SCREENING FOR SMALL MOLECULE INHIBITORS OF $p27^{Kip1}$ DEGRADATION

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SCREENING FOR SMALL MOLECULE INHIBITORS OF $p27^{Kip1}$ DEGRADATION

by

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
β -TRCP	β-transducin repeat-containing protein
CBB	Coomassie Brilliant Blue
CDK	Cyclin-Dependent Kinase
CHX	Cycloheximide
CKIs	Cyclin-Dependent Kinase Inhibitors
Cks1	CDK subunit 1
cm	centimeter
CS	Calf serum
Cul1	Cullin subunit
Cys	Cysteine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
FACS	Fluorescence Activated Cell Sorting
FBPs	F-box-proteins
FBW7	F-box and WD-40 domain protein 7
FCS	Fetal calf serum
Gly	Glycine
HA	Hemagglutinin
HBS	HEPES-buffered saline
HEK293T	Human embryo kidney 293T
HRP	Horseradish Peroxidase
HTS	High-Throughput Screening
IC_{50}	Half maximal inhibitory concentration
IPTG	Isopropyl β-D-1-thiogalactopyranoside
mAG	monomeric Azami Green
MAPK	Mitogen-Activated Protein Kinase
MEM	Minimum Essential Media
mg	miligram
µg	microgram
ml	mililiter
µl	microliter
mM	milimolar
µM	micromolar
mRNA	messenger Ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NaN_3	Sodium Azide
nm	nanometer

NPDepo	Natural Products Depository
NSCLC	Non-small-cell lung carcinoma
OD	Optical Density
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
pT187	phosphorylated Threonine residue at 187
PVDF	Polyvinylidene fluoride
$p27^{Kip1}$	p27 Kinase inhibitor protein
p/s	penicillin/streptomycin
Ras	Rat sarcoma
RB	Retinoblastoma
Rbx1	RING-finger protein
SCF	Skp1-Cul1-F-box-protein
SDS	Sodium Dodecyl Sulfate
Skp1	S-phase kinase-associated protein 1
Skp2	S-phase kinase-associated protein 2
siRNA	small interfering RNA
T187	Threonine residue at 187
TBS	Tris-Buffered Saline
TGF-β	Transforming growth factor-β
Tween20	Polyoxyethylene Sorbitan Monolaurate
Ub	Ubiquitin
UPS	Ubiquitin-Proteasome System

PENYARINGAN MOLEKUL KECIL PERENCAT DEGRADASI $p27^{Kip1}$

ABSTRAK

Dalam majoriti kanser manusia, perencat "cyclin-dependent kinase" (CDK) yang dikenali sebagai $p27^{Kip1}$, biasanya didapati didegradasikan secara aktif oleh laluan ubiquitin-proteasom yang terdiri daripada komponen SCF^{Skp2} E3 Ligase. Walaupun perencat proteasom bertindak untuk menstabilkan $p27^{Kip1}$, molekul kecil perencat yang berkebolehan untuk merencatkan degradasi $p27^{Kip1}$ oleh fungsi SCF^{Skp2} E3 Ligase seterusnya akan menyumbang kepada penghasilan ubat yang lebih spesifik dan efisien dengan kesan sampingan yang lebih rendah. Dalam kajian ini, satu sistem penyaringan daya pemprosesan tinggi berpandukan interaksi protein-protein yang bergantung kepada pemfosforilan dengan menggunakan sistem ekspresi protein baculovirus telah dibangunkan. Sistem ini telah meningkatkan daya pemprosesan untuk menyaringi perpustakaan sebatian kimia yang besar dalam Depositori Produk Semulajadi RIKEN (NPDepo) dan telah memudahkan pengenalpastian perencat molekul kecil degradasi $p27^{Kip1}$. Dalam sistem ekspresi protein baculovirus, protein 2 berdasarkan-kinase fasa-S (Skp2) yang ditag dengan pendarfluor monomerik Azami Hijau (mAG) dan subunit 1 CDK (Cks1) (mAGSkp2-Cks1) kompleks telah dihasilkan. Pengikatan kompleks mAGSkp2-Cks1 kepada $p27^{Kip1}$ fosfopeptida berdasarkan pemfosforilan telah disahkan melalui spektrofluorometri. Dengan pendekatan ini, kira-kira 20,000 sebatian kimia dengan keberatan molekul yang rendah yang disimpan dalam RIKEN NPDepo telah disaringi dan dua sebatian molekul kecil iaitu linichlorin A and gentian violet telah dikenalpasti merencat interaksi antara mAGSkp2-Cks1 dan $p27^{Kip1}$ fosfopeptida. Kajian

yang lanjut menunjukkan bahawa sebatian-sebatian tersebut telah merencat "ubiquitination" $p27^{Kip1}$ *in vitro*. Sebatian kecil tersebut juga didapati menstabilkan tahap $p27^{Kip1}$ dalam sel HeLa yang seterusnya menghalang degradasi pesat $p27^{Kip1}$. Kedua-dua sebatian itu juga menunjukkan aktiviti antiproliferatif yang lebih cenderung terhadap sel HeLa dan sel mencit terubahsuai, tsFT210 berbanding dengan fibroblast embrio mencit, sel NIH3T3 pada kepekatan 3.2 µM dan 1.6 µM untuk linichlorin A dan 0.4 µM dan 0.6 µM untuk gentian violet masing-masing untuk sel HeLa dan sel tsFT210. Selain itu, sebatian-sebatian tersebut juga merangsang perlambatan kemajuan fasa G_1 dalam sel tsFT210 pada kepekatan yang sama. Pengenalpastian molekul kecil perencat yang spesifik dan pendekatan yang digunakan dalam kajian ini mencadangkan strategi yang berpotensi untuk restorasi tahap $p27^{Kip1}$ protein dalam kanser manusia.

