

Thesis for doctoral degree (Ph.D.)
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The Many Faces of p57^{Kip2}

Acting in Apoptosis, Differentiation and Cytoskeleton Reorganisation

Pinelopi Vlachos

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Till Pappa och Mamma
Για τον μπαμπά και την μαμά

ABSTRACT

p57^{Kip2} is a known cyclin dependent kinase inhibitor, which has been suggested to be a tumor suppressor gene. Indeed various human cancers show a reduction in p57^{Kip2} expression, indicating that this protein might be of relevance in tumorigenesis. Mutated forms of p57^{Kip2} have rarely been detected in human tumors; rather epigenetic mechanisms are involved in inactivating the expression of the gene. Inactivation of the p57^{Kip2} gene correlates with disease progression and poor prognosis for the patient, indicating that reactivation of p57^{Kip2} in cancer cells could possibly inhibit tumor cell growth.

Prior research has primarily focused on p57^{Kip2}'s role as cell-cycle regulator, whereas the aim of this thesis is to study its function during other cellular events such as apoptosis, cytoskeleton reorganisation and differentiation of neuronal cells.

We report that selective expression of p57^{Kip2} sensitizes tumor cells to cell death induced by different drugs. This function is independent of its role as a cyclin dependent kinase inhibitor. Our studies also show that p57^{Kip2} primarily promotes the mitochondrial apoptotic pathway. In accordance, we found that Bcl-2 overexpression or VDAC inhibition were able to inhibit p57^{Kip2} cell death promoting effect. We also reveal that p57^{Kip2} is a direct target gene for p73 β , which is known for its pro-apoptotic properties. Moreover, the p73 β -induced p57^{Kip2} expression contributes to mitochondrial events related to apoptotic cell death.

Furthermore, we establish that p57^{Kip2} expression promotes actin stress fiber formation in cells. It interacts with, and activates the actin cytoskeleton modifying enzyme, LIM-Kinase-1. This activation resulted into increase phosphorylation, inactivation of cofilin and in a reduction of actin protein mobile fraction, ultimately, affecting negatively cell mobility. Remodelling of actin cytoskeleton plays a key role in cell migration and has implication for invasion and metastasis, supporting the proposal that p57^{Kip2} can act as a tumor suppressor gene by affecting the cytoskeleton.

We further investigated the role of p57^{Kip2} in neural stem cell differentiation. We show that p57^{Kip2} transiently accumulates in the nuclei of neural progenitors during early astrocyte differentiation and represses neuronal differentiation. It can interact with a subset of pro-neuronal bHLH factors, including Mash1. p57^{Kip2} inhibits the transcriptional activity of Mash1 and thereby represses neuronal differentiation, possibly to allow proper glial differentiation. These findings once again confirm the importance of p57^{Kip2} during development and differentiation.

In conclusion, our work shows that p57^{Kip2} plays essential roles during several cellular processes besides acting as a cell-cycle regulator. This thesis contributes to the understanding of p57^{Kip2}'s role in apoptosis, differentiation and cytoskeleton reorganisation.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which in the text will be referred to by their roman numerals (I-IV).

- I. **Pinelopi Vlachos**, Ulrika Nyman, Nabil Hajji and Bertrand Joseph.
The cell cycle inhibitor p57(Kip2) promotes cell death via the mitochondrial apoptotic pathway.
Cell Death Differ. 2007,14(8):1497-507.
- II. Ulrika Nyman*, **Pinelopi Vlachos***, Naveen Reddy Muppani and Bertrand Joseph.
The p73 β transcriptional target gene p57^{Kip2} promotes p73 β -mediated mitochondrial apoptotic cell death.
Submitted to Cell Death Differ.
- III. **Pinelopi Vlachos** and Bertrand Joseph.
The CDK inhibitor p57Kip2 controls LIM-Kinase-1 activity and regulates actin cytoskeleton dynamics.
Oncogene, In press
- IV. Bertrand Joseph *, Emma Andersson*, **Pinelopi Vlachos** , Erik Södersten, Lining Liu, Ana Teixeira and Ola Hermanson.
p57Kip2 is a repressor of Mash1 activity and neuronal differentiation in neural stem cells.
Cell Death Differ. 2009 16(9):1256-65.

* Authors contributed equally to this work

LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

- I. Nabil Hajji, Karolina Wallenborg, **Pinelopi Vlachos**, Ulrika Nyman, Ola Hermanson and Bertrand Joseph.
Combinatorial action of the HDAC inhibitor trichostatin A and etoposide induces caspase-mediated AIF-dependent apoptotic cell death in non-small cell lung carcinoma cells.
Oncogene. 2008, 27(22):3134-44.
- II. Karolina Wallenborg*, **Pinelopi Vlachos***, Sofi Eriksson, Lucas Huijbregts, Elias SJ Arnér, Bertrand Joseph, and Ola Hermanson.
Red wine triggers cell death and thioredoxin reductase inhibition: effects beyond resveratrol and SIRT1.
Exp Cell Res. 2009, 315(8):1360-71.
- III. Ulrika Nyman, **Pinelopi Vlachos**, Anna Cascante, Ola Hermanson, Boris Zhivotovsky and Bertrand Joseph.
Protein kinase C-dependent phosphorylation regulates the cell cycle-inhibitory function of the p73 carboxy terminus transactivation domain.
Mol Cell Biol. 2009, 29(7):1814-25.
- IV. Ulrika Nyman, Agnieszka Sobczak-Pluta, **Pinelopi Vlachos**, Thomas Perlmann, Boris Zhivotovsky and Bertrand Joseph.
Full-length p73alpha represses drug-induced apoptosis in small cell lung carcinoma cells.
J Biol Chem. 2005, 280(40):34159-69.
- V. Nabil Hajji, Karolina Wallenborg, **Pinelopi Vlachos**, Ola Hermanson and Bertrand Joseph.
Opposing effect of hMOF and SIRT1 control H4K16 acetylation and the sensitivity to topoisomerase II inhibitors
Revised version submitted to Oncogene

* Authors contributed equally to this work

LIST OF ABBREVIATIONS

AIF	apoptosis inducing factor
ALL	acute lymphoblastic leukemia
ANT	adenine nucleotide translocator
Apaf	apoptosis protease activating factor
Bak	Bcl-2 homologous antagonist
Bax	Bcl-2 associated protein X
Bcl-2	B-cell lymphoma-2
Bcl _{XL}	Bcl-2 related protein X long
bHLH	Basic-helix-loop-helix protein
Bid	BH3-interacting domain death antagonist
BWS	Beckwith–Wiedemann syndrome
CDK	cyclin dependent kinases
CKI	cyclin dependent kinases inhibitors
Cyt c	cytochrome c
Diablo	direct IAP-binding protein with low pI
DISC	death inducing complex
FADD	Fas-associated death domain
FRAP	fluorescence recovery after photo bleaching
GFP	green fluorescent protein
Hes1	hairy and enhancer of split 1
IAP	inhibitor of apoptosis protein
IGF2	insulin-like growth factor 2
IMM	inner mitochondria membrane
IMS	intermembrane space
JNK	c-jun NH ₂ -terminal protein kinase
Mash	mammalian achaete-scute homolog
MyoD	myoblast determination protein
NLS	nuclear localization signal
OMM	outer mitochondria membrane
PTP	permeability transition pore
ROCK	Rho-associated protein kinase

LIST OF ABBREVIATIONS

SCC	squamous cell carcinoma
siRNA	small interfering ribonucleic acid
Smac	second mitochondria-derived activator of caspases
STS	staurosporine
SVZ	subventricular zone
tBid	truncated Bid
TRADD	tumor necrosis factor receptor associated death domain
VDAC	voltage-dependent anion channel

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INTRODUCTION

INTRODUCTION

Human tumors develop following alterations of oncogenes and tumor suppressor genes, which confer a growth advantage for cancer cells. It has been proposed that down-regulation of differentiation genes may be the primary event in human carcinogenesis; this may be the initiating event that causes cells to become immortalized (Prasad *et al.*, 2001). Cell growth and tissue homeostasis are normally regulated by two major biological processes: cell proliferation and cell death. Each of these pathways can be defined by a unique set of molecular events. Deregulation in one of these pathways can have consequences in others leading at the end to, among other things, development disabilities and tumor formation.

p57^{Kip2}, MEMBER OF THE CIP/KIP FAMILY

p57^{Kip2} (where Kip refers to kinase inhibitory protein) is a member of the Cip/Kip family of proteins, together with p21^{Cip1} and p27^{Kip1}. p21^{Cip1} was the first family member to be identified (el-Deiry *et al.*, 1993; Harper *et al.*, 1993; Noda *et al.*, 1994), followed by p27^{Kip1} (Koff and Polyak, 1995; Polyak *et al.*, 1994). p57^{Kip2} was originally cloned simultaneously by two groups in 1995 when looking for homologues of p21^{Cip1} and p27^{Kip1} (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). The Cip/Kip family members were initially considered as tumor suppressor genes based on their ability to block cell proliferation by interacting with the cyclin- cyclin dependent kinases (CDK) complexes. Although they bind to and inhibit cyclin D, E, and A dependent kinases *in vitro* their main CDK inhibitory function seems to be mediated through cyclin E and A dependent CDK2, causing G1 cell-cycle arrest (Cheng *et al.*, 1999; LaBaer *et al.*, 1997; Sherr and Roberts, 1999). However it rapidly became clear that the situation was not as simple. p21^{Cip1}, p27^{Kip1} and p57^{Kip2} are also involved in the regulation of cellular processes beyond cell-cycle regulation, including apoptosis, differentiation and cytoskeleton reorganisation (Besson *et al.*, 2008). Early it was realized that p57^{Kip2} has a unique tissue distribution and distinctive structure compared with its family members, making it a very interesting protein to investigate (Lee *et al.*, 1995).

p57^{Kip2} and its role in Beckwith-Wiedemann syndrome

The p57^{Kip2} gene is located on human chromosome 11p15.5 and mouse chromosome 7. This is a region implicated in sporadic cancer and a familiar syndrome of cancer and overgrowth called Beckwith-Wiedemann syndrome (BWS), which gave rise to the hypothesis that p57^{Kip2} could play a role in the development of this syndrome (Matsuoka *et al.*, 1996). Furthermore, mice deficient in p57^{Kip2} displayed some BWS

associated phenotypes, such as omphalocele and cleft palate (Yan *et al.*, 1997; Zhang *et al.*, 1997). Like others genes situated in the same chromosomal region, for example *IGF2*, p57^{Kip2} was also found to be imprinted (Hatada *et al.*, 1996a; Hatada and Mukai, 1995; Hatada *et al.*, 1996b; Matsuoka *et al.*, 1996). p57^{Kip2} is expressed from the maternal allele, indicative of a complex regulation of imprinting in this region. In addition there are also some differences between the human and mouse p57^{Kip2} gene in regards of imprinting.

