2004). These authors showed that p27 absence is associated to higher RhoA activity, resulting in an increase of actin stress fibers and focal adhesions that in turn impairs motility in 2D experimental systems. An excessive adhesive potential is well known to reduce migration on 2D substrates, but in 3D environments, fully mature focal contacts are not usually observed (Friedl and Wolf, 2003). In 3D contexts, cells are less adhesive and can switch rapidly from a migration mechanisms to another, a phenomen called cell plasticity (Friedl and Wolf, 2003; Condeelis and Segall, 2003). Thus, in 3D matrices an up-regulation in RhoA activity could favour cell motility rather than impair it. RhoA is known for its ability to form actin stress fibers and focal adhesions through the recruitment and activation of its effectors mDia and the Rho-kinases, ROCK1 and ROCK2. But the recent analyses of motile behaviour in 3 dimensional environments have suggested that Rho-GTPases can display a major versatility and less “fixed” functions (Higashida et al., 2004). For example a localized RhoA activation is required for the induction of membrane ruffling, typical morphological trait of amoeboid cells (Kurokawa and Matsuda, 2005). Recently, an increase in RhoA activity, rather than impaired motility, was associated to the transition from a mesenchymal to an amoeboid motility in 3D systems (Sahai et al., 2007; Gadea et al., 2007).
Thus, in our model, we can speculate that the absence of p27 could induce high RhoA activity that in turn determines the switch to the amoeboid morph-dynamics observed in p27<sup>-/-</sup> v-src fibroblasts. Moreover, the acquirement of an amoeboid motility results more advantageous in a 3D environment because allows to easier pass through ECM lattices in vitro (Friedl and Wolf, 2003) and overcome tissue barriers in vivo. The data we collected evaluating the phosphorylation levels of cofilin (target protein phosphorylated on Ser3 by active RhoA) have highlighted a different kinetics of RhoA activity in p27<sup>-/-</sup> src cells compared to the wild type counterpart, following cell-ECM contact. Interestingly, in the same assay we observed a different kinetics of FAK, AKT, STAT3 and ERK1,2 activation, suggesting that in p27 null cells there is a general modification of the signal trasduction pathways activated following cell adhesion to ECM. One possible explanation for this observation resides in the recent demonstration that the MT network controls vesicular transport in adherent cells (Balasubramanian et al., 2007). It is thus possible that p27, by modifying MT stability through stathmin inhibition, control in turn the vesicular recycling of several proteins necessary for the proper control of cell growth and motility. Studies are in course in our laboratory to test this hypothesis in our model systems.

In conclusion, our results demonstrated that p27 expression is an important determinant both in the first steps of cell transformation and tumor establishment and in the following progression to tumor dissemination and colonization of distant sites. The fact that p27 in transformed cells controls not only cell cycle progression but also the regulation of cellular morph-dynamics makes it an attractive target for new anticancer therapies.
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