E3 ubiquitin ligase Skp2 as an attractive target in cancer therapy

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1. ABSTRACT

E3 ubiquitin ligase Skp2 attaches ubiquitin to its target proteins and marks them for destruction by the 26S proteasome. This mechanism participates in a number of important cellular processes such as cell proliferation, DNA replication, V(D)J recombination, gene transcription, cellular metabolism and senescence. Skp2 is oncogenic. It is overexpressed in various solid tumors and hematological malignancies. Due to the antagonistic role Skp2 plays against p27, Skp2 overexpression is frequently associated with down-regulation of p27. Importantly, Skp2 overexpression in cancer cells is prognostic of cancer progression and overall survival. Recent studies have shown that Skp2 suppression might be an excellent strategy to inhibit tumorigenesis in tumors in which tumor suppressor genes such as VHL, RB or TP53 are mutated. In this review, we also summarize early efforts in the development of Skp2 inhibitors. The implications of continued, long-term Skp2 suppression are discussed.

2. INTRODUCTION

Polyubiquitin proteasome system regulates multiple cellular processes through targeted protein degradation(1). The polyubiquitin system adds ubiquitin to proteins to be degraded through three steps mediated by three classes of enzymes. First, the ubiquitin-activating enzyme (E1), activates the ubiquitin carboxyl terminal glycine residue in an ATP dependent fashion, forms a linkage via a thiolester bond with the ubiquitin. The activated ubiquitin is then passed on to the ubiquitin-conjugating enzyme (E2), and finally the ubiquitin-ligase (E3) attaches the ubiquitin molecule to the lysine residue of the target protein and joins the ubiquitin with the target protein with an isopeptide bond. These steps are repeated multiple times before the polyubiquitinated target protein is funneled to the 26 S proteasome for destruction(2). During this process, the substrate specificity is determined by the E3 ubiquitin ligase.

Classification of E3 ubiquitin ligases is based on whether they possess the homologous
E6 associated protein carboxyl terminus (HECT) domain or Really Interesting New Finger (RING) domain that are involved in adding ubiquitin to the target protein(3, 4). Polyubiquitin E3 ligases usually function in a multi-component protein complex. Two well-known RING finger type E3 ubiquitin ligase complexes are Anaphase-promoting-complex (APC) (5) and Skp1 Cullin F-box (SCF) protein complex (Skp for S phase kinase associated protein) (6). Whereas APC/C is essential for mitosis, the SCF complex is involved in G1/S cell cycle progression, transcription, DNA replication, DNA repair, B and T cell development and apoptosis (see below). Most of these are intimately related to tumorigenesis and tumor progression.

Within the SCF family of E3 ubiquitin ligases, Cullin serves as a rigid scaffold recognizing Rbx1 (RING finger domain protein) and F box proteins. Skp1 in the complex is the adaptor protein that links Rbx1 to the F box proteins that determine substrate specificity. The F-box proteins control substrate recognition/recruitment through distinct protein-protein interaction. Therefore, the function of the SCF complex depends on the F box protein serving as the substrate recognition component. So far, 69 proteins are found to share the F-box motif, which is a 40 amino acid motif within the protein and are classified as Fbw, Fbl and Fbo subtypes. The F-box of Skp2 is of Fbw type and it contains a WD40 domain (7).

Since the revelation of its role in carcinogenesis, Skp2 has been found overexpressed in various cancer types. Overexpression of Skp2 predicts poor outcome, resistance to chemotherapy and radiation therapy, disease recurrence. Lately, Skp2 was found to participating in cellular senescence, metabolism, maintaining cancer cell stemness and autophagy activation that are deregulated in cancer (see below). Here we review the role of Skp2 in important cellular processes, its target proteins, studies of skp2 expression in various types of malignancies, and the implications of several recent studies in cancer therapy and prevention. Finally, we will review Skp2 inhibitors still in the early stages of drug development.