Mouse p57^{Kip2} is exclusively expressed from the maternal allele, whereas human p57^{Kip2} is predominantly expressed from the maternal allele and partially from the paternal allele, at low levels in most tissues and at levels comparable to the maternal allele in foetal brain and some embryonic tumors (Hatada and Mukai, 1995; Higashimoto *et al.*, 2006). Although mutations in the p57^{Kip2} gene have been detected, especially in familial case of BWS (Lam *et al.*, 1999), in sporadic cases of BWS these mutations are rare (Hatada *et al.*, 1996b; O'Keefe *et al.*, 1997). This indicates that BWS is a multifunctional disease potentially involving several genes in the 11p15.5 region.

Structure properties of p57^{Kip2} and its family members

The Cip/Kip family members show significant amino acid homology at their amino terminal domains, called the CDK binding/inhibitory domains. Both p21^{Cip1} and human p57^{Kip2} contains a proliferating cell nuclear antigen (PCNA) binding domain within their carboxyl-terminus (Luo 1995, Watanabe 1998). This domain, when separated from its amino-terminal CDK-cyclin binding domain, can prevent DNA replication *in vitro* and S phase entry *in vivo*. This demonstrates a dual function of these cyclin dependent kinase inhibitors (CKIs) as cell-cycle regulators. Interestingly, the PCNA inhibition domain is not conserved in mouse p57^{Kip2} and p27^{Kip1}. Furthermore, they all have a nuclear localization signal (NLS) near the carboxyl-terminus. Both p27^{Kip1} and p57^{Kip2}, but not p21^{Cip1}, have a similar carboxyl-terminal sequence, called the QT-domain, the function of which it's still not determined, but for p57^{Kip2} it is suspected to be important for protein interactions (Chang *et al.*, 2003). In addition to the mentioned domains, the mouse derived p57^{Kip2} contains a unique proline-rich and acidic domain in the middle of its sequence; whereas human derived p57^{Kip2} appears to have replaced the internal region with sequences containing proline-alanine (PAPA) repeats (Figure 1). These domains have not been found in p21^{Cip1} and p27^{Kip1}, implying that p57^{Kip2} has unique functions.

The Cip/Kip family of proteins have been under investigation during more than a decade. Much effort has been put in the investigation of the family members, p21^{Cip1} and p27^{Kip1}, and their role in various signalling pathways. p57^{Kip2} on the other hand has been ignored even if it is structurally the most complex family member and it's the only member required for proper development.

INTRODUCTION- p57^{Kip2}, member of the Cip/Kip family

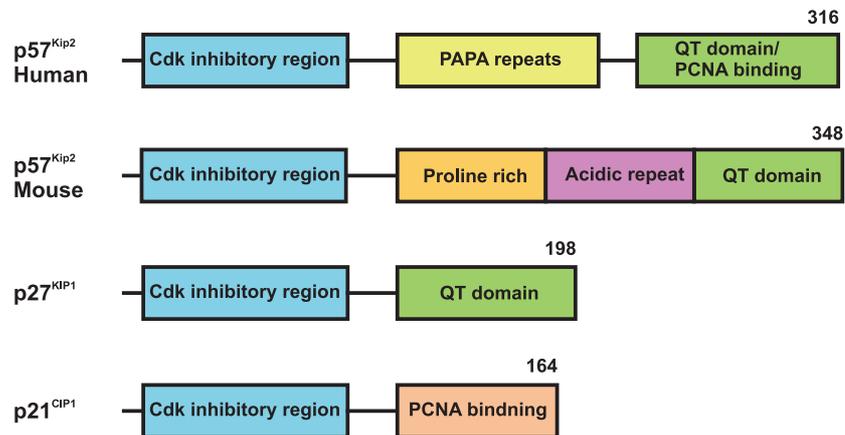


Figure 1. Structure properties of p57^{Kip2} and its family members.

p57^{Kip2} and its regulation

Despite the importance of p57^{Kip2} in the regulation of the cell-cycle, the regulation of its expression is still unclear. Mutations are rare for p57^{Kip2}, in contrast; down-regulation of p57^{Kip2} has been reported in a number of human malignancies (Kikuchi *et al.*, 2002; Kondo *et al.*, 1996). Abberant DNA methylation of a critical region in close proximity of the p57^{Kip2} promoter has been associated with gene inactivation, a phenomenon reversed by exposing methylated cell lines to a hypomethylating agent (Kikuchi *et al.*, 2002). Furthermore, aberrant DNA methylation has been observed in patients with acute lymphoblastic leukemia (ALL) (Canalli *et al.*, 2005).

Other studies show that p57^{Kip2} protein expression increase after TGF- β stimulated proliferation in hematopoietic cells (Scandura *et al.*, 2004). The control of p57^{Kip2} by TGF- β was dependent on the methylation status of the p57^{Kip2} promoter. p57^{Kip2} promoter methylated cells only responded to epigenetic modulation, such as the use of hypomethylating agent or histone deacetylase inhibitors, but not to TGF- β . In contrast, unmethylated cells responded to TGF- β stimulation (Kuang *et al.*, 2007). This indicate that the cellular consequences of the reintroduction of a particular gene, are dramatically different in cells where the target gene is inactivated by aberrant DNA methylation.

INTRODUCTION- p57^{Kip2}, member of the Cip/Kip family

p57^{Kip2} can also be regulated through the ubiquitin-proteasome pathway, phosphorylation at threonine residue (Thr-310) of human p57^{Kip2} is required for Skp2-mediated ubiquitylation and proteolysis (Kamura *et al.*, 2003).

The p57^{Kip2} gene was recently identified as a target of the transcription factors p73 β and p63, two members of the p53 family (Beretta *et al.*, 2005; Blint *et al.*, 2002). In the case of p73 β , silencing of p57^{Kip2} considerably reduced p73 β mediated cell death, suggesting that p57^{Kip2} is required for p73 β death promoting effect.

p57^{Kip2} is also induced by the muscle specific transcription factor myoblast determination protein (MyoD) through an indirect mechanism involving the p73 β protein as intermediate (Vaccarello *et al.*, 2006).

In addition, recent studies indicate that E47, a bHLH transcription factor, target p57^{Kip2} and induces its expression to cause cell-cycle arrest in developing neuroblast (Reyraud *et al.*, 2000; Rothschild *et al.*, 2006). In conclusion, little is known about the mechanism controlling p57^{Kip2} expression and increased knowledge may lead to better understanding of its role in different pathways.

APOPTOSIS

Apoptosis was first described by John Foxton Kerr in 1972 (Kerr *et al.*, 1972) and the term apoptosis was suggested by James Cormack. The word "apoptosis" is used in Greek to describe the "dropping off" or "falling off" of petals from flowers, or leaves from trees.

Programmed cell death or apoptosis is an evolutionary conserved process, which is important for normal development, maintenance of homeostasis and removal of unwanted and/or damaged cells (Clarke and Clarke, 1996; Lockshin and Zakeri, 2001). In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers undergo apoptosis; the result is that the digits are separated. Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult.

Many cancers show severe defects in the apoptotic machinery. If a cell is unable to undergo apoptosis because of genetic mutation or biochemical inhibition, it continues to divide and develop into a tumor. Apoptosis resistance may contribute not only to tumorigenesis, but could also explain the resistance of cancer cells to undergo apoptosis upon conventional treatment with chemotherapeutic drugs and irradiation. Mammalian cells respond to DNA damage signals by activating cell-cycle checkpoints, which arrest the cell-cycle, or by inducing apoptosis. There are two distinct molecular signalling pathways that lead to apoptotic cell death: (i) the extrinsic or receptor-mediated pathway and (ii) the intrinsic or mitochondrial-mediated pathway (Figure 2).

The extrinsic, receptor-mediated pathway

The extrinsic pathway begins outside the cell through the activation of specific death receptors on the cell surface. These are activated by specific molecules known as death receptor ligands. These ligands include CD95L/FasL and Apo2L/TRAIL and bind their cognate receptors CD95/Fas and DR4/DR5, respectively (Itoh *et al.*, 1991; LeBlanc and Ashkenazi, 2003).

Ligand binding induces receptor clustering and recruitment of the adaptor protein Fas-associated death domain (FADD) and the initiator caspases-8 or -10 as pro-caspases, forming a death-inducing signalling complex (DISC). Formation of the DISC brings pro-caspase molecules into close proximity of one another, facilitating their autocatalytic processing and release into the cytoplasm where they activate effector caspases, pro-caspase-3, -6, and/or -7 (Medema *et al.*, 1997). In some cells (known as

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type-I cells) caspase-8 is sufficient to activate effector caspases and execute apoptosis. Other cells (known as type-II cells) needs caspase-8 to cleave Bid to produce truncated Bid (tBid). The cleavage of Bid by caspase-8 results in its translocation to the mitochondria where it initiates the release of mitochondrial factors, like cytochrome c (cyt c)(Li *et al.*, 1998; Luo *et al.*, 1998), leading to the association of cyt c with adaptor apoptotic protease activating factor-1 (Apaf-1). The binding of cyt c to Apaf-1 results in a conformational change, allowing oligomerization of Apaf-1 into a multimeric complex, the apoptosome, and recruitment of pro-caspase-9 (Li *et al.*, 1997; Tsujimoto, 1998; Zou *et al.*, 1999). Pro-caspase-9 is then activated, initiating the cleavage of pro-caspase-3, -6, and/or -7. The activated caspases then cleave substrates in the cytosol as well as in the nuclei. This triggers chromatin condensation and DNA fragmentation, which in turn lead to cell death.

