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Proliferation unleashed: The role of Skp2 in vascular smooth muscle cell proliferation

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1. ABSTRACT

Vascular smooth muscle cell proliferation plays a major role in the development of numerous vascular pathologies. Understanding the molecular mechanisms that regulate smooth muscle cell proliferation is therefore essential for the development of new therapies for the treatment of these pathologies. Skp2 is an F-box protein component of the SCF^{Skp2} ubiquitin-ligase that controls cellular proliferation by regulating the ubiquitination and degradation of several cell-cycle regulatory proteins, including the cyclin-dependent kinase inhibitor, $p27^{Kip1}$. This review discusses the recent literature on the function and regulation of Skp2 in smooth muscle cells, which is emerging as a key player in the control of smooth muscle cell proliferation during vascular disease.

2. INTRODUCTION

The idea that vessel injury stimulates VSMC proliferation and neointima formation was originally proposed by Russell Ross in his 'Response to Injury' hypothesis (1, 2). Subsequent work amply confirmed that proliferation of locally-derived vascular smooth muscle cells (VSMCs) plays a major role in the development of numerous vascular pathologies characterized by neointimal thickening, including atherosclerosis, pulmonary artery hypertension and peripheral vascular disease (3, 4). VSMC proliferation is also a key factor limiting the long term success of veins used as arteriovenous fistulas, coronary or peripheral vein-grafts (5-7) and balloon angioplasty with or without stent implantation. In healthy adult arteries, VSMC have an extremely low rate of proliferation, existing in a

differentiated, contractile state where their main role is to regulate vessel tone. However, experimental vessel injury dramatically elevates VSMC proliferation and this contributes to the high rates of restenosis after angioplasty and stenting observed clinically (8, 9). Most importantly, these concepts encouraged the development 'drug-eluting stents' or DES, which elute anti-proliferative agents designed to block VSMC proliferation. DES markedly reduces angiographic and clinical restenosis rates (10, 11). However, the use of currently-available DES is associated with a statistically significant increased risk of acute stent thrombosis attributed to delayed endothelialisation, probably as a result on the non-discriminatory anti-proliferative effects of the agents used on endothelial proliferation (12, 13). After three years, late stent thrombosis was associated with a significantly increased mortality rate in one landmark study (14). The problems of DES and our inability so far to extend the anti-proliferative treatment strategy to other vascular pathologies highlights the need for a more detailed understanding of the molecular mechanisms governing VSMC proliferation during neointima formation. A primary goal is to enable the development of more cell-selective therapies to treat these pathologies without causing adverse events.

3. $p27^{KIP1}$ PUTS THE BRAKE ON PROLIFERATION

Anyone who has cultured VSMC in a dish will know that these cells can be induced to proliferate rapidly simply with the addition of a growth factors. There is immediate activation of signal transduction pathways, leading ultimately to phosphorylation of the extracellular receptor-related kinases ERK1/ERK2 (15, 16) and cell cycle progression. Progression through the G_1 phase of the cell-cycle is controlled by cyclin D and E, which associate and activate their catalytic partners, the cyclin-dependent kinases (cdk4 and cdk2 respectively) (17). Active CDKs hyper-phosphorylate retinoblastoma protein (Rb), leading to its inactivation and release of E2F transcription factor (see Figure 1) (17). In turn, this initiates S-phase specific gene expression and progression through the G_1 restriction point, beyond which proliferation becomes mitogen independent. The activity of the cyclin:cdk complexes is also subject to negative regulation by the Cip/Kip ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) and Ink ($p15^{INK4a}$, $p16^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) families of cyclin-dependent kinase inhibitors (CKIs). The levels of these inhibitors are typically down-regulated in mid-G1, thus relieving the inhibition of CDK activity (18). Important growth factors for VSMC include platelet derived growth factor (PDGF) (19), basic fibroblast growth factor (bFGF) (20) and thrombin (21).