3. SKP2 CONTROL OVER IMPORTANT CELLULAR PROCESSES RELATED TO ONCOGENESIS

Skp2 was first cloned during characterization of a kinase complex essential for S phase entry in 1995 (8). Skp2 mRNA level was found the highest during S phase and there was increased abundance in transformed cells. In normal fibroblasts, Cdk2, Cyclin A, PCNA and p21 form complexes. However, p21 disappears from the complex in transformed cells. Skp1(p19) and Skp2 (p45) form complexes in significant quantities with cyclin A and CDK2 instead in transformed cells. Depletion of Skp2 protein by microinjection of antibodies to p45 or antisense mRNA prevented the cells from entering into the S phase. This was the first clue pointing out that Skp2 might be oncogenic. Skp2 gene was mapped to chromosome 5 (9) and found to be associated with karyotypic alteration and known amplification. It has soon become clear that Skp2 is the F-box containing protein that functions as a E3 polyubiquitin ligase, working together with Skp1, Culin to target cell cycle regulators for ubiquitination-mediated proteolysis(10-13).

3.1. Cell proliferation

One of the hallmarks of cancer is its ability to maintain sustained proliferation signal(14). This is often times achieved by up-regulation of positive cell cycle regulators (oncogenes) and down regulation of negative cell cycle regulators (tumor suppressor genes). p27Kip1 is a well-known negative cell cycle regulator. p27 needs to be degraded for cells to transition from quiescent to proliferating state. It is destabilized in many types of tumors and this destabilization correlates with tumor aggressiveness and metastasis (15-17). Plenty of evidences point out that Skp2 plays an essential role in cell proliferation. Skp2 is the ubiquitin E3 ligase that targets p27 for degradation (18-20). Skp2 recognizes p27 that is phosphorylated at T187 and target it for degradation. The Cyclin A or Cyclin E/Cdk2 complex is then freed from p27 inhibition and able to transit from G1 to S phase and start DNA synthesis. Importantly, overexpression of Skp2 in fibroblasts was able to drive quiescent cells into S phase, an event that has happened in only a few instances with E2F, c-Myc and CyclinE/Cdk2 (18, 19). Skp2 up-regulation is now thought to be the major mechanism of p27 down regulation in cancers. In the mouse model, knockout of Skp2 is not lethal (21). However, the Skp2-/- mouse had reduced growth rate and increased apoptosis. Levels of cyclin E and p27Kip1 were markedly increased. Cyclin E level does not drop during S and G2 phase, highlighting the role of Skp2 in cell proliferation and regulation of cell cycle. Regulation of other negative cell cycle regulators by Skp2 has also been extensively documented (for review see Frescas and Pagano (22)).
3.2. DNA replication

Skp2 participates in DNA replication control. As part of the mechanism to ensure that DNA replication only happens in S phase of the cell cycle, human origin recognition complexes (hOrc1) is degraded immediately after the initiation of S phase following assembly onto the chromosome right after mitosis. Degradation of the hOrc1 complex is mediated by Skp2 (23). Skp2 also control degradation of DNA replication licensing factor Cdt1 (24). Consistent with this finding, Skp2-/- mice had markedly enlarged nuclei with polyploidy. Melanoma cells displayed 8N DNA content when Skp2 was knocked down (21, 25).

3.3. V(D)J recombination

Skp2 is involved in V(D)J (Variable (V), Diversity (D) and Joining (J) gene segments) recombination in the immune system and DNA repair(26). V(D)J recombination is an essential step in the development of vertebrate immune system (27). This step enables the completion of DNA rearrangement that is required for B and T cell development. Defect in V(D)J recombination results in combined immune deficiency (SCID) (28, 29). V(D)J recombination is initiated by RAG1 and RAG2 that recognize the recombination sequence signal (RSS) and make double strand break (DSB) and is completed by non-homologous end joining (NHEJ). V(D)J recombination is coupled to the cell cycle. Periodic phosphorylation and destruction of RAG2 takes place during the G1/S transition. RAG2 mutant that cannot be phosphorylated uncouples DNA recombination from cell cycle transition (26). As a result, aberrant DNA recombination happens and results in genomic instability manifested by clonal chromosomal translocation. Skp2 is involved in the destruction of RAG2 during G1/S transition (30) and mice that are deficient in Skp2 (Skp2-/-) had similar phenotype under a p53-mutated background (26).