The intrinsic, mitochondrial-mediated pathway

As its name suggests, the intrinsic pathway is initiated within the cell. This initiation is usually in response to cellular signals resulting from DNA damage, a defective cell-cycle, and detachment from the extracellular matrix, loss of cell survival factors, or other types of cellular stress. This pathway involves the release of pro-apoptotic proteins from the mitochondria that activate caspase enzymes leading ultimately to apoptosis.

Activation of this pathway relies on the disturbance of the permeability of the outer mitochondrial membrane (OMM). The mitochondria are composed of an OMM and an inner mitochondria membrane (IMM). The disruption of the mitochondrial membrane potential leads to the release of multiple proteins from intermembrane space (IMS) into the cytosol. The mechanism of OMM permeabilization is not fully established, but there are several different mechanisms that may explain this phenomenon.

The intrinsic apoptotic pathway depends on the balance between pro- and anti-apoptotic members of the Bcl-2 superfamily of proteins, which act to regulate the permeability of the OMM (Sharpe *et al.*, 2004). The Bcl-2 family contains numerous proteins that are either pro-apoptotic (*e.g.* Bid, Bad, Bak, Bax, Bcl_{XS}) or anti-apoptotic (*e.g.* Bcl-2, Bcl_{XL})(Gross *et al.*, 1999; Tsujimoto, 1998).

The pro-apoptotic proteins Bax and/or Bak have been shown to form heterotetrameric channels through which mitochondrial proteins such as, cyt c are released. The anti-apoptotic Bcl-2 proteins, Bcl-2 and Bcl_{XL} act to prevent permeabilization of the OMM by inhibiting the oligomerization of the pro-apoptotic Bcl-2 proteins Bax and/or Bak (Oltvai *et al.*, 1993; Sharpe *et al.*, 2004).

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One of the oldest models for OMM permeabilization describes that cyt c is released as a result of the opening of the permeability transition pore (PTP), a large pore that is between the OMM and the IMM. The components of this pore are the adenine nucleotide translocator (ANT) protein found in the IMM and the voltage-dependent anion channel (VDAC) protein, located in the OMM. Several Bcl-2 family members can bind to and regulate the channels activity. As the pore size of this channel is too small to allow larger proteins to pass through, this model assumes that VDAC undergo a significant conformational change upon binding to Bcl-2 family members, allowing larger proteins like cyt c to pass through (Cheng *et al.*, 2003).

Another phenomenon that was described thirty years ago, by Haworth and Hunter, showed that Ca^{2+} uptake by mitochondria could stimulate severe changes in mitochondrial morphology and activity, due to opening of the PTP in the IMM, leading to osmotic swelling of the mitochondria and OMM permeabilization, causing the release of cyt c etc (Hunter and Haworth, 1979).

Regardless of which mechanism that trigger OMM permeabilization, cyt c and the pro-apoptotic protein SMAC/DIABLO are then able to translocate from the IMS into the cytosol. As part of the intrinsic apoptotic pathway, the SMAC/DIABLO protein released from the mitochondria promotes apoptosis by directly interacting with inhibitors of apoptosis proteins (IAPs) and neutralizing their ability to inactivate the caspase enzymes (Deveraux *et al.*, 1997; Du *et al.*, 2000; Verhagen *et al.*, 2000). Cyt c binds Apaf-1 and pro-caspase-9 forming a large multiprotein structure known as the apoptosome (as described above). This in turn activates the downstream effector caspases -3, -6, and/or -7, eventually leading to cell death.

Depending on the mode of OMM permeabilization, in addition to the release of cyt c there might also be release of other proteins influencing apoptosis progression. One of these proteins is called apoptosis-inducing factor (AIF) and is able to induce apoptosis independently of caspase activation (Susin *et al.*, 1999). This protein is normally located in the IMS. When OMM permeabilization is triggered, AIF is released from the mitochondria and translocates into the nucleus; induces nuclear chromatin condensation, large scale DNA fragmentation, and exposure of phosphatidyl serine on the plasma membrane surface (Lorenzo *et al.*, 1999).

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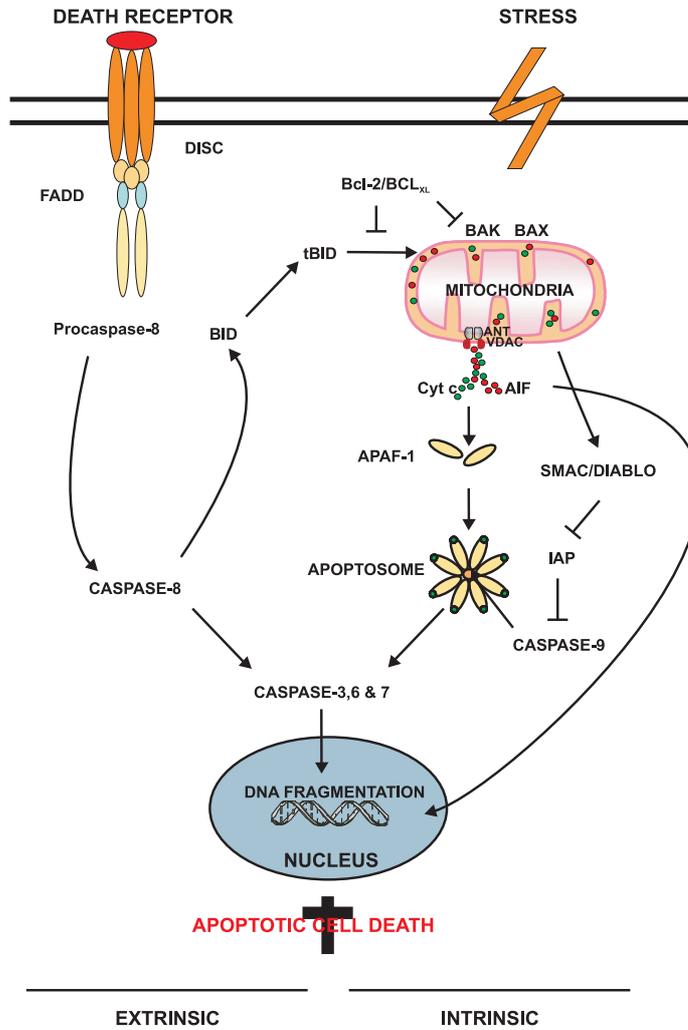


Figure 2. Signalling pathways leading to apoptotic cell death.

p57^{Kip2}'s role during apoptosis

CDK inhibitors belonging to the Cip/Kip family have been assumed to play a role in apoptosis and therefore, they provide a potential link between the cell-cycle and apoptosis.

p57^{Kip2} is structurally the most complex member of the Cip/Kip family and it is most likely involved in processes additional to its role in cell-cycle control. Our lab and others have shown that p57^{Kip2} deficient mice exhibit an increase in the rate of cell death in several cell populations during embryogenesis (Joseph *et al.*, 2003; Yan *et al.*, 1997; Zhang *et al.*, 1997). Whether this increased apoptosis is a direct or indirect effect due to aberrations in cell proliferation and differentiation is not known. Importantly, the p57^{Kip2} gene, located on chromosome 11p15.5, has been suggested to be a tumor suppressor gene, being inactivated in various types of human cancers. However, little is known concerning p57^{Kip2}'s function during apoptotic cell death during adulthood and its possible implication for cancer.

A single report has shown that p57^{Kip2} has a stimulatory effect on apoptosis induced by staurosporine, suggesting a role for p57^{Kip2} in the response of tumors to cytotoxic drugs (Samuelsson *et al.*, 2002). Several apoptotic stimuli, including treatment with hypomethylating agents, histone deacetylase inhibitors or p73 β overexpression induce p57^{Kip2} expression. In the case of p73 β mediated apoptosis, silencing p57^{Kip2} considerably reduced cell death, suggesting that p57^{Kip2} is required (Gonzalez *et al.*, 2005). On the other hand, p57^{Kip2} was found to bind via its QT domain to the stress-activated kinase JNK1/SAPK, inhibiting its kinase activity, and expression of the QT domain was sufficient to block UV- or MEKK1-induced apoptosis, mediated by JNK1 (Chang *et al.*, 2003). These observations indicate that p57^{Kip2}'s role during apoptosis is still unclear and has to be investigated more extensively.

Involvements of p21^{Cip1} and p27^{Kip1} in apoptosis have been reported before. p21^{Cip1} has been shown to antagonize apoptosis. It can prevent stress-induced apoptosis mediated by the JNK and p38 signalling pathways by acting at several distinct levels. First, p21^{Cip1} binds to and inhibits the activity of the MAPKKK ASK1/MEKK5 (Huang *et al.*, 2003). Second, like p57^{Kip2}, p21^{Cip1} can bind to JNK kinases through its cyclin-CDK binding domain, which both inhibits JNK activity and prevents JNK activation by upstream kinases (Shim *et al.*, 1996). Third, p21^{Cip1} might also promote survival by binding and blocking caspase-3 activation, thereby preventing Fas-dependent apoptosis (Suzuki *et al.*, 1999a). The binding of p21^{Cip1} with caspase-3 occurs at the mitochondria, suggesting that cytoplasmic p21^{Cip1} exert anti-apoptotic properties (Suzuki *et al.*, 1999b). Given that p21^{Cip1} antagonize apoptosis signalling it is not surprising to discover that multiple mechanisms exist to block its protective effect. p21^{Cip1} effectiveness to protect cells during apoptosis is lost when it is attacked by active caspase-3 (Gervais *et al.*, 1998), and is cleaved in its caspase-3 sensitive site in

INTRODUCTION- p57^{Kip2}'s role during apoptosis

its carboxyl-terminus (Park *et al.*, 1998). It was suggested that caspase-3 mediated cleavage of p21^{Cip1} and p27^{Kip1} were associated with an increased cyclin A/Cdk2 activity and apoptosis in SK-HEP-1 cells (Jin *et al.*, 2000). The function of p27^{Kip1} in the apoptotic process is still unclear. Overexpression of this CKI has been shown not only to protect HeLa cells from STS-induced apoptosis but also to induce apoptosis in several transformed mammalian cell lines (Denicourt and Dowdy, 2004). Thus, indicating that the function of p27^{Kip1} could be cell type specific.