SCREENING FOR SMALL MOLECULE INHIBITORS OF $p27^{Kip1}$ DEGRADATION

ABSTRACT

In a majority of human cancers, the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ is commonly found to be deregulated due to proteolysis by the ubiquitin-proteasome pathway involving the SCF^{Skp2} E3 ligase. Although proteasome inhibitors act to stabilize $p27^{Kip1}$, small molecules agents inhibiting the degradation of $p27^{Kip1}$ by the function of SCF^{Skp2} E3 ligase may contribute to the development of a specific targeted drug that could prove to be more efficacious with less side effects. In this work, we have developed a high-throughput screening system based on phosphorylation dependent protein-protein interaction using the baculovirus protein expression system. This system has provided an increased throughput in screening of large libraries of compounds in RIKEN Natural Products Depository (NPDepo) and has facilitated the identification of small molecule inhibitors of $p27^{Kip1}$ degradation. In the baculovirus protein expression system, S-phase kinase-associated protein 2 (Skp2) tagged with fluorescent monomeric Azami Green (mAG) and CDK subunit 1 (Cks1) (mAGSkp2-Cks1) complex was constructed. The binding of mAGSkp2-Cks1 complex to $p27^{Kip1}$ phosphopeptides in a phosphorylation dependent manner was confirmed by spectrofluorometry. With this approach, we have screened approximately 20,000 low molecular weight compounds stored in RIKEN NPDepo and have identified two small molecule compounds namely; linichlorin A and gentian violet that were shown to inhibit the interaction between mAGSkp2-Cks1 and $p27^{Kip1}$ phosphopeptides. Further studies

have shown that the compounds inhibited the *in vitro* ubiquitination of $p27^{Kip1}$. It was discovered that these small molecule compounds stabilized the level of $p27^{Kip1}$ in HeLa cells, thus preventing the rapid degradation of $p27^{Kip1}$. Both of the compounds exhibited preferential antiproliferative activity against HeLa cells and transformed mouse cells, tsFT210 relative to the mouse embryonic fibroblast, NIH3T3 cells at concentrations of 3.2 μM and 1.6 μM for linichlorin A and 0.4 μM and 0.6 μM for gentian violet, respectively, in HeLa cells and tsFT210 cells. Moreover, the compounds also induced delay in the G_1 phase progression using tsFT210 cells at the same concentrations. The identification of these specific small molecule inhibitors and the approaches taken in our study suggest a potential strategy for the restoration of $p27^{Kip1}$ levels in human cancers.

CHAPTER ONE

Introduction

1.1 General Introduction

The applications of chemical principles and techniques to biological systems have had remarkable advancements in the fields of chemistry and biology. This emerging multidiscipline approach, also known as chemical biology, has notably contributed in the elucidation of many important underlying biochemical structures and functions including proteins and DNA (Martin and Schultz, 1999). Indeed, the deeper understanding has enabled the manipulation of nucleic acid and protein in living organism, providing platforms in various applications such as proteomics, genetic engineering and pharmacology.

In the past few years, the approach of chemical biology has been extensively applied on many biological studies by the use of small molecule probes. These small molecules had been proven to be useful in exploring protein function and cell biology. Recently, high-throughput screening (HTS) has become a routine method used in many research laboratories in the effort to discover such small molecules in chemical biology studies. The flexibility and high-throughput speed of the HTS process have facilitated the rapid screening of small molecules for novel drug discovery (Inglese *et al.*, 2007). Due to the higher molecular weight of natural compounds, synthetic compounds are more preferable and suitable to develop medicines for oral administration. This has motivated pharmaceutical companies to explore new small-molecule drug candidates through HTS screening systems. One of the screening systems available is based on the protein-protein interaction for the discovery of cell cycle inhibitors (Osada, 2009).

Generally, most eukaryotic cell cycle progression is regulated by regulatory proteins. An unregulated cell cycle will lead to abnormal cell growth and thus, the occurrence of malignant cancers. Normal cells proliferate healthily in response to mitogenic signals, and on the other hand, the proliferation of cancer cells proceeds unchecked due to defects found in both the internal and external proliferation inhibitory signals as well as the negative-feedback systems (Nakayama and Nakayama, 2006). Therefore, insights in the fields of cell cycle regulation and cancer may provide a strategy in the development of novel therapeutic interventions (Frescas and Pagano, 2008).

It is well-known that in a normal cell cycle, a series of kinases control the cell cycle progression from one phase to another. In the cell cycle progression, the phosphorylation of proteins by the different members of the cyclin-dependent kinase (CDK) is crucial. The activation of CDK is dependent upon the association with a particular cyclin. Besides that, the activity of CDK is also controlled by the expression of the CDK inhibitors (CKIs). The ubiquitin-proteasome system (UPS) has an essential role in maintaining and regulating cellular homeostasis. This system controls the cellular abundance of a variety Cyclin/CDKs and CKIs of cellular proliferation which are known to be oncoproteins or tumour suppressors. Hence, these two types of post-translational-protein modification namely phosphorylation and ubiquitination regulate the cell cycle (Bloom and Pagano, 2003; Nakayama and Nakayama, 2006).

The close link between deregulated cell cycle and cancer was initially discovered when findings showed that genes encoding retinoblastoma (Rb) and p53 tumor suppressor proteins that inhibit cell-cycle progression appeared to be frequently mutated or deleted in human cancers (Nakayama and Nakayama, 2006; Chu *et al.*, 2008).

Moreover, deregulated expression or lost of one of the important CKIs, $p27^{Kip1}$ that is commonly found in many human cancers is also associated with poor prognosis (Bloom and Pagano, 2003). Unlike the aforementioned tumor suppressors, $p27^{Kip1}$ is rarely mutated or deleted in human cancers but is usually degraded through enhanced proteolysis (Chu *et al.*, 2008).

Clinical findings indicated that $p27^{Kip1}$ is targeted for degradation by SCF^{Skp2} which is an ubiquitin ligase complex, that consequently resulted with an overexpression of the S-phase kinase-associated protein 2 (Skp2) in many human cancers (Nakayama and Nakayama, 2005). In addition, numerous studies also showed that Skp2 exhibits oncogenic activity (Hershko, 2008). Thus, the involvement of Skp2 in many aggressive cancers suggests that targeting this protein through ubiquitin-proteasome pathway to restore $p27^{Kip1}$ levels are poised to advance the field of cancer biology. With chemical biology approach, this thesis attempts to target the protein-protein interaction by small molecule inhibitors as a fundamental approach for future cancer therapy.

1.2 Hypothesis and Aims of Thesis

In UPS, the involvement of the F-box protein known as Skp2 in the degradation of $p27^{Kip1}$ that promoted tumorigenesis in human has not been fully elucidated and is still being extensively studied in many ongoing researches. Although studies have shown that proteasome inhibitors act to stabilize $p27^{Kip1}$ (Adams and Kauffman, 2004), however, the discovery of small molecule inhibitors for the degradation of $p27^{Kip1}$ to prevent cancers may contribute to the development of a specific targeted drug that could prove to be more efficacious with lesser side effects.