3.4. Gene transcription

Skp2 participates in transcriptional regulation of many genes that are intimately related to oncogenesis. Genes under Skp2 regulation that are closely associated with carcinogenesis include but not limited to MLL, MYC, FOXO1 and MEF. Fusion of the MLL (mixed lineage leukemia) N-terminal 1400 aminio acid with ~70 partners at the C terminus through chromosomal translocation at 11q23 is characteristic of all MLL leukemia (31). This type of leukemia occurs predominantly in the pediatric population and has very poor prognosis. MLL encodes a histone methyl-transferase necessary for efficient gene transcription. It regulates gene expression through the MLL-E2F axis (32). During the cell cycle, MLL is destructed by SCFSkp2 in G1/S and by APCCdc20 in G2/M. The MLL fusion proteins are however resistant to degradation by the ubiquitin proteasome system (UPS) containing Skp2 (33). MYC (myelocytomatosis i.e. leukemia and sarcoma) protooncogene encodes short-lived transcription factors that are important in cancer development. MYC translocation causes Burkitt’s lymphoma in human. MYC also contributes to genesis of many cancers (for review see (34)). Overexpression of MYC has been linked to genomic instability (35). Together with three other genes SOX2, OCT4 and KLF4, MYC could reprogram the fibroblasts to a pluripotent stem cell state (36). Skp2 interacts with c-Myc and participate in its degradation. Skp2 enhances c-Myc induced S phase transition and activates c-Myc target gene in a c-Myc dependent manner. Furthermore, Myc induced transcription is Skp2 dependent emphasizing the interdependent nature between Skp2 and c-Myc (37). Foxo1 (Forkhead transcription factors) plays an important role in tumor suppression by inducing programmed cell death and growth arrest. Loss of Foxo1 protein function by protein degradation has been implicated in cancer. Foxo1 is degraded by Skp2. Loss of Foxo1 correlates with Skp2 overexpression in a lymphoma model, which strongly suggests a role for Skp2 in tumorigenesis through Foxo1 down-regulation (38, 39). Lastly, Skp2 also takes part in ubiquitin-mediated proteolysis of MEF (40), a ETS family of transcription factors originally cloned from human megakaryocytes leukemia cell lines (40). MEF is repressed by several leukemia associated fusion transcription factors (PML-Retinoic receptor alpha and AML-ETO) known to cause acute myeloid leukemia in adults(41, 42). Studies in mice showed that MEF is required for normal NK and NK-T cell development (43). It is also essential for maintaining hematopoietic stem cell quiescence (44).

3.5. Cellular senescence

Cellular senescence mechanism has been proven to restrict tumor initiation and promotion (45-47). This mechanism is induced either by expression of oncogene or loss of tumor suppressor gene and is thought to be dependent on the induction of p19Arf-p53 pathway (48). Skp2 knock down was shown to induce cellular senescence in cells having aberrant proto-oncogenic signals or loss of tumor suppressor genes. Importantly, the process was shown to be independent of p19Arf-p53 but instead dependent on p21, p27 and ATF4. Since
Skp2 encodes an E3 ubiquitin ligase and knockdown of Skp2 is compatible with life, this raised the interesting possibility of using Skp2 inhibitor in cancer prevention (49).

### 3.6. Cellular metabolism

Akt is a kinase that regulates a number of important cellular processes such as metabolism and tumorigenesis (50, 51). Activation of Akt by growth factors is a two-step process involving membrane recruitment and K63-linked ubiquitination (52). Akt supports Warburg effects (preferential use of aerobic glycolysis for energy need and biosynthesis by the cancer cells) by increasing glucose uptake/glucose influx. Activation of Akt causes cells to become dependent on aerobic glycolysis. Skp2 is the E3 ubiquitin ligase in EGF mediated activation of Akt. While Skp2 overexpression is associated with Akt activation, Skp2 deficiency impairs Akt activation, glucose uptake and glycolysis (53).

### 4. STUDIES OF SKP2 IN VARIOUS CANCER

#### 4.1. Head and neck cancer

In a study of 37 cases of oral squamous cell cancer (SCC), it was found that 49% of them overexpressed Skp2. Skp2 overexpression correlated well with poor prognosis and inversely with p27 expression(54). In 102 cases of laryngeal cancer, Skp2 was an independent prognostic factor for poor disease free survival (DFS) and overall survival (OS)(55). In mucoepidermoid cancer, Skp2 expression correlated with tumor size, microscopic grade and worse prognosis(56). The same conclusion was reached in studies of 62 cases of head and neck cancer(57). Skp2 overexpression correlated with poor OS. Skp2 expression in 42 cases of glottis cancer was associated with stage, metastasis, poor prognosis. Negative correlation was found between PTEN and Skp2(58).