Although VSMC in culture rapidly respond to mitogens by proliferating, this is not the case for those in their natural environment in the vessel wall. VSMC in intact healthy vessels are refractory to growth factor stimulation unless a concomitant injury is also present (16, 22-24). The clearest demonstration of this comes from organ culture studies comparing intact pieces of artery with isolated VSMC. In these studies, serum stimulation, which potently stimulates proliferation in isolated smooth muscle cells, fails to elicit any proliferation in the intact artery segments (16, 22). This occurs despite almost identical activation of ERK1/2, up regulation of cyclin-dependent kinase subunits and cyclin D and E proteins in both artery segments and isolated cells (16). Importantly, cdk complexes remain inactive in intact artery but not in isolated cells. This is associated with constitutively elevated levels of the cyclin dependent kinase inhibitor $p27^{Kip1}$, suggesting that an inability to down-regulate this inhibitor is an important mechanisms restraining VSMC proliferation and maintaining quiescence in healthy uninjured vessels. The implication is that down-regulation of $p27^{Kip1}$ is a prerequisite for VSMC proliferation. Consistent with this, immunohistochemical staining studies show reduced levels of $p27^{Kip1}$, concomitant with increased VSMC proliferation, in response to vessel injury (25). Forced expression of $p27^{Kip1}$ in these injured vessels potently suppresses VSMC proliferation and inhibits neointima formation *in vivo* (26). Furthermore, mice lacking $p27^{Kip1}$ are larger than their wild-type counterparts, display a generalised hyper-proliferative phenotype, organomegaly, exaggerated atherosclerosis and increased VSMC proliferation and neotimima formation after vessel injury (27-30). Taken together, these observations indicated a major role for $p27^{Kip1}$ in maintenance of VSMC quiescence and the induction of VSMC proliferation after vessel injury.

4. SKP2 PROMOTES $p27^{kip1}$ UBIQUITINATION AND DEGRADATION

The identification of $p27^{Kip1}$ as an important negative regulator of the cell cycle focussed attention on the mechanisms controlling its levels. Research in mid-1990's established that $p27^{Kip1}$ in several cell types is largely regulated by its rate of degradation rather than modulation of $p27^{Kip1}$ gene transcription or translational (31). The mRNA and translational rates remain relatively constant in mid to late G1 phase, whereas the protein levels of $p27^{Kip1}$ are rapidly down regulated. Furthermore, low levels of $p27^{Kip1}$ in rapidly proliferating cells are associated with increased $p27^{Kip1}$ ubiquitination and proteasome inhibitors can block $p27^{Kip1}$ down regulation, implying a central role for the Ubiquitin Proteasome System (UPS) in $p27^{Kip1}$ regulation (31). The cell-cycle periodicity of numerous other cell-cycle proteins is controlled in a similar manner by the UPS and their degradation is often dependent on phosphorylation by a CDK (32). This prompted the question whether $p27^{Kip1}$ degradation is also dependent on its phosphorylation. Using site-specific mutagenesis, Vlach *et al* demonstrated that $p27^{Kip1}$ degradation is in fact dependent on it association with active cyclin E:cdk2 and its phosphorylation on Thr187 (33). In this model, phosphorylation of $p27^{kip1}$ on Thr 187 triggers covalent ligation of multiple ubiquitin proteins via the action of UBC3, which targets $p27^{kip1}$ for degradation at the proteasome.

Around the same time, an independent line of research identified a 45kD protein that interacted with the cyclinA cdk2 complex (34). This protein was termed S phase kinase associated protein 2 (Skp2) and was quickly