In view of the effort to identify specific small molecule inhibitors of $p27^{Kip1}$ degradation, several compounds have been reported by several groups with various biochemical studies on Skp2-dependent degradation of $p27^{Kip1}$ by those compounds have been confirmed (Chen *et al.*, 2008; Rico-Bautista *et al.*, 2010). This thesis expands upon the studies on the discovery of novel small molecule inhibitors of $p27^{Kip1}$ degradation by developing a HTS system to facilitate the search of the inhibitors from a large library of compounds. Furthermore, the thesis attempts to study the mode of action of the identified inhibitors.

The flow of this thesis is outlined in the following:

- **CHAPTER THREE** describes the construction of a novel screening system by baculoviruses through the approach of phosphorylation-dependent protein-protein interaction to identify small molecule inhibitors of $p27^{Kip1}$ degradation. This chapter also reports on the importance of several proteins involved in the protein-protein interaction in a phosphorylation-dependent manner. Moreover, the efficiency of the screening system to be further employed in HTS was evaluated.

- Upon the construction of the baculovirus-based screening system, HTS for the inhibitors of $p27^{Kip1}$ degradation was performed by exploring the large chemical library in RIKEN Natural Products Depository (NPDepo) as described in **CHAPTER FOUR**. In the same chapter, the confirmation of the inhibitors activity identified from HTS in affecting the protein-protein interaction was outlined by the *in vitro* beads pull-down assay.
- After that, **CHAPTER FIVE** expands the study of the inhibitor's mode of action in the *in vitro* $p27^{Kip1}$ ubiquitination by SCF^{Skp2} E3 Ligase. The importance of the involvement of several enzymes or reagents in this assay was also examined in this chapter.
- **CHAPTER SIX** highlights the study of the inhibitors in a cellular context. This chapter describes the effect of the inhibitors on $p27^{Kip1}$ stabilization in cancer cells in the effort to restore the level of $p27^{Kip1}$ in human cancer.
- Lastly, **CHAPTER SEVEN** summarizes on the examination of the activity of the inhibitors on cancer, transformed and normal cell growth. Those activities exhibited by the inhibitors were compared to evaluate the selectivity of the inhibitors in the cells. Furthermore, the study of the inhibitor's effect on cell cycle progression was also included in this chapter.

Specific aims of this thesis were:

1. To construct a screening system by baculovirus protein expression system for the identification of small molecule inhibitors of $p27^{Kip1}$ degradation.

2. To screen for the inhibitors of $p27^{Kip1}$ degradation by HTS system in a phosphorylation-dependent protein-protein interaction manner and to confirm the inhibitory activity obtained by *in vitro* beads pull-down assay.
3. To examine the effect of the small molecule inhibitors on SCF^{Skp2}-mediated *in vitro* ubiquitination of $p27^{Kip1}$.
4. To examine the effect of small molecule inhibitors on $p27^{Kip1}$ stabilization in cancer cells.
5. To study the selectivity of the small molecule inhibitors on the growth of cancer, transformed and normal cells as well as their effect on cell cycle progression.

CHAPTER TWO

Literature Review

2.1 Cell Cycle and Cancer

Recent insights in the field of cell cycle regulation have provided a better understanding as a platform for cancer research. Both cell cycle regulation and cancer are known to be closely related. A dysfunctional cell cycle regulation will lead to abnormal cell proliferation and these are the fundamentals of cancer development. Therefore, the understanding in the molecular mechanism underlying the deregulation of cell cycle progression in cancer is important to study how normal cells become tumorigenic and how cancer treatments can be designed.

Cancer cells and normal cells differ from one another with a number of important alterations in cell physiology that include several acquired capabilities namely; evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential and sustained angiogenesis as depicted in Figure 2.1 (Hanahan and Weinberg, 2000). These acquired capabilities are found in most and perhaps all types of human tumours. Each of the physiological changes as aforementioned represents the break through of an anticancer defense mechanism erratic into cells and tissues (Hanahan and Weinberg, 2000).

Other than that, it has long been interpreted and suggested that tumorigenesis increased accordingly with age due to the gain of mutations and epigenetic abnormalities in the expression of multiple genes with highly diverse functions (Hanahan and Weinberg, 2000; Park and Lee, 2003). These important groups of genes were thought to involve in the cell cycle checkpoints (Park and Lee, 2003).