#### 4.2. Lung cancer

Skp2 was first studied in 22 small cell lung cancer (SCLC) cell lines and found to be amplified on a region of chromosomal 5, resulting in overexpression of Skp2. Amplification and overexpression was found in 44% and 83% of the primary SCLC samples. Down-regulation of Skp2 expression significantly suppressed cell growth (59). Skp2 was overexpressed in 88% (N=16) of non-small cell lung cancer (NSCLC) cell lines and 65% of the primary tumors (N=163). In tumors with Kras mutation, Skp2 overexpression was an independent poor prognostic marker for survival (60). In a separate study of 138 NSCLC tumor specimens, Skp2 was found overexpressed in smokers, male, squamous cell and poorly differentiated carcinoma. Skp2 overexpression correlated with cancer aggressiveness and was an independent prognostic marker for poor prognosis with an inverse relationship with p27 expression (61). Studies of 128 surgically resected NSCLC concluded that Skp2 overexpression was involved in progression of squamous cell lung cancer alone (62). In an effort to develop molecular markers for early detection of lung cancer, it was found that the copy number change of Skp2 along with 5 other genes, could diagnose stage I NSCLC with a sensitivity of 86.7% and specificity of 93.9% from the sputum samples. This test worked better for squamous subtype than adenocarcinomas (90).

#### 4.3. Breast cancer

Skp2 was found expressed at a higher level in estrogen receptor (ER) and Her2/neu negative tumors, the so called basal like subtype(63). These tumors are of higher grade and have low p27 levels. Overexpression of Skp2 increased resistance to antiestrogen therapy to ER positive cells. A more recent study found Skp2 overexpression correlated with Akt activation and tumor metastasis, served as a marker for poor prognosis in Her2 positive breast cancer patients. Silencing Skp2 sensitized these patients to Herceptin treatment(53). Overexpression of Skp2 mRNA and protein were especially prominent in young and poor prognosis patients with breast cancer (N=169)(64). The expression levels independently predicted poor DFS.

#### 4.4. Prostate cancer

In a study of 622 prostate cancers treated with radical prostatectomy (65), Skp2 levels were found higher compared to the normal tissue, premalignant lesions and prostate intraepithelial neoplasms. Skp2 labeling frequencies correlated with preoperative PSAs, Gleason scores. The Skp2 labeling Indices correlated with extraprostate extension, clinical stage and Gleason scores. A Skp2 index score of >10 was associated with shorter biochemical recurrence survival. A similar study confirmed that Skp2 overexpression was associated with shorter biochemical recurrence survival(66).

#### 4.5. GI Cancers

In resectable esophageal cancers, high Skp2 expression was associated with poor OS(67). Negative Skp2 expression in primary resected
esophageal squamous cancer was an independent factor for better survival (N=157). For gastric cancer, overexpression of Skp2 at the mRNA and protein levels was associated with poor OS(68). In cells stably transfected with Skp2, p27 and apoptotic levels were down. However, cell growth rate was up. These cells showed more invasive behavior. The number of cells labeled with BrdU increased compared to the control even when the cells were starved for serum. Skp2 labeling indices increased progressively when gastric lesions became increasingly malignant(69). Skp2 labeling indices were significantly elevated as hepatocarcinogenesis advanced (70). Increases in Skp2 levels were most frequently observed in less differentiated tumors and the Kaplan-Meier survival analysis showed poor prognosis in these patients. Skp2 nuclear stainings were associated with shorter DFS and cytoplasmic stainings were associated with less aggressive disease(71). Similarly in colon cancer, overexpression of Skp2 and Cks1 strongly correlated with loss of p27, loss of differentiation. A significant decrease in OS was observed in tumors expressing higher levels of Skp2 and Cks1 particularly in stage II and III patients(72). Skp2 expression was studied in rectal cancer patients who have received neoadjuvant chemo and radiation. Higher levels of Skp2 were associated with advanced post treatment nodal status, lower degree of tumor regression rate. Skp2 levels were independent prognostic factor for local recurrence free survival. Thus higher Skp2 levels were associated with poor therapeutic response and adverse outcome in rectal cancer treated with neoadjuvant chemoradiation therapy (73).