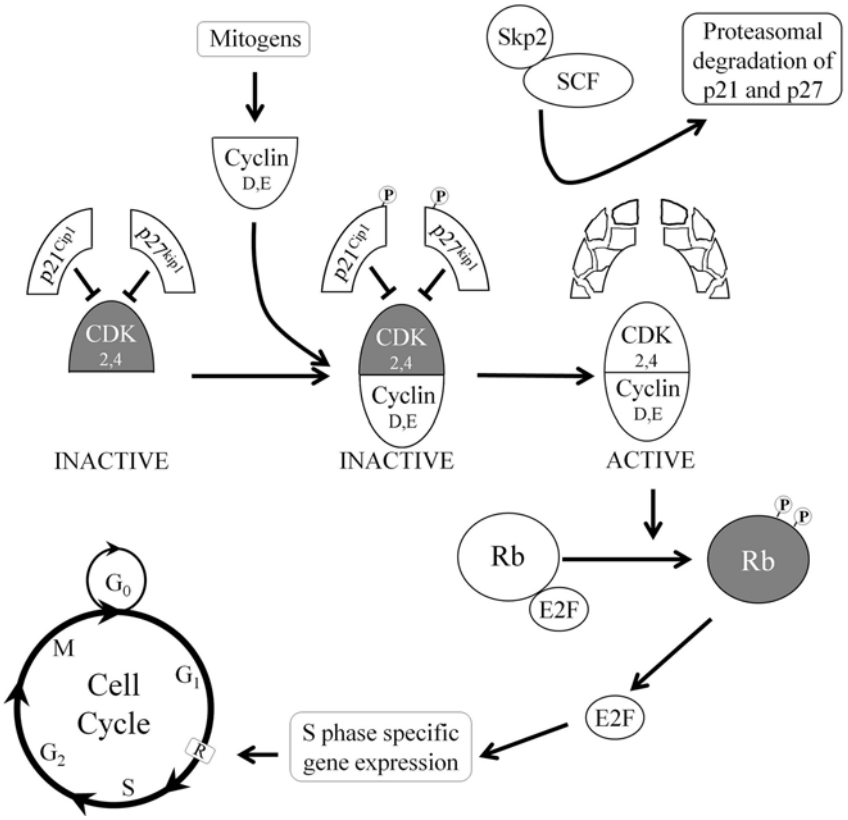

Figure 1. Regulation of G1 phase of the cell-cycle by Skp2. VSMC in healthy vessels exist in a quiescent G_0 state but enter the cell-cycle in response to vascular injury or insult. Progression through the G_1 phase of the cell-cycle is regulated primarily by the cyclin-dependent kinases (cdk 2/4). Cdk activation is dependent complex formation with D and E type cyclins, synthesis of which is induced by growth factor stimulation. Cdk:cyclin complex activity is also subject to negative regulation by the cyclin dependent kinase inhibitors (such as $p21^{Cip1}$ and $p27^{Kip1}$) the levels of which are down regulated in mid- to late- G_1 by SCF^{Skp2} dependent ubiquitination and subsequent degradation by the proteasome. CDK-dependent phosphorylation of CDKIs enhances their ubiquitination by SCF^{Skp2}. Active cyclin:cdk complexes hyper phosphorylate and inactivate retinoblastoma (Rb) protein. This is a critical step in transition through the G_1 restriction point R, triggering release of transcription factors such as E2F1 that promote expression of genes required for S-phase.

identified as a member of the large F-box family of proteins that are all components of Skp-Cullin-F-box family of E3 ubiqutin-ligase complexes (35). Initial studies showed that Skp2 was unable to directly modulate cdk activity but that its inhibition with neutralising antibodies or antisense oligonucleotides resulted in growth arrest via an unknown mechanism (34). Since SCF complexes act together with UBC3 and preferentially ubiquitinate phosphorylated substrate proteins, it was not long before Skp2 was identified as a key factor responsible for promoting ubiquitination and subsequent degradation of $p27^{Kip1}$ by the proteasome during late-G_1 (36). More recently, it was shown that the Skp2-dependent ubiquitination of $p27^{kip1}$ requires the accessory protein Cks1, which binds to Skp2 and increases its affinity for phosphorylated $p27^{kip1}$.