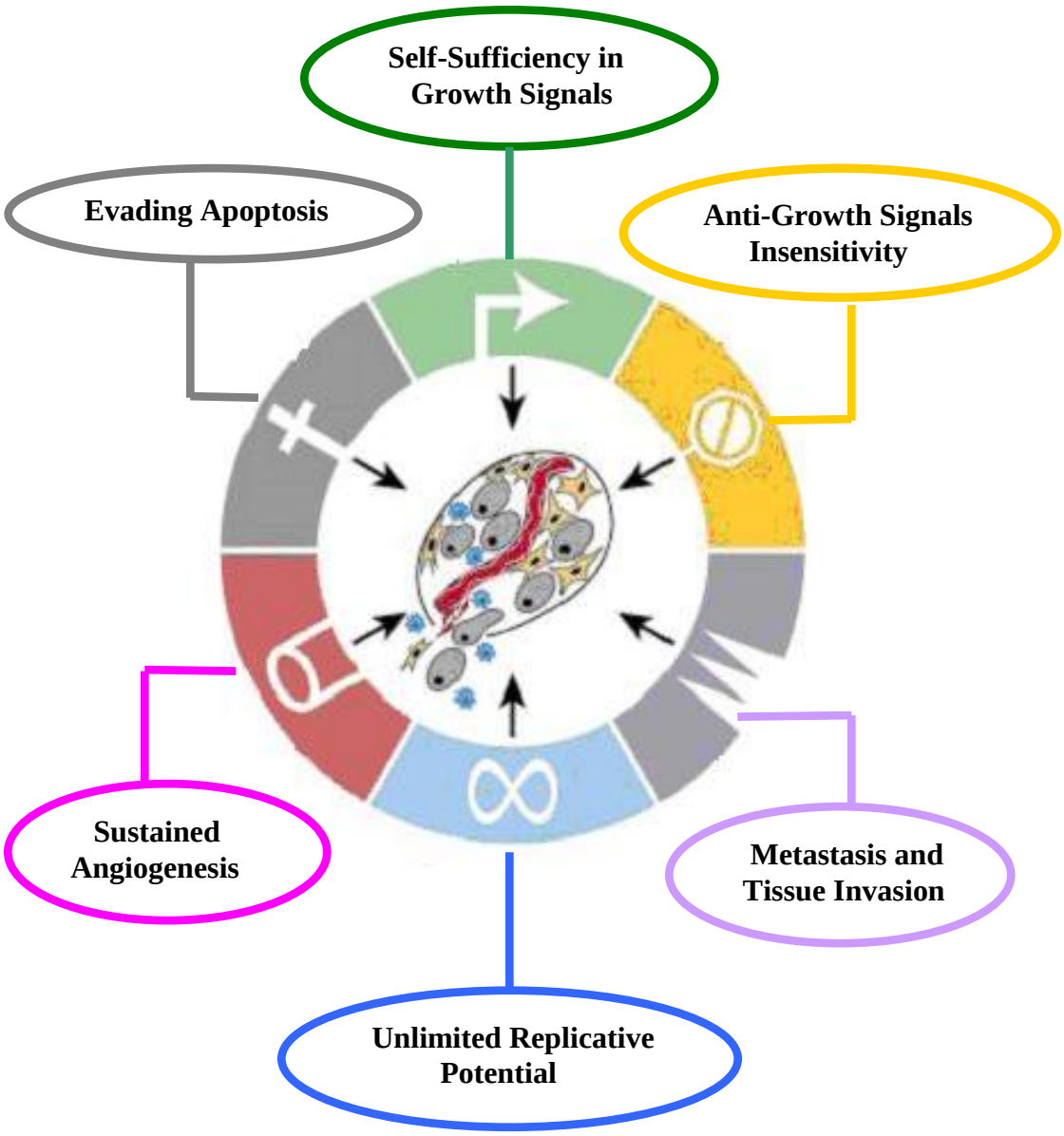

Figure 2.1 Capabilities acquired by most of the cancer cells

Six acquired capabilities shown by most of the human cancers (Hanahan and Weinberg, 2000).

Cell cycle progression is a highly-ordered and a tightly-regulated process with multiple checkpoints. These checkpoints are essential in the cell cycle machinery to sense flaws in important events such as DNA replication as well as to assess extracellular growth signals and cell size (Park and Lee, 2003). Figure 2.2 shows the somatic cell cycle which is divided into four phases namely G_1, S, G_2 and M phase.

In each cell cycle, chromosomes are replicated once during the S-phase, followed by segregation of the chromosomes thereafter to generate two genetically identical daughter cells during the M-phase. These events are spaced by 2 gap phases; G_1 and G_2 phase where the cells prepare for the successful completion of the S-phase and M-phase respectively.

When cells stop cycling after division because of the presence or absence of a certain antimitogenic signals, cells enter a state of quiescence (G_0) which is a non-dividing state. Cells may also be arrested at G_1 or G_2 checkpoints for the evaluation on cell size, extracellular growth signals as well as the DNA integrity (Park and Lee, 2003).

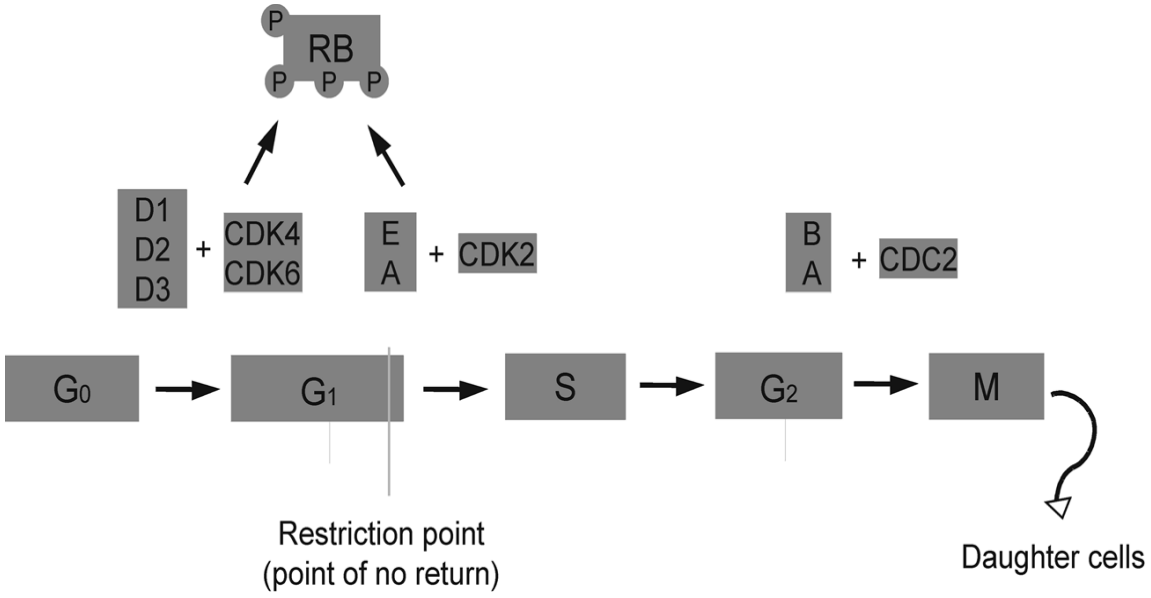

Figure 2.2 A schematic representation of the somatic cell cycle

The cell cycle is divided into four distinct phases: i) initial growth (G_1-phase), ii) DNA synthesis (S-phase), iii) a gap/interval (G_2-phase), and iv) mitosis (M-phase). The restriction point is the critical point of the cell cycle control where the cell cycle is irreversibly progressing to the next cell division (Park and Lee, 2003).

A proper maintenance of the cell cycle through its regulator is essential to prevent human cancers. This is because in a majority of human tumours, cell cycle regulators are commonly found to be mutated. Recently, abnormalities or mutations in Cyclins, CDKs and CKIs have frequently been associated with the process of tumour progression in human cancers (Sgambato *et al.*, 1998). However, to understand the causes of mutations or abnormalities of the cell cycle regulatory proteins which have eventually led to the occurrence of many human cancers, studying and understanding the basic knowledge on the normal cell cycle is important and required (Park and Lee, 2003).