4.6. Leukemia, lymphoma and myeloma

Studies in acute myelogenous leukemia found that Skp2 expression significantly correlated with unfavorable cytogenetics. There was no inverse relationship between Skp2 and p27. High frequency of PTEN phosphorylation was seen in those expressing higher levels of Skp2. Skp2 expression level was an independent prognostic factor for DFS and OS(74). In Diffused large B cell lymphoma treated with CHOP or Rituximab plus CHOP (R-CHOP)(75), adding rituximab was not beneficial in patients whose tumors were expressing higher levels of Skp2 and lower levels of p27. Higher levels of Skp2 or lower levels of p27 were associated with poor PFS and OS in 671 patients treated with either regimen. Higher levels of Skp2 or lower levels of p27 were independent of factors included in the international prognostic index (IPI). Higher Skp2 levels and lower p27 levels in combination predicted the worst prognoses. Finally, CKS1B was overexpressed in aggressive multiple myelomas. Skp2 mediated the CKS1B effects at least partially (76, 77).

In summary, Skp2 has been studied in various solid tumors or hematological malignancies including head and neck, lung, breast, prostate, GI, GU, sarcoma, leukemia, lymphoma, myeloma, melanoma and brain cancer. So far, all studies have defined Skp2 as oncogenic. Skp2 overexpression is associated with poor prognosis (Table 1).

5. SKP2 DOWN REGULATION MIGHT BE A USEFUL STRATEGY FOR CANCER PREVENTION

VHL (von Hippel Landau) protein is part of the ubiquitin ligase complex that destructs HIF1 (hypoxia inducible factor) under well oxygenated condition. Germline VHL mutation results in haemangioblastoma, renal cell carcinoma and pheochromocytoma. VHL mutation also occurs in sporadic renal cell carcinoma. VHL inactivation induces a senescence-like phenotype in vitro and in vivo. This phenotype is independent of p53 and Hif1. It is dependent on SWI2/SNF2, p400 and Rb. Down regulation of Skp2 by siRNA increased p27 levels and induced cell senescence (78). Therefore, down-regulating Skp2 may be an effective way of targeting VHL mutated cancers in vivo.

Skp2 was required for survival of aberrantly proliferating Rb1 deficient cells (Rb1 +/-) and for tumorigenesis in the mouse model (79). Deletion of Rb1 in melanotrophs ablated the entire pituitary intermediate lobe when Skp2 was also ablated. This prevented tumorigenesis completely in Rb1 +/- cells. Cell proliferation in those cells was normal but they went into apoptosis resulting in cell elimination. Skp2 inactivation in Rb1 deficient retinoblastoma cells produced the same phenotype. These implicate that Skp2 inactivation halts tumorigenesis in the pituitary glands or retinoblastoma and Skp2 inhibition can create a scenario of synthetic lethality in Rb1 deficient cells.

Oncogenic signaling or loss of tumor suppressor genes induces cellular senescence. In the mouse model, this process is dependent on the p19Arf/p53 pathway(80, 81). Depletion of Skp2, in combination with oncogenic signaling or inactivation of tumor suppressor gene triggers cellular senescence in mice without activation of the p19Arf/ p53 or DNA damage response (DDR) pathway. This process is dependent on p27, p21 and ATF4 and...
occurs even in cells with impaired p19Arf/p53 or p53/PTEN pathways (49). In light of the chemo resistant nature of cells with p53 mutation, Skp2 inhibition may provide an alternative approach in cancer control in a p27, p21 and ATF4 unmutated background. It also has implications in cancer prevention since it provides a way to eliminate carcinogenesis in those cells with oncogenic signaling by mutated ras or E1A regardless of the p53 status.

In the pituitary cancer or prostate cancer models, Skp2 inactivation in the Rb and TP53 doubly deficient mice triggered a surge in p27 levels. Although this surge was not able to block DNA synthesis, cells entered into endoreplication and eventually triggered apoptosis resulting in cell elimination (82). Therefore Skp2 deletion blocked tumorigenesis in pituitary or prostate tumorigenesis in Rb and TP53 doubly deficient cells. Skp2 inactivation in TP53 mutated background triggered Rb induced cellular senescence. This implicated that cells need Skp2 when either TP53 or TP53 and RB1 both are inactivated. Given the high prevalence of TP53 and/or Rb, Skp2 inhibition may provide a way for cancer prevention.