DEVELOPMENT AND CELL DIFFERENTIATION

During embryogenesis, precursor cells initially proliferate to give rise to sufficient amount of cells; subsequently these cells stop to proliferate and start to differentiate. The proper timing of transition from proliferation to differentiation is critical for normal development since premature or delayed transition leads to abnormal cell numbers as well as abnormal morphology of the tissues. Recent studies revealed that these developmental processes are regulated positively or negatively by multiple basic helix-loop-helix (bHLH) genes (Kageyama *et al.*, 2005).

Proper development of an organism is the result of an integrated network of differentiation programs and signalling pathways that control cell-cycle exit and the terminal differentiation of a cell. The precise control of cell-cycle progression is believed to be critical for normal development. The Cip/Kip family members are involved during embryonic development, acting as cell-cycle inhibitors but also in the differentiation of cells that already exited from the cell-cycle.

p57^{Kip2}'s role during development and cell differentiation

p57^{Kip2} has a more restricted pattern of expression than the other Cip/Kip family members. It is expressed in skeletal muscles, brain, heart, lungs, kidneys, pancreas, testis and eyes (Lee *et al.*, 1995). Embryos show high expression levels of p57^{Kip2}, but the levels tend to decline in adults. Only p57^{Kip2} has been shown to be required for normal development, a function that the other Cip/Kip members cannot compensate for. Mice deficient in the p57^{Kip2} gene show altered cell proliferation, differentiation and apoptosis (Yan *et al.*, 1997; Zhang *et al.*, 1997). Mice died upon birth and showed macroglossia, omphalocele, gigantism, various levels of limb shortening, and an important modification in the skeletal muscle distribution, establishing that p57^{Kip2} has an important role during development.

Mice lacking p21^{Cip1} develop normally and do not display any predisposition to cancer development (Deng *et al.*, 1995). In contrast, p27^{Kip1} knockout mice appeared to have a greater body size than the wild-type (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). Moreover, they show hyperplasia of several organs, this phenotype indicates clearly that p27^{Kip1} is a key molecule that negatively regulates cell proliferation in those organs. The loss of each of the Cip/Kip members leads to significantly different results, which may reflect the functional diversity of each member. Thus, the developmental defects observed from the knockout mice may reflect their importance during development; p21^{Cip1} may not be essential, whereas p27^{Kip1} and p57^{Kip2} play important roles. Furthermore, the knockout mice gave indications that p27^{Kip1} functions may be, at least in part compensated by p57^{Kip2} whereas, p27^{Kip1}

INTRODUCTION- p57^{Kip2}'s role during development and cell differentiation

cannot fully compensate for the lack of p57^{Kip2}'s function. The organs that express both p27^{Kip1} and p57^{Kip2} include the brain, lung, liver, kidney, gastrointestinal tract, muscle, cartilage and lens and do not seem to be affected in p27^{Kip1} knockout mice, regardless of the high expression levels of p27^{Kip1} in normal mice (Nakayama, 1998).

As discussed above, mice lacking p21^{Cip1} develop normally, despite the fact that induction of p21^{Cip1}, by the muscle specific transcription factor myoblast determination protein (MyoD), has been implicated in terminal differentiation of skeletal muscle (Halevy *et al.*, 1995; Parker *et al.*, 1995). These observations indicate that there are mechanisms compensating for the lack of p21^{Cip1}. In support of this hypothesis, mice deficient in both p21^{Cip1} and p57^{Kip2} display developmental defects that are more severe than the effects observed in mice lacking either CKI alone (Zhang *et al.*, 1999). Moreover, *in vitro* studies have shown that MyoD is capable to induce the expression of p57^{Kip2} (Figliola and Maione, 2004). Intriguingly, this ability is restricted to cells lacking p21^{Cip1}, suggesting that p57^{Kip2} can compensate for p21^{Cip1} in muscle differentiation. These studies indicate that p57^{Kip2} has a more diverse and complicated role than its family members, being able to compensate for the loss of its family members.

The role of p57^{Kip2} in brain development has not been characterized extensively despite the fact that p57^{Kip2} is the only member of the Cip/Kip family that is absolutely required for survival (Yan *et al.*, 1997). It has been implicated in the control of neural precursor proliferation at several levels of the developing nervous system, and recent studies have established that in the absence of p57^{Kip2}, excess numbers of neurons are generated in the developing spinal cord (Gui *et al.*, 2007). p57^{Kip2} has also been shown to play a direct role in regulating the activity of transcription factors implicated in neuronal differentiation. For example, p57^{Kip2} interacts directly with the nuclear receptor Nurr1 and promote maturation of dopaminergic neurons in postmitotic precursors (Joseph *et al.*, 2003). However, during development, p57^{Kip2} is also expressed in mitotic multipotent neural progenitors in the ventricular and subventricular zones (SVZs) of specific regions of the forebrain, including the telencephalon. In the retina, p57^{Kip2} is involved in cell-cycle exit at early time, and later, in differentiation of a subset of amacrine interneurons (Dyer and Cepko, 2000; Dyer and Cepko, 2001). In addition, recent studies using peripheral neural crest-derived neuroblastoma cell lines indicate that E47, a bHLH transcription factor, target p57^{Kip2} to induce cell-cycle arrest in developing neuroblast (Reynaud *et al.*, 2000; Rothschild *et al.*, 2006). Additionally, it was recently shown that p57^{Kip2} in cooperation with p27^{Kip1} regulate cell migration in the developing neocortex (Itoh *et al.*, 2007). Thus, the expression and the possible roles of p57^{Kip2} in developing CNS are yet not established and the understanding of the mechanisms underlying the diverse effects of p57^{Kip2} in different contexts is still limited.

CYTOSKELETAL REORGANISATION AND CELL MIGRATION

Cytoskeleton reorganisation and cell migration play an essential role during a lifetime. During embryonic development it is important for tissue formation. In a fully established human, it is important during several moments, for example when an effective immune response is needed or during the repair of damaged tissues. Errors made in this integrated process, leading to failure of migration, or migration to an inaccurate location, will have serious consequences for the embryonic development or in the adult tissue. This could lead to mental retardation, vascular disease, rheumatoid arthritis, tumor formation and metastasis. A less dynamic cytoskeleton is not able to respond effectively to signals from the surroundings, like growth factors or stress signals, which could result in decreased cell motility and reduced reaction during injury.

During cell migration, the actin cytoskeleton is dynamically remodelled by actin polymerization/depolymerization cycles, and this reorganisation produces the force necessary for cell migration. Because inhibition in any of the two processes decreases cell motility, elucidation of the molecular mechanisms of actin organisation is important for cancer therapeutics.

The actin cytoskeleton undergoes rearrangement under the control of various cascades involving the Rho A small GTPase (Chrzanowska-Wodnicka and Burridge, 1996; Ridley *et al.*, 1992). The Rho-family of small GTPases Rho, Rac and Cdc42 are important regulators of the actin and tubulin cytoskeleton, which control actin filament dynamics and focal adhesion assembly in response to extra- and intracellular stimuli. Each member of the Rho family induces distinct patterns of actin organisation. Rho A induces the formation of actin stress fibers, Rac the assembly of lamellipodia and membranes ruffles, and Cdc42 regulates filopodia protrusions (Hall, 1998).

Early breakthrough in our understanding of the regulation of actin stress fibers came with the identification of downstream effectors of Rho A, in particular the ROCK/ROK protein kinases (Ishizaki *et al.*, 1996; Leung *et al.*, 1995). ROCK is a serine/threonine kinase that is activated by Rho A binding and cause prominent stress fiber formation (Leung *et al.*, 1996). ROCK in turn phosphorylates and activates the serine/threonine kinase LIMK-1, at the Thr-508 residue (Ohashi *et al.*, 2000). It is noteworthy that activation of LIMK-1 by VEGF is independent of its phosphorylation at Thr-508 (Kobayashi *et al.*, 2006), indicating that activation of LIMK-1 can be done in different ways then through the ROCK dependent pathway. LIMK-1 regulates actin polymerization by phosphorylating and inactivating its substrate, the actin depolymerization factor, cofilin (Arber *et al.*, 1998). Cofilin regulates actin dynamics by severing actin filaments and sequestering the actin monomers from the pointed end

of actin filaments. However, once phosphorylated at serine-3 by LIMK-1, cofilin can no longer bind to actin, resulting in accumulation of actin polymers, stabilization of the actin and reduction of cell mobility (Arber *et al.*, 1998; Sidani *et al.*, 2007) (Figure 3). Several recent studies suggest that proteins linking cytoskeleton reorganization with migration of cells are upregulated/downregulated in invasive and metastatic cancer cells. For example, LIMK-1 has been shown, depending of the cellular context, to either promote or inhibit cell mobility. Ectopic expression of LIMK-1 has been reported to increases migration and invasiveness of breast and prostate cancer cells (Davila *et al.*, 2003; Yoshioka *et al.*, 2003), and its over-expression decreases the mobility and invasion of Ras-transformed fibroblasts and mammary tumor cells (Wang *et al.*, 2006; Zebda *et al.*, 2000).

A better understanding of the mechanism behind cell motility and cytoskeletal organisation may provide novel therapeutic strategies which would block metastatic progression and increase patient survival.