Basically, the cell cycle control mechanism from G_1 to M phase has been previously elucidated by several groups (Pardee, 1989; Morgan, 1995; Hwang and Muschel, 1998). According to their studies, the cell cycle progression involved a highly regulated kinase family known as the Cyclin-Dependent Kinases (CDKs). Kinase activation generally requires the association with another protein called cyclin, to create an active complex (Pardee, 1989; Morgan, 1995; Hwang and Muschel, 1998). Table 2.1 and Figure 2.2 show the complexes formed between the CDKs and cyclins, as well as their involvements in cell cycle regulation.

Table 2.1 CDK-cyclin complexes

Cyclins	CDKs
Cyclin A and B	Cdc2
Cyclin A, E and D	Cdk2
Cyclin D1, D2, D3	Cdk4, Cdk6
Cyclin E	Cdk3
Cyclin C	Cdk8
Cyclin H	Cdk7
Cyclin T	Cdk9
p35	Cdk5

Active complexes formed between mammalian CDKs and their regulatory cyclins (Park and Lee, 2003).

CDKs and their cyclin partners are known to be the positive regulators of the cell cycle. They act as an accelerator in inducing the cell cycle progression. Contradictorily, the negative regulators of the cell cycle for example the inhibitors of the cyclin-dependent kinase (CKIs) (Sherr and Roberts, 1995; Sherr and Roberts, 1999), act in an opposite manner. They act to halt the cell cycle progression depending on the regulatory signals.

There are two families of CKIs which have been characterized namely; the Ink4 family and the Kip/Cip family. Members of the Ink4 family which include $p15^{Ink4b}$, $p16^{Ink4a}$, p18 and p19 inhibit the progression of the cell cycle by binding to either CDK4 or CDK6 and blocking the action of cyclin D. On the other hand, the members of Kip/Cip family of CKIs which include $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ inhibit specifically the activity of CDK2 by binding to both complexes of CDK2/Cyclin E and CDK2/Cyclin A (Park and Lee, 2003).

Therefore, controlling the regulation of these positive and negative regulators of the cell cycle is crucial as a major role in determining a proper progression of a cell cycle in order to avoid uncontrollable and abnormal proliferation of cells which are known to induce the occurrence of cancer. Recently, many works have been done to investigate the role of CKIs and meanwhile, this thesis focuses on one of the CKIs, $p27^{Kip1}$ which is usually found to be deregulated in a majority of human cancers.

2.2 Cell Cycle Functions and Regulation of $p27^{Kip1}$

It is known that the control of eukaryotic cell cycle progression is governed by the cyclin dependent kinases (CDKs) which is activated upon cyclin binding and inhibited by the CDK inhibitors (CKIs) (Morgan, 1995; Sherr and Roberts, 1995; Sherr and Roberts, 1999; Chu *et al.*, 2008). One of the CKIs, $p27^{Kip1}$, which is a 198 amino acid protein that was originally discovered in cells arrested by transforming growth factor-β (TGF-β), by lovostatin and contact inhibition, (Polyak *et al.*, 1994; Slingerland *et al.*, 1994) has pivotal roles in the control of cell proliferation, cell motility, senescence and apoptosis (Sherr and Roberts, 1995; Sherr and Roberts, 1999; Chu *et al.*, 2008). In the cell cycle, a $p27^{Kip1}$ level is found to be high in G_0 and early G_1 to target inhibition on the G_1 CDK/Cyclin primarily on the E-type cyclin/CDK2 complexes that eventually causes G_1 cell cycle arrest.

Studies have shown that a variety of abnormalities were observed in the $p27^{Kip1}$ knockout mice. When compare to the control animals, it was observed that mice lacking $p27^{Kip1}$ developed multi organ enlargement that eventually led to an increase in the body size, multi organ hyperplasia, retina dysplasia and pituitary tumours (Slingerland and Pagano, 2000). These observations hence supported the importance of $p27^{Kip1}$ in cell proliferation and differentiation (Fero *et al.*, 1996; Nakayama *et al.*, 1996).

Although the levels of $p27^{Kip1}$ oscillate during the cell cycle, being at the highest level during G_1, such a fluctuation in protein levels during the cell cycle is not similarly observed in the $p27^{Kip1}$ mRNA levels. It has been reported that the mRNA levels of $p27^{Kip1}$ remained constant throughout the cell cycle (Hengst and Reed, 1996). Instead, the $p27^{Kip1}$ protein levels are known to be mainly regulated by a sequential degradation

system known as the ubiquitin-mediated proteolysis system (Pagano *et al.*, 1995; Bloom and Pagano, 2003) which will be described in detail in the following section of 2.3.2.

The functional inactivation of $p27^{Kip1}$ can occur in several ways by the activation of tumour-associated oncogenes. For example, the overexpression of *c-myc* can induce a heat-labile factor that binds $p27^{Kip1}$ and inhibits the association of $p27^{Kip1}$ with CDK2/Cyclin E (Vlach *et al.*, 1996). This effect is said to be independent of $p27^{Kip1}$ degradation. In certain cell types, it is reported that the upregulation of Cyclin D1 and D2 by *c-myc* leads to sequestration and inactivation of $p27^{Kip1}$ (Slingerland and Pagano, 2000).

Other than that, several factors have been shown to cause the loss of $p27^{Kip1}$ (Slingerland and Pagano, 2000). *Ras* is one of the important factors reported for the loss of $p27^{Kip1}$. According to Aktas *et al.* (1997) and Takuwa and Takuwa (1997), $p27^{Kip1}$ accumulation and G_1 cell cycle arrest were observed in the fibroblast cells transfected with a dominant negative *ras* allele. This suggests that for the progression of cell cycle from G_1 to S phase, *ras* is required for the degradation of $p27^{Kip1}$.

However, another study has shown that *ras* itself is not sufficient for the loss of $p27^{Kip1}$ but rather requiring co-expression of *ras* and *myc* (Leone *et al.*, 1997). Furthermore, overexpression of mitogen-activated protein kinase (MAPK) in fibroblast has been reported to cause an increase in $p27^{Kip1}$ degradation (Kawada *et al.*, 1997).