6. EFFORTS IN TARGETING THE SKP2 MEDIATED UBIQUITINATION: SUMMARY OF LEAD COMPOUNDS

Using an *in vitro* reconstituted system with CDK2/Cyclin E, Skp1, Skp2, Roc1, Cul1, a high
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throughput screening (HTS) was performed (123). As a result, CpdA was identified based on its ability of interfering with Skp2 function resulting in accumulation of p21 and other Skp2 substrate but without activating the heat shock protein response. CpdA prevented Skp2 incorporation into the ubiquitin enzyme complex, induced cell cycle arrest and programmed cell death in the form of autophagy. The compound was able to decrease cancer cell resistance to dexamethasone, doxorubicin, melphalan and bortezomib. It was active against both the myeloma and leukemia cells. However, there was no in vivo data reported. The lack of potency was a problem as the author pointed out. The drug was used at 5-25 μM concentration in the assays. Celgene is not developing this compound (personal communication).

One of the substrate of Skp2 is p27, which is important for cell cycle arrest. Binding of p27 to Skp2 results in p27 degradation. A structural based approach called in silico Virtual Ligand Screening was used for HTS of a library (124). A total of 96 hits were found, of which 4 compounds were tested and found to inhibit Skp2, p27 binding (C1, C2, C16 and C20). These compounds inhibited binding between p27 and Skp2, caused G1 and G2/M cell cycle arrests in various cancer cell lines. No animal experiments were shown. The concentration needed to achieve the drug effects was between 5-10 μM.

Based on the crystal structure of the Skp2-SCF complex, two pocket-like structures along the Skp1 and Skp2 interaction surface important for Skp1 and Skp2 binding were identified. Using a similar approach described above, another in silico HTS of 120,000 compounds was performed. Twenty-five compounds inhibited the interaction between Skp1 and Skp2. C25 was selected for further study due to its potency. It blocked Skp1 and Skp2 interaction completely in vitro at 5 μM(125). Binding of C25 physically blocked Skp1 and Skp2 interaction, inhibited the E3 ligase activity. C25 restricted cancer cell survival by triggering cellular senescence that is p53 independent and inhibited glycolysis. In xenograft models of lung (A549 cell line) and prostate (PC3 cell line) cancer, injection intraperitoneally at 40-80 mg/kg achieved dose dependent decrease of Skp2 levels, simultaneous increase in p27 and p21 levels and tumor suppression. Intra-tumoral Akt and Glut1 levels were also decreased.

A high throughput AlphaScreen assay singled out two compounds named NSC689857 and NSC681152 as the inhibitors interrupting interactions between Skp2 and Cks1. As a result, p27 ubiquitination was inhibited, resulting in p27 accumulation. The compounds were validated in a structural-activity relationship analysis(126).

7. POTENTIAL PROBLEMS OF EXTENDED SKP2 DOWN-REGULATION

Skp2 suppression maybe a useful strategy in cancer prevention as reviewed earlier. However, there are some concerns as whether extended Skp2 suppression in cells will cause genomic instability resulting in cancer promotion. First, as discussed above, The V(D)J recombination process normally only occurs in the lymphoid tissue since RAG1 and RAG2 are lymphoid specific recombinases. They are not expressed in other tissues except in the brain (28). Coexpression of RAG1 and RAG2, however can lead to test substrate recombination in non-lymphoid tissue where it would not occur normally. This means that all other key factors required for the process are available and functional in other tissues (127, 128). Cancer cells are well known for reprogramming of gene expression. Hence, many genes that are normally only expressed in the testis are found expressed in the cancer cells. Indeed, IgG (129), RAG1 and RAG2 (130) are all found expressed in the cancer cells. Aberrant expression of RAG1 and RAG2 could contribute to genomic instability, which is another hallmark of cancer (14, 131). Alternatively, in cells where p27 is silenced or mutated, Skp2 down regulation would cause unrestricted cyclin E expression since Skp2 targets p27 and cyclin E for degradation. It is well known that cyclin E overexpression drives oncogenesis (132). Mouse model of lung cancer has been established successfully by cyclin E overexpression (133, 134). Accumulation of cyclin E under the context of p27 and Skp2 silencing were linked to genomic instability(135). Thus the long term benefit of exclusive Skp2 down regulation remains to be settled. Careful monitoring of genomic stability is warranted under the situation.

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