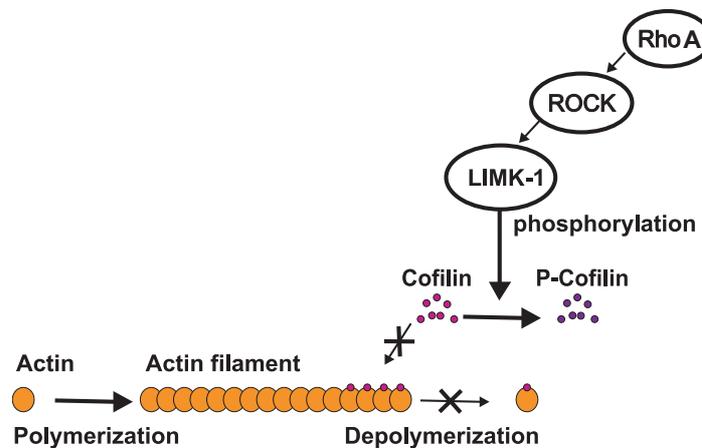


Figure 3. Mechanism of actin dynamics

Rho A activates ROCK which in turn can activate LIMK-1 by phosphorylation. LIMK-1 regulates actin dynamics by phosphorylating and inactivating the actin depolymerization factor, cofilin. Cofilin is severing actin filaments and sequestering the actin monomer from the pointed end of actin filaments. Though, once phosphorylated by LIMK-1, cofilin cannot longer bind to actin, resulting in increase of actin polymers, actin stress fiber formation and a reduction in cell mobility.

p57^{Kip2} during cytoskeletal reorganisation and cell migration

All cancers are characterized by an abnormal cell proliferation, caused by mutation or misregulation of cell-cycle regulatory genes and proteins. Tumors can acquire aggressive phenotype by an enhanced ability to invade adjacent tissues and migrate to distant sites. The motility of a cancer cell is governed by the regulators of cytoskeletal dynamics, in particular the Rho family of GTPases (Chrzanowska-Wodnicka and Burridge, 1996). It now seems that these two fundamental features of tumor cells might be connected by direct crosstalk between cell-cycle proteins and cytoskeletal regulatory proteins. In particular, the Cip/Kip family members can regulate the Rho signalling pathway and thereby affect functions that are sensitive to cytoskeletal organization, including cell migration.

p57^{Kip2} has been shown to play part in migration of cells during development and cancer invasion. Little is known about p57^{Kip2}'s function and mechanism in the regulation of the actin cytoskeleton dynamics and its possible implication for cancer cell mobility. One report proposed that p57^{Kip2} negatively regulates actin stress fiber formation by binding and translocating LIMK-1 to the nucleus (Yokoo *et al.*, 2003). This report did not include sufficient evidence and more adequate investigations are needed to clarify the role of p57^{Kip2} in the regulation of the actin cytoskeleton.

Several studies have correlated poor patient outcomes with loss or low levels of p57^{Kip2} in multiple human cancers. In patients with laryngeal squamous cell carcinoma (SCC), advanced tumor size, clinical stage and the recurrence of disease were associated with reduced p57^{Kip2} expression. The occurrence of lymph node metastasis was also related to negative expression of p57^{Kip2}. The overall 5-year disease-free survival rate of patients with laryngeal SCC was significantly higher in the p57^{Kip2}-positive than in the p57^{Kip2}-negative group (Fan *et al.*, 2006). Furthermore, expression of p57^{Kip2} is significantly decreased in human prostate cancer, and the overexpression of p57^{Kip2} in prostate cancer cells significantly suppressed cell proliferation and reduced the invasive ability (Jin *et al.*, 2008). Another report in astrocytoma cell lines shows that inducible expression of p57^{Kip2} is able to reduce cell motility and invasion (Sakai *et al.*, 2004). In addition, it was recently shown that p57^{Kip2} in cooperation with p27^{Kip2} regulates cell migration in the developing neocortex (Itoh *et al.*, 2007). All these reports give indications that p57^{Kip2} has a vital role in the migration of cells during development and cancer. Mutation of p21^{Cip1} and p27^{Kip1} are rarely found in cancer cells, and inactivation preferentially occurs through down-regulation of the protein in the nucleus or relocalization to the cytoplasm. Furthermore, increased levels of p21^{Cip1} and p27^{Kip1}, or its cytoplasmic localization has been correlated with high tumor grade and poor prognosis for patients with tumor types like breast, cervix, lymphomas and leukaemia. In the cytosol, p21^{Cip1} has been shown to bind and inhibit the Rho kinase ROCK1, promote neurite extension of neuroblastoma cells and hippocampal neurons, and lead to

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loss of actin stress fibers in NIH3T3 cells (Lee and Helfman, 2004; Tanaka *et al.*, 2002). Cytoplasmic p27^{Kip1} can bind to Rho A, preventing its activation by its guanine-nucleotide exchange factors, leading to decreased actin stress fiber, thus resulting in increased migration, invasion, and metastasis in several cell types (Besson *et al.*, 2004; McAllister *et al.*, 2003).

The upregulation of the Cip/Kip family members in the cytoplasm of many cancer cells may indicate that although loss of nuclear protein is important for cancer cells proliferation, gain of cytoplasmic protein function might be involved in different ways in tumor invasion and metastasis

AIM OF THE THESIS

The aim of the thesis was to investigate the role of p57^{Kip2} in the cell beyond its well-known role as a cell-cycle regulator.

Specific aims of the study were:

- Investigate the role of p57^{Kip2} in apoptotic cell death. (Paper I and II)
- Elucidate if selective induction of p57^{Kip2} by the p73 β isoform account for its pro-apoptotic activity. (Paper II)
- Elucidate if p57^{Kip2} acts on the actin cytoskeleton and thereby affect the cytoskeleton reorganisation. (Paper III)
- Investigate the role of p57^{Kip2} in neuronal differentiation. (Paper IV)

RESULTS

PAPER I - The cell cycle inhibitor p57^{Kip2} promotes cell death via the mitochondrial apoptotic pathway

The aim with this study was to elucidate the role of p57^{Kip2} during apoptosis. It has been observed that p57^{Kip2} deficient mice exhibit an increase in the rate of cell death in several cell populations. Whether this increased apoptosis is a direct or indirect effect due to aberrations in cell proliferation and differentiation is not known. In addition, little is known concerning p57^{Kip2}'s possible interplay with the apoptotic cell death machinery and its possible implication for cancer.

In this paper we report that selective p57^{Kip2} expression sensitize tumor cells to cell death induced by drugs such as staurosporine (STS), etoposide and cisplatin. This function did not require the ability of p57^{Kip2} to mediate inhibition of cyclin-dependent kinases. Our study reveals that p57^{Kip2} translocates to the mitochondria and sensitizes cells to drug induced apoptosis. It favors loss of mitochondrial transmembrane potential, release of cytochrome-c into cytosol, and the subsequent activation of caspase-9 and caspase-3. Further experiments revealed that p57^{Kip2}-expressing cells lost their mitochondrial transmembrane potential upon STS treatment faster than the non-p57^{Kip2} expressing cells. An increase of Bax activation and its translocation to the mitochondria was also observed. Furthermore, we found that Bcl-2 overexpression or VDAC inhibition were able to inhibit p57^{Kip2}'s cell death promoting effect. These results establish that p57^{Kip2} accelerate the apoptotic process and act upstream of the mitochondria.

Thus, in addition to its established function during control of proliferation, this study reveals a mechanism where p57^{Kip2} influences the mitochondrial apoptotic cell death pathway in cancer cells. p57^{Kip2} has been considered a candidate tumor suppressor gene, because of its location in the genome, biochemical activities and imprinting status. These studies provide evidence that p57^{Kip2} is an important gene in cancer tumorigenesis and that p57^{Kip2}'s involvement in the mitochondrial pathway may be a potential target for cancer prevention and therapy. Many anticancer drugs are mediating their cytotoxic effects via the apoptotic pathways. It is common that tumor cells are chemoresistant, thus having the ability to avoid the effect of multiple classes of anticancer drugs. As mentioned before, mitochondrial membrane permeabilization is a critical event in the process leading to apoptosis. A better understanding of p57^{Kip2}'s role in the regulation of the mitochondrial cell death pathway may provide insights into sensitivity or resistance of tumor cells. This knowledge could be further exploited to restore efficient apoptotic pathways in tumor cells downregulated in their p57^{Kip2} expression, thus increasing their sensitivity to therapy. By reintroducing p57^{Kip2} in cancer cells one might inhibit cancer progression, thus resulting in better prognosis for the patients.

PAPER II - The p73 β transcriptional target gene p57^{Kip2} promotes p73 β -mediated mitochondrial apoptotic cell death

p73 is a transcription factor, which has been shown to elicit cell-cycle arrest, apoptosis and differentiation. The *p73* gene encodes several isoform variants that have different functions in cell-cycle progression, differentiation and apoptosis. Expression of the full length p73 α isoform has been reported to be increased in different types of tumors (Frasca *et al.*, 2003; Kang *et al.*, 2000; Niyazi *et al.*, 2003; Novak *et al.*, 2001; Yokomizo *et al.*, 1999). Importantly, p73 α overexpression appears to significantly correlate with poor prognosis for patients (Niyazi *et al.*, 2003; Novak *et al.*, 2001). This suggests that p73 α might be implicated in tumorigenesis and possibly functions as a dominant oncogene to enhance tumor progression and therapeutic resistance. In contrast, the p73 β isoform is constantly reported as a pro-apoptotic factor and thereby as a tumor suppressor gene (Das and Somasundaram, 2006; Tuve *et al.*, 2006).

It was discovered earlier in our lab that the p73 α isoform inhibits drug-induced apoptosis whereas the p73 β isoform promotes it (Nyman *et al.*, 2005). The different functions of p73 might be due to different characteristics of its isoforms to regulate these processes due to different ability to transactivate unique target genes. Interestingly, given good correlation with our finding on the pro-apoptotic function of p57^{Kip2} in paper I, we and others found that p73 β , but not p73 α , induces p57^{Kip2} expression. The main aim of this study was to elucidate if the pro-apoptotic function of p73 β could be explained by its ability to induce p57^{Kip2} expression.