In contrast, these effects of *ras* on the loss of $p27^{Kip1}$ have also been shown to be antagonized by the action of several chemicals (Slingerland and Pagano, 2000). For instance, effect of *ras* on $p27^{Kip1}$ was abolished by the inhibitor of mitogen-activated protein kinase, PD9859 (Kawada *et al.*, 1997). Other studies as reported in the previous publications by several groups also showed the effect of *ras* to be abrogated by

wortmannin (Ui *et al.*, 1995) and LY294002 (Vlahos *et al.*, 1994), which are the PI3K inhibitors.

Generally, $p27^{Kip1}$ is a phosphoprotein and the phosphorylation of $p27^{Kip1}$ is known to be cell cycle regulated. Very commonly, phosphorylation of $p27^{Kip1}$ is the signal for ubiquitination which is the process responsible for the degradation of $p27^{Kip1}$ (Alessandrini *et al.*, 1997). The regulation of $p27^{Kip1}$ degradation is related to its phosphorylation by Cyclin E/CDK2 complex (Sheaff *et al.*, 1997; Vlach *et al.*, 1997 Montagnoli *et al.*, 1999; Slingerland and Pagano, 2000). Therefore, understanding the pathways or mechanisms that trigger the phosphorylation of $p27^{Kip1}$ followed by the degradation meachanisms may provide mechanistic links for further evaluation between the deregulated cell cycle in cancers and the activation of oncogenes and tumor suppressors (Slingerland and Pagano, 2000).

2.3 The Ubiquitin-Proteasome System (UPS)

About more than three decades ago, it has been reported that many regulatory proteins indeed have a short life and a majority of them are degraded through the ubiquitin proteasome pathway (Schimke, 1973). Therefore, the ubiquitin-proteasome pathway is the principal mechanism and selective degradation of various short-lived proteins in eukaryotic cells (Pickart, 1997; Hershko and Ciechanover, 1998). This pathway controls the degradation of a vast majority of proto-oncogenes, tumour suppressors as well as the components responsible for the signal transduction system which determine the cell growth and the cell proliferation. Because of that, abnormalities or dysfunctional ubiquitin-mediated processes will eventually cause

pathological conditions including malignant transformation (Hershko and Ciechanover, 1998).

In UPS, degradation of a particular targeted protein occurred through covalent ligation to ubiquitin, which is a 76 amino acid residue protein. Ubiquitin acts as a degradative tag of the targeted proteins upon the recognition of the targeted proteins by the 26S proteasome for degradation. Together, the function of ubiquitin as a covalent degradation signal and the properties of the 26S proteasome as an enzyme created novel features of UPS (Pickart, 1997).

The overview of UPS is illustrated in Figure 2.3. Briefly, a targeted protein is degraded through a sequential degradation system, requiring the action of three enzymes. Step one of the UPS begins with the activation of the entire cellular pool of the ubiquitin molecules at the C-Terminal Gly residue in an ATP-dependent manner by a specific activating enzyme called E1. This step involves the binding of the ubiquitin molecule to a Cys residue to E1 through thiolester linkage, releasing AMP.

Then, step two of the UPS continues the system with the ubiquitin-conjugating enzyme known as E2, to inherit the activated thiolester-bonded ubiquitin from the E1 enzyme. After that in the third step of this system, the E3 ligase enzyme binds both the target substrate and the E2 complex which then transfers ubiquitin to the target protein. This step features the selectivity of protein degradation which is determined by the specificity of a certain class of cellular proteins that bind to a specific E3 ligase enzyme.

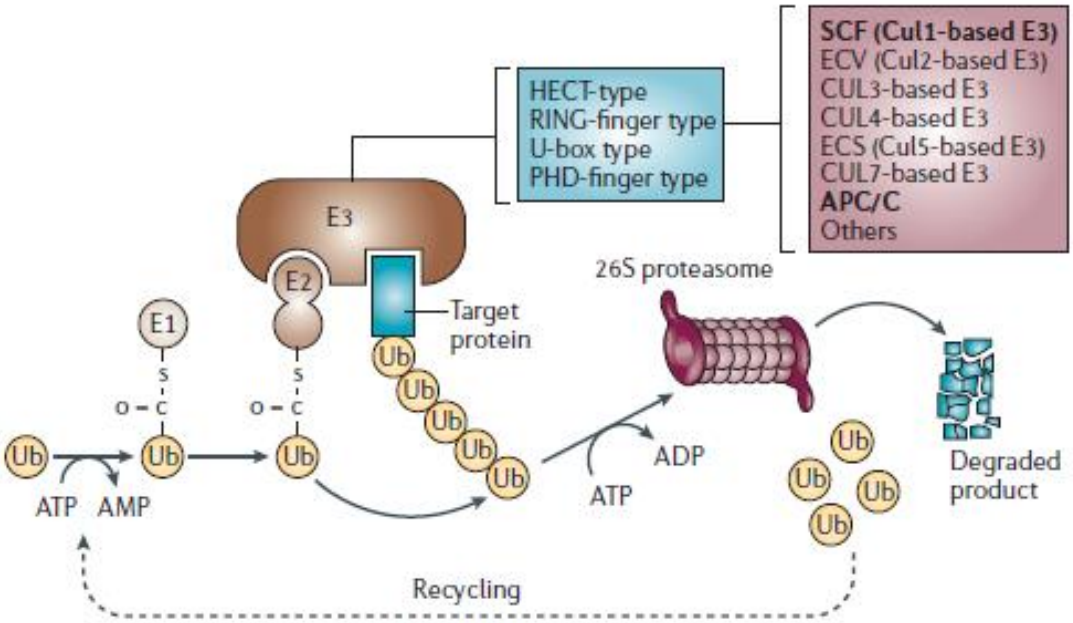

Figure 2.3 Schematic representation of the ubiquitin-proteasome pathway

The pathway begins with the transferring of ubiquitin (Ub) protein in an ATP-dependent manner to the ubiquitin-activating enzyme (E1). The activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme (E2), followed by the covalent attachment of ubiquitin to the target protein by the ubiquitin ligase (E3), forming a polyubiquitin chain. Upon polyubiquitination of the target protein, the polyubiquitinated protein is recognized by the 26S proteasome and degraded (Nakayama and Nakayama, 2006).

Several rounds of the ubiquitin conjugation produce polyubiquitination (long chains of ubiquitin moieties) of the target protein. The polyubiquitinated target protein will then be recognized by the 26S proteasome prior to its degradation. The ubiquitin molecules are then freed and released upon the degradation of the target protein (Hershko and Ciechanover, 1998; Nakayama and Nakayama, 2006).