5. THE SCF^{Skp2} UBIQUITIN LIGASE

E3 ubiquitin ligases can be classified into two families, those based on HECT (Homologous to the E6-AP Carboxyl Terminus)-domain proteins and those based on RING (Really Interesting New Gene)-domain proteins. Many of the RING-ligases contain a cullin (Cul) protein, so-called because the proteins 'cull' i.e. selectively degrade certain cellular proteins. The cullin-containing Cul-RING-ligases (CRLs) represent the largest family of E3 ligases and have been implicated in the degradation of numerous proteins with functions in cell signalling, gene transcription and cell-cycle regulation. Several sub-families of CRLs have been identified in mammals but the best characterised are the Skp-Cul-F-box (SCF) ubiquitin ligases. As their name suggest, these are multi-subunit ligases consisting of a Cul1 backbone, which binds the RING-domain protein Roc1/Rbx1 at its C-terminus and an adaptor protein Skp1 at its N-terminus (see Figure 2). Roc1/Rbx allows recruitment of the E2 enzyme UBC to the ligases, while Skp1 serves to bridge the gap between the cullin backbone and a substrate-specificity subunit represented by one of a family of sixty nine F-box proteins of which Skp2 is a member. The interaction between Skp1 and the F-box protein occurs via a conserved F-box motif of ~40 amino acids that was first identified in cyclin F, hence the name F-box protein (Bai 1996). F-box proteins also contain additional protein-interaction domains, which allow

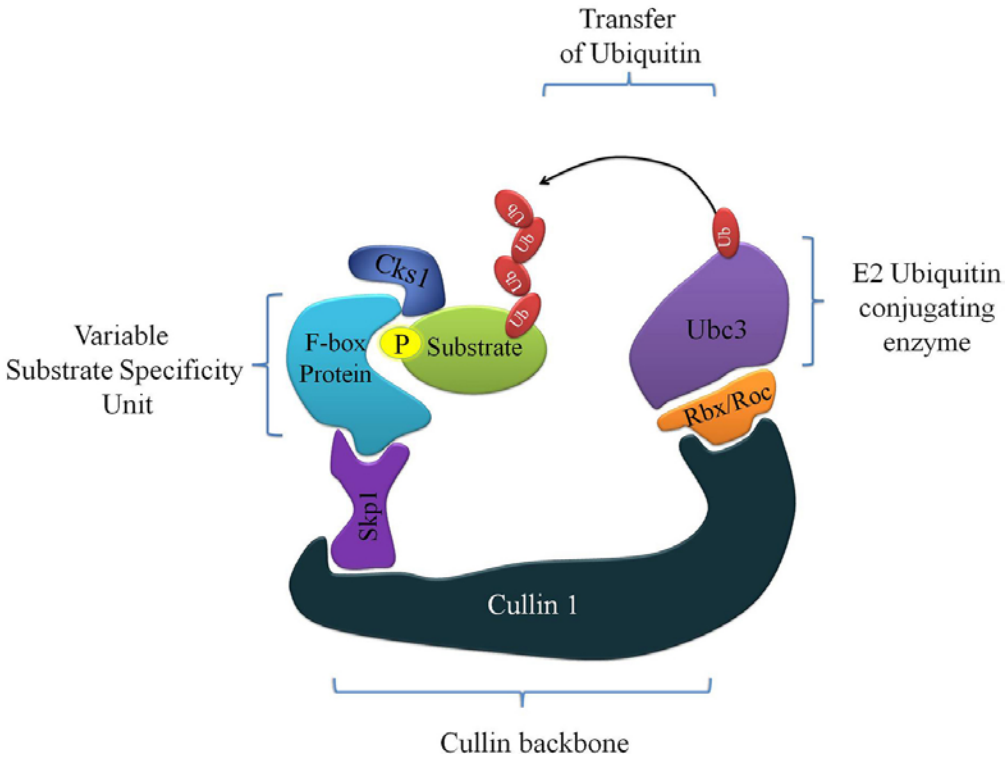

Figure 2. Structure of the SCF^{Skp2} E3 ubiquitin-ligase. The SCF (Skp1-Cul1-F-box) E3 ubiquitin -ligase is composed of invariable subunits of Skp1, Cul1 and Rbx1/Roc1 and a variable F-box protein component that functions as a substrate specificity factor. In SCF^{Skp2}, the F-box protein component is represented by Skp2. Cul1 forms the backbone of the ligase, binding Skp1 at it N-terminus and the RING-domain protein Rbx1/Roc1 at its C-terminus. Skp1 functions as an adaptor protein that recruits F-box proteins such as Skp2 via interactions with their F-box domains. Rbx1/Roc1 is essential for recruitment of UBC E2-ubiquitin conjugating enzyme which is required for transfer of activated ubiquitin to the substrate protein. Ubiquitination of $p27^{Kip1}$ by SCF^{Skp2} requires cdk-dependent phosphorylation of $p27^{Kip1}$ on Thr-187. In addition, ubiquitination of $p27^{Kip1}$ requires the accessory protein cks1 (CDK1 subunit 1).