In this paper we established that p73 β can induce p57^{Kip2} expression in four different cell lines, in addition to previously reported cell lines (Gonzalez *et al.*, 2005; Blint *et al.*, 2002). Altogether, these data indicate that the control of p57^{Kip2} expression by p73 β is not cell type specific but rather a general mechanism. It is worth noting that a previous study showed that p57^{Kip2} induction was required for p73 β -mediated apoptosis (Gonzalez *et al.*, 2005). However, the precise mechanism by which p73 β control p57^{Kip2} gene expression and the contribution of p73 β -induced p57^{Kip2} expression to the different cell death pathways was still unclear. Our study reveals that p57^{Kip2} is a direct transcriptional target of p73 β but not of p73 α . Further experiments showed that p73 α , in a dose dependent manner, inhibited the transcriptional effect of p73 β on the p57^{Kip2} promoter. In order to unravel the importance of p57^{Kip2} in the p73 β death promoting effect, the influence of gene silencing of p57^{Kip2} with siRNA was investigated. This demonstrated that a reduction of p57^{Kip2} significantly reduced p73 β induced apoptosis through the mitochondrial pathway via activation of Bax. These results establish that p73 β is partially dependent on p57^{Kip2} expression to promote drug induced apoptosis via the intrinsic mitochondrial pathway. Since, both p73 β and p57^{Kip2} are suggested to be tumor suppressor genes; this finding brings additional evidence in support of their tumor suppressor activity.

RESULTS

PAPER III - The CDK inhibitor p57^{Kip2} controls LIM-Kinase-1 activity and regulates actin cytoskeleton dynamics

Reorganisation of the actin cytoskeleton plays a central role in the migration of cells from conception to death. This process is fundamental for tissue formation during embryonic development. Cell migration is needed to establish an effective immune response and during the repair of damaged tissue in the adult body. Occasionally this complex system does not work properly leading to migration of cells to inappropriate location which can have serious consequences. In fact, the dynamic remodelling of the actin cytoskeleton in response to growth factors or stress signals is an essential process in eukaryotic cells; as a less dynamic cytoskeleton is unable to respond effectively to those signals and result in reduced cell mobility. Understanding the mechanism controlling cancer cell invasion and metastasis constitutes a fundamental step in setting new strategies for diagnosis, and therapy of metastatic cancers.

Even if p57^{Kip2} has been implicated in different kind of tumors before, both in metastatic and invasive tumors, its role during cytoskeleton reorganisation and cell migration has not been explored in detail. A reduction of p57^{Kip2} protein expression has been correlated with an occurrence of lymph node metastasis (Fan *et al.*, 2006). In human prostate cancer the expression of p57^{Kip2} is significantly decreased, and the overexpression of p57^{Kip2} considerably suppressed cell proliferation and reduced invasive ability of the cancer (Jin *et al.*, 2008). In paper III we explored the potential role of p57^{Kip2} in the regulation of cytoskeleton dynamics and cell migration in cancer cells.

We observed, upon p57^{Kip2} expression, the formation of actin stress fibers. We also monitored p57^{Kip2}'s effect on actin dynamics using the fluorescence recovery after photobleaching (FRAP) technique. FRAP experiments provided information about the mobility of a fluorescent molecule in a defined compartment. Experiments were performed in p57^{Kip2} expressing cells that are transfected with GFP-actin. We showed that when a region is bleached, the fluorescence recovery over time for GFP-actin is slower in the p57^{Kip2} expressing cells, indicating that the actin cytoskeleton is more stable as compared to control cells. Furthermore, we observed that p57^{Kip2} is translocated to the cytosol where it interacts with LIMK-1, an actin cytoskeleton modifying enzyme and enhances its activity. The enhanced activity was independent of phosphorylation at Thr-508 by ROCK and lead to the phosphorylation and inactivation of cofilin. The mobility of cells was inhibited when cells expressed p57^{Kip2} as compared with control cells, indicating that it may have a role in cell migration.

Our findings define a novel mechanism for coupling cytoplasmic p57^{Kip2} to the regulation of actin polymerization by the control LIMK-1 activity. These studies suggest that localization of p57^{Kip2} to the cytoplasm in cancer cells is involved in pathways that favour not only cell death (paper I and II), but also prevent cell motility independently of the Rho A/ROCK signalling pathway. These data also support the

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proposal that p57^{Kip2} can act as a tumor suppressor gene. Since remodelling of actin cytoskeleton play a key role in cell migration and has implication for invasion and metastasis, uncovering its biological regulators and better understanding of p57^{Kip2}/LIMK-1/cofilin signalling pathways may provide new insights into tumor development and progression.

RESULTS

PAPER IV - p57^{Kip2} is a repressor of Mash1 activity and neuronal differentiation in neural stem cells

Recent studies revealed that developmental processes in embryogenesis are regulated positively or negatively by multiple basic helix-loop-helix (bHLH) genes. For example, the bHLH genes *MyoD* and mammalian achaete-scute homolog 1 (*Mash1*) positively regulate muscle and neuronal differentiation, respectively (Lo *et al.*, 2002; Lu *et al.*, 1999). The bHLH gene *Hes1* antagonises *MyoD* and *Mash1* and keeps cells at the precursor stage (Chen *et al.*, 1997; Sasai *et al.*, 1992). bHLH proteins play key roles in the control of the gene expression during development of various tissue types, including the nervous system. One member of the bHLH family of proteins is *Mash1*. *Mash1* was shown to be involved in the determination and differentiation of neurons. The aim of this study was to investigate putative interactions of p57^{Kip2} with bHLH factors involved in neural differentiation, and whether p57^{Kip2} exerted any influence on the differentiation of neural stem cells (NSCs).

The expression of p57^{Kip2} is believed to play an essential role for the maturation of several tissues such as skeletal muscle, lung alveoli, retina and bone. p57^{Kip2} influence differentiation, cell death of several cell types (paper I and II) and cytoskeletal organisation (paper III), implying that it have a broader scope of cellular action than only being a cell-cycle regulator. Previous studies have indicated that p57^{Kip2} can interact with *Nurr1* to promote differentiation of dopamineurergic neurons, with the bHLH factors *MyoD*, acting positively on myogenic differentiation and with *E47* inducing cell-cycle arrest in developing neuroblast (Reynaud *et al.*, 2000; Rothschild *et al.*, 2006).

In this paper we found that p57^{Kip2} interacts with a subset of neurogenic bHLH proteins, including *Mash1*, *NeuroD*, and *Nex/Math2*. p57^{Kip2} transiently accumulates in the nuclei of neural progenitors during early astrocyte differentiation and repress neuronal differentiation by inhibiting the transcriptional activity of *Mash1*. The repression of activity as well as the interaction turned out to be independent of p57^{Kip2}'s role as a cell-cycle regulator. p57^{Kip2} interaction with *Mash1* represses neuronal differentiation, possibly to allow proper glial differentiation.

Our results expand the understanding of p57^{Kip2}'s function in neural differentiation and strengthen the hypothesis that p57^{Kip2} provide a direct molecular link between cell-cycle and differentiation, in addition to previous reports on its role in cell migration during development.

CONCLUSIONS AND FUTURE PERSPECTIVES

p57^{Kip2} is typically viewed as a cell-cycle regulator but during the years different ways of looking at p57^{Kip2} have started to emerge. Indeed, in this thesis we show that it has other functions. These new functions of p57^{Kip2} have to be studied further in the near future to establish its significance in different processes and diseases. We established that it has a pronounced role in differentiation, apoptosis and cytoskeleton reorganisation. We believe that these different roles of p57^{Kip2} are not separated but intertwined into each other, implying that deregulation of p57^{Kip2} can have consequences in these processes (Figure 4). Furthermore, p57^{Kip2} is considered as a tumor suppressor gene, since it is often found inactivated in various types of human cancers. In fact, loss or low levels of p57^{Kip2} protein correlates with poor survival for the patient in multiple human cancer malignancies like lung, kidney, stomach, pancreas, liver, thyroid and urinary bladder (Fan *et al.*, 2006; Jin *et al.*, 2008).

The studies presented in the current thesis are aimed at elucidating the importance of p57^{Kip2} in different processes in the cell.

In paper I we uncovered that p57^{Kip2} has a role during cell death by sensitizing cells to apoptosis through the mitochondrial apoptotic pathway. One protein that was found to be partially dependent on p57^{Kip2} to induce cell death is the transcription factor p73 β discussed in paper II. Both p57^{Kip2} and p73 β are important during development, cell differentiation (Danilova *et al.*, 2008; Yan *et al.*, 1997; Zhang *et al.*, 1997) and they play a central part in cell-cycle regulation (Ichimiya *et al.*, 2000). In this paper we demonstrate the induction of p57^{Kip2} expression by p73 β . Silencing of p57^{Kip2} significantly reduced p73 β induced apoptosis through the mitochondrial pathway. Studies indicate that both proteins are not usually mutated but silenced by epigenetic mechanisms. This is apparent in ALL, where both proteins are usually silenced and associated with a poor prognosis for the patient (Canalli *et al.*, 2005). Both proteins are considered to be tumor suppressor genes, thus the conclusion from this study confirm their tumor suppressor activity.