Studies have also shown that there is only a single E1 but multiple species of E2 and E3 which involve in the ligation to ubiquitin of different proteins (Hershko and Ciechanover, 1998). As the specificity of an ubiquitin system is determined by the E3 ligase enzyme, therefore majority of the studies on UPS concentrated on the E3 ubiquitin ligases (Nakayama and Nakayama, 2005).

Generally, the E3 ubiquitin ligases are categorized into four classes; RING-finger type, HECT-type, U-box-type and PHD-finger-type. The RING-finger-type E3 ligases are further divided into subfamilies including the cullin-based E3 ligase. In fact, there are seven cullin-based E3 ligases as shown in Figure 2.3 (Nakayama and Nakayama, 2006). Two major cullin-based E3 ligases which have a central role in cell-cycle regulation are the Skp1-Cul1-F-box-protein (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C) (Nakayama and Nakayama, 2006). This thesis involves the examination on the degradation of one of the CKIs, $p27^{Kip1}$ through ubiquitination by the SCF complex with Skp2 as the specific F-box protein.

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2.3.1 SCF and F-Box Protein (FBP)

In UPS, E3 components are primarily responsible for the specific recognition of target proteins (Hershko, 1983). Referring to Figure 2.3 in the previous section, E3 ligases are divided into four major classes according to their specific structural motif, in which RING-finger-type E3 ligases appeared to be the largest family with subfamilies. Cullin-based E3 subfamily, for example the Skp1-Cul1-F-box-protein (SCF) complex is involved in the proteolysis of the components of the cell-cycle machinery (Nakayama and Nakayama, 2006).

In this complex, Cul1 (Cullin subunit) functions as a molecular scaffold protein that interacts simultaneously with an adaptor protein, Skp1 (S-phase kinase-associated protein 1) at the amino-terminus, and while at the carboxyl-terminus, it interacts with a RING-finger protein called Rbx1 which is also known as Roc1 or Roc2 (Cardozo and Pagano, 2004).

Skp1 binds to one of the many F-box-proteins (FBPs) and was thought to be important for the ubiquitin-mediated proteolysis of the Cdk inhibitor, Sic1. It was discovered that Skp1 was able to bind to many protein for example cyclin F and Cdc4 through a conserved 40-amino-acid domain. Since Skp1 was first noted in cyclin F, hence this has led to the introduction of the F-box family of proteins. Therefore, cyclin F and Cdc4 were known as the first F-box proteins identified (Bai *et al.*, 1996). Following that, further studies have found that Sic1 ubiquitination involved an E3 ligase which is formed by Cul1, Skp1 and Cdc4. Thus, this complex was later called as the SCF ubiquitin ligase (Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Cardozo and Pagano, 2004).

The SCF complex is constituted of common subunits and a variable substrate-recognition subunit known as the FBPs. Three FBPs in the SCF complex which

appeared to be the most important key players in cell cycle regulation are the S-phase kinase-associated protein 2 (Skp2), F-box and WD-40 domain protein 7 (FBW7) and β-transducin repeat-containing protein (β-TRCP). These FBPs target known substrates, implicating their functions in the control of the cellular proliferation. On the other hand, the functions of most of the other F-box proteins are still preliminary or remained unknown (Cardozo and Pagano, 2004; Nakayama and Nakayama, 2006).

Skp2 is known to target the negative regulators of the cell-cycle namely, $p27^{Kip1}$, $p21^{Cip1}$ and $p57^{Kip2}$ for degradation, promoting cell cycle progression from G_1 to S phase. Because of uncontrolled cell proliferation, hence Skp2 level is frequently found to be upregulated in a majority of human cancers. Another example of FPBs which is FBW7 is often found to be mutated in a subset of human cancers. FBW7 on the other hand, targets the positive regulators of the cell cycle such as MYC, JUN, cyclin E and Notch for degradation. Whereas for β-TRCP, it targets β-catenin and IκB as well as recognizes a number of cell cycle regulators for instance; EMI1/2, WEE1A and CDC25A/B for degradation (Nakayama and Nakayama, 2006).

Based on the fact that the expression level of Skp2 is usually found to be inversely correlated to that of $p27^{Kip1}$ (a tumour suppressor) with overexpression of Skp2 and deregulation of $p27^{Kip1}$ commonly observed in human tumours, hence Skp2 is proven to act as an oncogene. The involvement of Skp2 in promoting tumorigenesis therefore requires the development of small molecule inhibitors against the interaction of Skp2 and $p27^{Kip1}$ as the fundamental approach for future cancer therapeutics (Frescas and Pagano, 2008).

2.3.2 Regulation of $p27^{Kip1}$ by the Ubiquitin-Proteasome System (UPS)

Unlike other tumor suppressors proteins for example p53, $p27^{Kip1}$ is rarely mutated in human cancers but is usually deregulated in cancers even in the presence of high or constant $p27^{Kip1}$ mRNA levels (Catzavelos *et al.*, 1997; Slingerland and Pagano, 2000; Chu *et al.*, 2008). In cell cycle, $p27^{Kip1}$ negatively regulates G_1 to S phase transition, and its levels are found to be highest at G_1 phase causing G_1 cell cycle arrest (Sherr and Roberts, 1995; Coats *et al.*, 1996; Hengst and Reed, 1996; Chu *et al.*, 2008).

However, such a fluctuation in protein levels during the cell cycle is not similarly observed in the $p27^{Kip1}$ mRNA levels (Hengst and Reed, 1996; Alessandrini *et al.*, 1997). Thus, this suggests that down-regulation of $p27^{Kip1}$ in human cancers which is associated with many aggressive phenotypes and a poor prognosis in a variety of cancers (eg. breast, colon, prostate, lung and gastric cancers) is mainly due to post transcriptional events rather than by transcription (Hengst and Reed, 1996; Chu *et al.*, 2008).

The cell cycle levels of Skp2 and $p27^{Kip1}$ are known to be inversely correlated to each other. In the early/mid G_1 of the cell cycle, the level of Skp2 expression is low while the level of $p27^{Kip1}$ is high. When the cell cycle reaches the late G_1 phase, an inverse effect occurs whereby the expression of Skp2 increases in contrast to a decrease in $p27^{Kip1}$ levels (Slingerland and Pagano, 2000).