recruitment of proteins destined for ubiquitination to the SCF-ligase. The identity of these additional substrate specificity domains allows the F-box proteins to be divided into three sub-families or classes: FBXW, FBXL and FBXO, depending on whether the proteins contain WD40, leucine rich repeat (LLR) or 'other' substrate-specificity domains respectively (37). Skp2 contains an LLR motif and hence is also referred to as FBXL1. Although 69 mammalian F-box proteins have been identified to date, the vast majority of these remain 'orphans' that have no known binding partners apart from SKP1. The small number of F-box proteins that have well-established binding partners includes FBXL1 (Skp2) (36), FBXL3 (38), FBXL5 (39) FBXL6 (40), FBXW1 (B-TRCP1) (41), FBXW7 (42, 43), FBXW8 (44), β-FBXW11 (B-TRCP2), FBXO4 (45, 46), FBXO7 (47).

6. SKP2 TARGETS MULTIPLE PROTEINS FOR DEGRADATION

The best characterised Skp2 'substrate' is $p27^{Kip1}$, degradation of which appears to mediate many of the biological functions of Skp2. Ubiquitination of $p27^{Kip1}$ can be reconstituted *in vitro* by addition of purified Skp2, together with other components of the SCF ligase and an UBC, while inhibition of Skp2 in cultured cells with dominant-negative mutants or antisense oligonucleotides prevents $p27^{Kip1}$ degradation and results in G1 growth arrest. Consistent with these *in vitro* observations, homozygous genetic deletion of the Skp2 gene in mice also results in elevation of $p27^{Kip1}$ levels and is associated with reduced size, markedly enlarged nuclei with polyploidy and multiple centrosomes, reduced growth rate and increased apoptosis. Importantly, almost all of these phenotypic abnormalities present in Skp2 -/- mice are rescued in Skp2 -/-; $p27^{Kip1}$ -/- doubly deficient mice, indicating that $p27^{Kip1}$ represents the most important target for Skp2 (48, 49).

Notwithstanding the above, a common feature of F-box proteins is that they each target more than one protein for ubiquitinylation and degradation. Consistent with this other protein targets of Skp2 have been identified and may contribute to the biological functions of Skp2 (reviewed in detail elsewhere (50)). These include several cell cycle ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, cyclin E) and transcriptional regulators (FOXO1, TOB1, c-Myc, E2F). Many of these are targeted for degradation between late-G_1 and G_2 phases, the window in which Skp2 levels are elevated in rapidly cycling cells (see Figure 3). Many of these substrates are also tumour suppressor proteins (see