During paper I, we came to the conclusion that the function of p57^{Kip2} was cell type specific. This can be explained by different effects; it can partially be attributed to cell- and tissue-specific differences in the function of apoptotic and antiapoptotic signal transduction pathways. The abundance of p57^{Kip2} is thought to be controlled by the ubiquitin–proteasome pathway. Phosphorylation at threonine residue (Thr-310) of human p57^{Kip2} is required for Skp2-mediated ubiquitylation and proteolysis (Kamura *et al.*, 2003). Therefore, potential differences in p57^{Kip2} post-translational modifications could in principle also influence its function in a cell type-specific manner. To notice, cell type specificity effects on apoptosis have also been reported for another member of the Cip/Kip family, p27^{Kip1}. Perhaps, in future treatments one could reintroduce p57^{Kip2} in tumor cells lacking its expression, making them more sensitive to drug induced apoptosis. This could result in better treatment for the patients in the future. Findings in paper III indicated that p57^{Kip2} also is involved in the remodelling of the

CONCLUSIONS AND FUTURE PERSPECTIVES

cytoskeleton. By interacting with LIMK-1 in the cytoskeleton it promoted changes in cytoskeleton reorganisation, an indication that it also could affect inhibition of cell migration and cancer metastasis. Moreover, p57^{Kip2} has been implied in cases of lymph node metastasis (Fan *et al.*, 2006) and in human prostate cancer (Jin *et al.*, 2008) where its expression is significantly decreased. p57^{Kip2} regulation of the LIMK-1 signalling pathway could have implications in the coordination of cytoskeletal changes during the different phases of the cell-cycle. This could also be the case for the regulation of cell migration and for the migratory and invasive capacity of tumor cells.

In paper IV we concluded that p57^{Kip2} acts as a transcriptional repressor, by inhibiting neuronal cells to differentiate; and thereby controls neurogenesis. Furthermore, it shows that this protein is important in embryogenesis and during the differentiation of specific cell types. This is not surprising since p57^{Kip2} is the only Cip/Kip member required during embryogenesis (Yan *et al.*, 1997). Embryonic development requires cell proliferation, cell-cycle exit, differentiation, and migration of cells in a highly coordinated manner. p57^{Kip2} is important as it regulates cell-cycle exit, but also influences processes of cell differentiation. Several studies have also indicated that it is important for the migration of cells to their ultimate destination. In the future it would be worthwhile to investigate p57^{Kip2}'s role in development, cell differentiation and cell migration, in particular to study the localization and functional role in different areas of the developing individual.

Is there any connection between p57^{Kip2} stabilization of actin and its role in apoptosis?

It would be interesting to explore different proteins with roles in the organization of actin as well as apoptosis and investigate their possible interactions with p57^{Kip2}.

One of these proteins is gelsolin that inhibit apoptosis by enhancing actin depolymerization through its ability to block mitochondrial VDAC activity (Kusano *et al.*, 2000). Mapping the part of p57^{Kip2} that is of significance for actin regulation would also give interesting information.

In a future perspective, it would be worthwhile to address the question if p57^{Kip2} might regulate mitochondrial apoptotic functions through its role in regulating actin dynamics.

It is known that a reduction in actin dynamics can lead to reduced mitochondrial membrane potential ($\Delta\psi_m$) and sensitivity to apoptotic insult (Gourlay and Ayscough, 2005). Interestingly, studies conducted previously indicate that actin is involved in the regulation of the VDAC, which functions in the mitochondrial apoptotic pathway as mentioned in the introduction of this thesis. Opening and closure of the VDAC is related to the regulation of the permeability of the outer mitochondrial membrane. In an open, or pro-apoptotic, state, which occurs when the actin cytoskeleton is stabilized, the VDAC allows the release of apoptogenic proteins, such as cyt *c*, from the mitochondria. Closure of the VDAC, in response to a dynamic actin cytoskeleton, is related to resistance to apoptotic stimuli.

CONCLUSIONS AND FUTURE PERSPECTIVES

As we have shown in paper I, p57^{Kip2} translocates to the cytosol and it is acting there as a pro-apoptotic protein.

In paper III, we show that p57^{Kip2} is involved in cytoskeleton remodelling, inducing stress fiber formation. We hypothesize that these different functions of p57^{Kip2} could be associated. By promoting stabilization of the actin and opening state of the VDAC, p57^{Kip2} promotes the release of pro-apoptotic proteins making the cell more sensitive to apoptotic stimuli.

Any modifications of cytoplasmic p57^{Kip2}?

Interestingly, we established that p57^{Kip2} is shuttled out of the nucleus into the cytoplasm, where it carries out its diverse functions. In fact, cytoplasmic localization for p57^{Kip2} has been reported in non-small-cell lung carcinoma (Pateras *et al.*, 2006) and oesophageal squamous cell carcinoma (Matsumoto *et al.*, 2000). Still, several details about the cytoplasmic localization and mechanism remain unknown; is p57^{Kip2} phosphorylated before shuttled out? Phosphorylation of various amino acids controls many aspects of Cip/Kip protein biology, not only by altering the Cip/Kip protein's affinity for specific cyclin-CDK complexes and other proteins, but also their stability (reviewed in (Borriello *et al.*, 2007; Child and Mann, 2006), and their subcellular localization. Phosphorylation of p21^{Cip1} at two sites, Thr-145 and Ser-153, by PKB/Akt and PKC, respectively, promotes the cytoplasmic retention of p21^{Cip1} (Child and Mann, 2006; Rodriguez-Vilarrupla *et al.*, 2005; Zhou *et al.*, 2001).

Phosphorylation of p27^{Kip1} at Thr-157 or -198 by Akt/PKB, inhibits nuclear import of p27^{Kip1}, resulting in the accumulation of p27^{Kip1} in the cytoplasm (Liang *et al.*, 2002; Shin *et al.*, 2002; Viglietto *et al.*, 2002). Phosphorylation at Ser-10 seems to play an important role in the exit of the protein from the nucleus. The nuclear-cytoplasmic shuttling of p27^{Kip1} upon Ser-10 phosphorylation requires the binding of p27^{Kip1} with CRM1/exportin (Connor *et al.*, 2003; Ishida *et al.*, 2002). p27^{Kip1} has also been shown to exit from the nucleus after its association with the protein Jab1, which interacts with CRM1 (Tomoda *et al.*, 1999).

Although we are far from understanding the control of p21^{Cip1} and p27^{Kip1} molecular biology by phosphorylation, it is evident that these modifications have profound consequences for the function of the members of the Cip/Kip family. Further analysis of p57^{Kip2} and its phosphorylation status at different residues could give important information about its subcellular localization.

Furthermore there is a conserved caspase cleavage site in the p57^{Kip2} sequence, suggesting that it is a potential substrate for caspases. It would be interesting to characterize the importance of this site for its different functions and its importance for the cytoplasmic localization. p21^{Cip1} and p27^{Kip1} is cleaved by caspase-3 and

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inactivated during the apoptotic process (Gervais JL, 1998; Zhang Y, 1999). In this regard, caspase-3 cleaves the p21^{Cip1} nuclear localization signal to relocalize the inhibitor to the cytoplasm. Furthermore, it was suggested that caspase-3 mediated cleavage of p21^{Cip1} and p27^{Kip1} associated with cyclin A/Cdk2 was required for apoptosis in SK-HEP-1 cells, leading to induction of cyclin A/Cdk2 activity and apoptosis (Jin *et al.*, 2000). Many aspects still remain to be investigated with regards to p57^{Kip2} localization in the cytoplasm.

Why is p57^{Kip2} special?

The functions of the unique domains in the middle of the structure of p57^{Kip2} are a total enigma. These additional domains may shape the specific characteristics of p57^{Kip2}. It seems that the most straightforward way of elucidating their functions is to identify the molecules associated with them. Identification and characterization of the putative binding molecules might further help our understanding of the specific function of p57^{Kip2}. Results obtained from this thesis will help to unravel p57^{Kip2}'s function in apoptosis, and migration as well as in cell differentiation. In particular, how one and the same gene, p57^{Kip2}, depending of the cell context, can interfere with different biological processes. This thesis demonstrates that p57^{Kip2} and its family members should not be viewed only as cell-cycle regulators, but as factors regulating various signalling pathways.

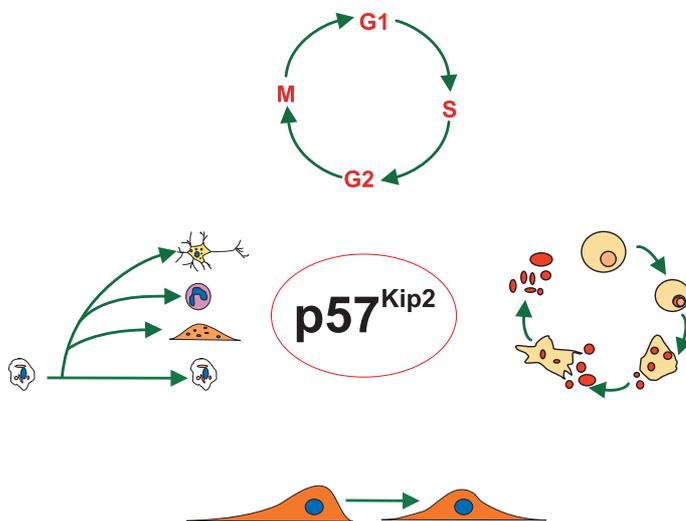


Figure 4. Summary of p57^{Kip2}'s functions.

We established that p57^{Kip2} has a pronounced role in differentiation, apoptosis and cytoskeleton reorganisation besides acting a cell-cycle regulator.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Var tredje person i Sverige drabbas någon gång under sin livstid av cancer. Det finns cirka 200 olika cancersjukdomar som alla har varierad svårighetsgrad. Cirka 20 000 personer om året dör i cancer i Sverige, vilket gör cancer till den näst vanligaste dödsorsaken, efter hjärt- och kärlsjukdomar.

Cellerna är kroppens minsta byggstenar. Människokroppen består av mellan 50 000 och 100 000 miljarder celler. Celler dör och ersätts hela tiden av nya celler. I kroppen bildas ständigt nya celler, detta sker genom att cellerna delar sig. När en frisk cell blir gammal och dör, ersätts den strax med en ny precis likadan cell som utför samma arbete.

Det finns ca 200 olika typer av celler, alla med olika funktioner. Cellerna är uppdelade i vävnader och vävnaderna bildar i sin tur organ. Tre olika processer påverkar celltillväxt: **cellproliferation**, **celldifferentiering** och **celldöd**. Alla tre processerna är aktiva under människans hela livsperiod, från embryo till vuxen människa.