It is known that $p27^{Kip1}$ is degraded through a sequential degradation system called the ubiquitin-proteasome system (UPS) (Pagano *et al.*, 1995). This system begins with the small ubiquitin protein being transferred and covalently attached to the target proteins by a cascade of enzymatic reaction followed by degradation of the marked target proteins by proteasome as described in the previous section (Hershko and Ciechanover, 1998).

Biochemical studies have shown that $p27^{Kip1}$ is ubiquinated and degraded *in vitro* and *in vivo* mainly by the SCF-type (Skp1-Cullin-F-box protein) ubiquitin ligase complex that contains S-phase kinase-associated protein 2 (Skp2) as the specific substrate-recognition subunit (SCF^{Skp2}) (Carrano *et al.*, 1999; Sutterluty *et al.*, 1999; Tsvetkov *et al.*, 1999; Nakayama *et al.*, 2000). The mechanism of degradation of $p27^{Kip1}$ through UPS by SCF^{Skp2} is illustrated in Figure 2.4.

As shown in Figure 2.4, $p27^{Kip1}$ is recognized by Skp2 only when it is phosphorylated by cyclinE/Cdk2 on Threonine-187 (T-187) (Sheaff *et al.*, 1997; Vlach *et al.*, 1997; Montagnoli *et al.*, 1999). Moreover, recognition of $p27^{Kip1}$ by SCF^{Skp2} also requires an accessory protein, CDK subunit 1 (Cks1) that binds to both Skp2 and phosphorylated $p27^{Kip1}$ (Ganoth *et al.*, 2001; Spruck *et al.*, 2001). After the recognition of $p27^{Kip1}$ by Skp2 and Cks1, the mechanism is then followed by the stimulation of $p27^{Kip1}$ targeting for ubiquitination by the SCF^{Skp2} complex. The ubiquitinated $p27^{Kip1}$ is then rapidly destroyed by the proteasome, thus releasing active cyclinE/Cdk2 and allowing the progression of the cell cycle to S-phase (Hershko, 2008).

In conclusion, $p27^{Kip1}$ proteolysis is regulated in response to the cooperation of a number of events initiated by a mitogenic stimulus. For example, the increase in the levels of cyclin E, Skp2 and Cks1 has resulted in the $p27^{Kip1}$ phosphorylation and degradation. Although the function of $p27^{Kip1}$ is rarely disrupted at the genetic level, however the enhanced ubiquitin-mediated proteolysis that caused the rapid degradation of $p27^{Kip1}$ has promoted various human malignancies (Slingerland and Pagano, 2000).

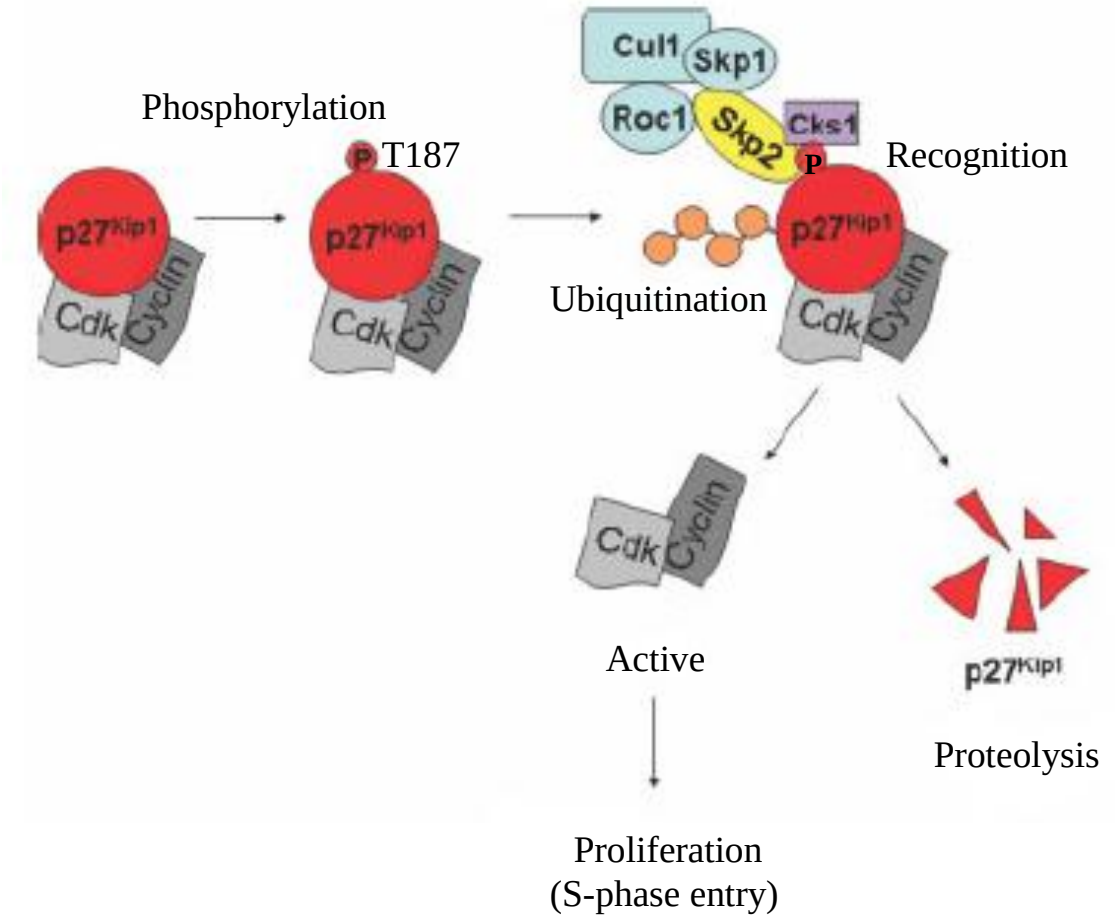

Figure 2.4 Mechanism of $p27^{Kip1}$ degradation by SCF^{Skp2} complex

The Cdk/cyclin complex as shown in this figure is the Cdk2/cyclin E complex. After the phosphorylation of $p27^{Kip1}$ by Cdk2/cyclin E at T187, $p27^{Kip1}$ is then recognized by Skp2 and Cks1 which triggers the ubiquitination of $p27^{Kip1}$ by the SCF^{Skp2} complex. The ubiquitinated $p27^{Kip1}$ eventually undergo proteolysis by the proteasome, thus allowing the release of active Cdk2/cyclin E and progression to the S-phase (Hershko, 2008).