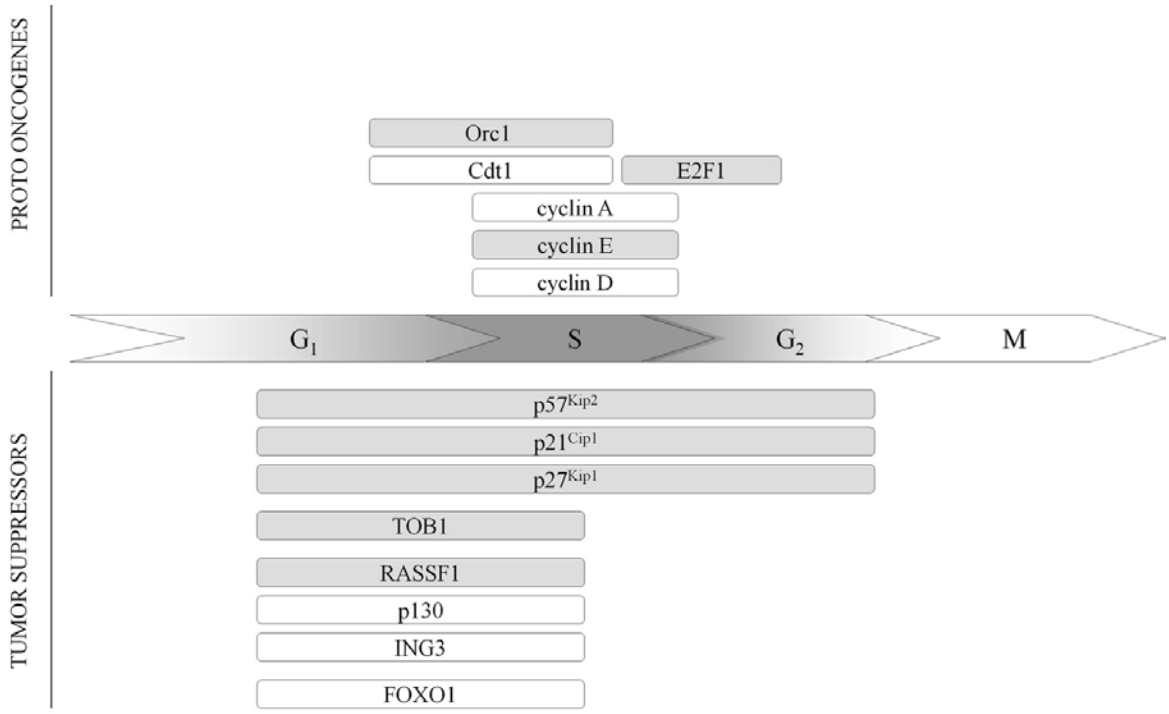

Figure 3. Skp2 targets multiple substrates for degradation during G1 and S phase. The window of Skp2 protein expression during the cell-cycle is illustrated by the shaded region. Proto-oncogenic SCF^{skp2} substrates are indicated above the cell-cycle line and tumour suppressor substrates and indicated below the line. The position and length of the boxes indicates the approximate timeframe of SCF^{Skp2}-mediated degradation of that substrate protein. Shaded boxes indicate substrates whose levels have been shown to be elevated in Skp2 -/- MEFs.

Figure 3) and their degradation by Skp2 in late G_1-S phase is thought to be an important mechanism relieving their inhibitory effects on cell-cycle progression, either by permitting CDK activation or by permitting changes in gene expression patterns required for S-phase progression. The degradation of many of these substrate proteins around the G_1 and S phase boundary underlines the important role played by Skp2 in controlling S-phase entry.

Interestingly, not all of the identified Skp2 substrates are growth inhibitors. For example, cyclin E is targeted for degradation by Skp2 in late S-G_2 phase of the cell cycle and this is disrupted in Skp2 -/- MEFs, indicating that Skp2 may also play an important function in controlling the periodicity and timing of key cell-cycle regulatory proteins. Skp2 also promotes ubiquitination and degradation of the proto-oncogene c-Myc. Interestingly, this ubiquitination of c-Myc by Skp2 appears to promote c-Myc transcriptional activity, suggesting Skp2-mediated ubiquitination acts as a 'licensing' mechanism linking c-Myc activity to its degradation (51). This suggests a second type of mechanism through which Skp2 controls cell growth. As cells progress through G_1 phase, Skp2 not only promotes degradation of growth-inhibitors such as $p27^{Kip1}$ but also transiently promotes c-Myc-dependent gene expression. Skp2 has also been reported to control ubiquitination of E2F1 during S and G_2 phases of the cell cycle (52). Degradation of E2F1 by Skp2 in late S phase is thought to be important for correct S-phase exit and entry into G_2. It is tempting to speculate that Skp2-dependent ubiquitination of E2F1 may also play a role in enhancing E2F1 activity, in a similar manner to c-Myc.

Taken together, these observations show convincingly that Skp2 plays a central role in controlling cell-cycle progression, in large part by coordinating the degradation and activity of several G_1-S phase regulators.