Cellproliferation innebär att celler växer och delar sig, d.v.s. att cellantalet ökar med bevarad cellstorlek. Här finns det olika gener; ”broms gener”, som säger till cellen att sluta dela sig och ”gasgener”, som sätter igång celldelning och förnyelse. Cancer är ofta ett resultat av en okontrollerad cellproliferation. Det kan uppstå på grund av en genetisk skada som slår ut ”bromsgenerna” eller en skada som snabbar upp ”gasgenerna”.

Celldifferentiering innebär att varje cell har egenskaper som är specifika för just denna celltyp och gör att cellen kan utöva sina funktioner. Det finns olika typer av gener som sätter igång utveckling av en speciell celltyp.

Celldöd kan ske när cellen utsätts för en skada ifrån omgivningen. Utöver detta finns något som kallas **programmerad celldöd** (s.k. självmord) som är reglerad, inte slumpmässig, och till nytta för kroppen. Cancer kan också uppstå om den programmerade celldöden är lägre än normalt, då växer cellerna obehindrat och tumörer kan uppstå. Om den programmerade celldöden är större än normalt kan degenerativa tillstånd uppstå, t.ex. Alzheimers sjukdom och andra neurologiska sjukdomar som kännetecknas av att nervceller dör.

Det är nödvändigt att förstå hur cellproliferation, celldifferentiering och celldöd regleras. Bara då kommer man att kunna förstå människans normala biologiska utveckling och uppkomsten av många sjukdomstillstånd.

Ett flertal gener deltar i regleringen av cellproliferation, celldifferentiering och celldöd. Några av dem har en roll i alla tre processerna och de fungerar alla inom ett komplext nätverk av signalsystem, en av dessa är p57.

p57 är en gen som länge visat sig ha en viktig funktion i cellproliferation d.v.s. den verkar som en ”broms gen”, och informerar cellen att sluta dela sig. Låg nivå eller

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frånvaro av p57 protein kan observeras i tumörer från olika typer av vävnader, såsom lunga, njure, mage, bukspottskörtel, lever, sköldkörtel och urinblåsa. I dessa tumörer korrelerar låg nivå av p57 med sjukdomens progression och en dålig prognos för patienten.

Målet med den här avhandlingen var att studera p57 och dess roll i celldöd och celldifferentiering. Sammanfattningsvis kan vi säga att p57 inte bara har en roll som ”broms” men också en funktion vid celldöd. Genom dessa upptäckter har vi kunnat aktivera p57 i cancerceller och på så sätt gjort dem mer benägna att dö. Detta kan troligtvis också begränsa cancers förmåga att sprida sig till andra platser i kroppen. Utöver detta har vi också visat att p57 har en betydande roll vid differentiation av celler i hjärnan.

Genom dessa studier har vi fått nya sätt att studera p57 vilket i framtiden kan leda till nya behandlingsformer för cancer och bättre förutsättningar för patienter med låga nivåer av detta protein.

ΕΠΙΣΤΗΜΟΝΙΚΟΙ ΠΕΡΙΛΗΨΗ

Κάθε τρίτος άνθρωπος στη Σουηδία επηρεάζεται σε κάποια χρονική στιγμή κατά τη διάρκεια της ζωής του από καρκίνο. Υπάρχουν περίπου 200 διαφορετικές μορφές καρκίνου, οι οποίες έχουν ποικίλη σοβαρότητα. Περίπου 20 000 άτομα το χρόνο πεθαίνουν από καρκίνο στην Σουηδία, η οποία καθιστά τον καρκίνο τη δεύτερη κυριότερη αιτία θανάτου μετά τις καρδιαγγειακές παθήσεις.

Τα κύτταρα είναι τα μικρότερα δομικά στοιχεία του σώματος. Το ανθρώπινο σώμα αποτελείται από 50 000 και 100 000 δισεκατομμύρια κύτταρα. Τα κύτταρα πεθαίνουν και αντικαθίστανται συνεχώς από νέα κύτταρα. Όταν ένα υγιές κύτταρο γίνεται παλιό και πεθαίνει, το αντικαθιστά αμέσως ένα νέο ομοειδή κύτταρο που κάνει την ίδια δουλειά. Υπάρχουν περίπου 200 διαφορετικά είδη κυττάρων, κάθε ένα με διαφορετικές λειτουργίες.

Τρεις διαφορετικές διαδικασίες επηρεάζουν την ανάπτυξη των κυττάρων: **ο πολλαπλασιασμός των κυττάρων**, **η κυτταρική διαφοροποίηση** και **ο θάνατος των κυττάρων**. Και οι τρεις διαδικασίες έχουν έντονη δραστηριότητα σε όλη την διάρκεια ζωής των ανθρώπων, από το έμβρυο μέχρι τον αναπτυγμένο άνθρωπο.

Πολλαπλασιασμός των κυττάρων σημαίνει ότι, τα κύτταρα αναπτύσσονται και διαιρούνται, δηλαδή ο αριθμός των κυττάρων αυξάνεται με κύτταρα του ίδιου μέγεθου και τύπου. Υπάρχουν διάφορα γονίδια, "γονίδια φρένο", που λένε στο κύτταρο να διακόψει την αύξηση, και "γονίδια γκάζι", που ενεργοποιούν την κυτταρική διαίρεση, την αναγέννηση και το πολλαπλασιασμό. Ο καρκίνος είναι συχνά το αποτέλεσμα του ανεξέλεγκτου πολλαπλασιασμού των κυττάρων, η λόγω γενετικών βλάβων που απενεργοποιούν τα "γονίδια φρένο" ή λόγω ζημίας που επιταχύνει τα "γονίδια γκάζι".

Κυτταρική διαφοροποίηση σημαίνει ότι κάθε κύτταρο έχει χαρακτηριστικά που είναι ειδικά για αυτό το συγκεκριμένο είδος κυττάρου, και επιτρέπει την άσκηση των καθηκόντων του. Υπάρχουν διάφορα είδη γονιδίων που δίνουν το έναυσμα για την ανάπτυξη ενός ειδικού τύπου κυττάρων.

Ο θάνατος των κυττάρων μπορεί να συμβεί όταν το κύτταρο είναι εκτεθειμένο σε ζημιές από τον εξωτερικό χώρο. Επιπλέον, υπάρχει και κάτι που ονομάζεται προγραμματισμένος κυτταρικός θάνατος (ονομάζεται αυτοκτονία), η οποία δεν είναι

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τυχαία αλλά προγραμματισμένη, και προς όφελος του οργανισμού. Εάν ο προγραμματισμένος θάνατος των κυττάρων είναι μεγαλύτερος από το κανονικό εκφυλιστική κατάσταση μπορεί να προκύψει, π.χ. η της νόσου του Alzheimer και άλλες νευρολογικές διαταραχές που χαρακτηρίζονται από νευρικά κύτταρα που πεθάνουν.

Ο καρκίνος μπορεί επίσης να προκύψει εάν ο προγραμματισμένος θάνατος των κυττάρων είναι λιγότερος από τον κανονικό, τότε αυξάνεται και ο απρόσκοπτος όγκος κυττάρων.

Είναι απαραίτητο να κατανοήσουμε τον τρόπο που ρυθμίζεται ο πολλαπλασιασμός των κυττάρων, η κυτταρική διαφοροποίηση και ο θάνατος των κυττάρων. Μόνο τότε θα είμαστε σε θέση να κατανοήσουμε την ανθρώπινη φυσιολογική βιολογική εξέλιξη και την εμφάνιση πολλών ασθενειών.

Διάφορα γονίδια εμπλέκονται στην κανονική ρύθμιση του πολλαπλασιασμού των κυττάρων, της κυτταρικής διαφοροποίησης και του θάνατο των κυττάρων. Ένα από τα γονίδια που έχει ένα ρόλο και στις τρεις διαδικασίες είναι το p57.

p57 είναι ένα γονίδιο που έχει από καιρό ένα σημαντικό ρόλο στον πολλαπλασιασμό των κυττάρων, δηλαδή είναι ένα "γονίδιο φρένο" και ενημερώνει τα κύτταρα να διακόψουν την αύξηση τους. Χαμηλό επίπεδο ή έλλειψη πρωτεϊνών p57 παρατηρείται σε όγκους από διάφορα είδη ιστών, όπως του πνεύμονα, των νεφρών, του στομάχου, του παγκρέατος, του ήπατος και του θυρεοειδούς. Σε αυτούς τους όγκους συσχετίζονται το χαμηλό επίπεδο p57 με την εξέλιξη της νόσου και φτωχή πρόγνωση για τον ασθενή.

Ο στόχος αυτής της διδακτορικής διατριβής είναι η μελέτη και ο ρόλος του p57 στον θάνατο των κυττάρων και τη διαφοροποίηση των κυττάρων. Εν κατακλείδι, μπορούμε να πούμε ότι το p57, δεν έχει μόνο έναν ρόλο ως "γονίδιο φρένο" αλλά και μια λειτουργία στο θάνατο των κυττάρων. Μέσω αυτών των ανακαλύψεων, θα είμαστε σε θέση να ενεργοποιήσουμε το p57 στα καρκινικά κύτταρα και έτσι να είναι πιο πιθανό να πεθάνουν. Αυτό ίσως μπορεί επίσης να περιορίσει τη δυνατότητα του καρκίνου για να εξαπλωθεί και σε άλλα μέρη του σώματος. Εκτός από αυτό, έχουμε επίσης δείξει ότι το p57 παίζει σημαντικό ρόλο στην ανάπτυξη των κυττάρων του εγκεφάλου.

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Μέσα από αυτές τις μελέτες, θα βρεθούν νέοι τρόποι για τη μελέτη του p57 που μπορούν στο μέλλον να οδηγήσουν σε νέες θεραπείες για τον καρκίνο ειδικά για ασθενείς με χαμηλά επίπεδα αυτής της πρωτεΐνης.

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