7. SKP2 CONTROLS VSMC PROLIFERATION AND NEOINTIMA FORMATION

The importance of Skp2 as a key regulator of $p27^{kip1}$ degradation suggests its involvement in the initiation of VSMC proliferation following vascular injury or inflammation. Less obviously, our laboratory hypothesised that VSMC quiescence in healthy arteries, which is associated with constitutively elevated levels of $p27^{kip1}$ (16), is due to a deficiency in Skp2 expression. In support of this hypothesis, *in vitro* and *in vivo* experiments demonstrated that Skp2 protein and mRNA expression is undetectable in healthy uninjured arteries, even though growth factors are present (53).This suggested that lack of Skp2 explained the inability of VSMC in these arteries to degrade $p27^{kip1}$ and enter the cell cycle. In effect, the 'brake' on proliferation was locked on. Consistent with this, we showed that forced expression of Skp2 in healthy arteries drives S phase entry both *ex vivo* and *in vivo* (53, 54). Similar experiments in quiescent fibroblasts

demonstrated that forced expression of Skp2 alone can drive S-phase entry, even in the absence of growth factors. Forced expression of Skp2 was associated with increased $p27^{kip1}$ degradation and cdk activation (55) and over expression of $p27^{kip1}$ blocked S-phase entry induced by Skp2. This unusual trait of a single gene being able to drive cells all the way from quiescence (G_o) into S-phase is shared by only a few other gene products; it underlines the importance of the Skp2-$p27^{Kip1}$ pathway in controlling release from quiescence and cell-cycle entry.

These observations imply that up regulation of endogenous Skp2 levels should be required for induction of VSMC proliferation following vascular injury. Indeed, balloon injury to the rat carotid artery stimulates a dramatic and rapid increase in Skp2 mRNA and protein levels in the medial VSMCs and in cells of the developing neointimal lesion at later time points, in a pattern that is both temporally and spatially associated with smooth muscle cell proliferation (54). Moreover, this increase in endogenous Skp2 levels by vessel injury appears to be essential for the proliferative response since adenovirus-mediated gene transfer of F-box deleted dominant negative mutants of Skp2, effectively blocked injury-induced degradation of $p27^{Kip1}$ and VSMC proliferation. More importantly, Skp2 inhibition in this way leads to development of significantly smaller neointimal lesions, presumably due to the early inhibition of VSMC proliferation. This data indicates that injury induced Skp2 expression is an essential contributor to VSMC proliferation and lesion development, whereas the effects of over expression of Skp2 described above demonstrates this pathway is sufficient to drive proliferation. To address this question, our laboratory used adenoviral-mediated gene transfer to restore Skp2 expression to uninjured but de-endothelialised rat carotids *in vivo*. Gently de-endothelialisation on its own does not induce significant levels of medial smooth muscle cell proliferation or Skp2 expression. However, exogenous expression of Skp2 in these vessels is able to trigger $p27^{Kip1}$ degradation and increase smooth muscle cell proliferation to levels similar to those induced by balloon-injured vessels. Hence Skp2 is both necessary and sufficient for induction of smooth muscle cell proliferation and neointimal lesion formation. A further confirmation of the importance of Skp2 in lesion development comes from experiments in Skp2 $^{-/-}$ mice, which develop significantly smaller lesions after carotid ligation than their wild-type counterparts (27). Taken together, these observations highlight the role of Skp2 in triggering release from quiescence and driving smooth muscle cell proliferation in response to vessel injury.

8. MULTIPLE PATHWAYS REGULATE SKP2 EXPRESSION

8.1. Skp2 protein stability

The importance of Skp2 as a regulator of smooth muscle cell proliferation raises the question of how Skp2 itself is regulated. The levels of many cell-cycle regulators fluctuate during the cell-cycle, being controlled by waves of stabilization and ubiquitin-mediated de-stabilisation; this appears also to apply to Skp2. In rapidly cycling cells, Skp2 levels are elevated in mid-G_1 phase and remain elevated until late G_2, when Skp2 levels begin to subside as cells enter M phase. These changes in Skp2 levels appear to be brought about by changes in the half-life of the Skp2 protein and rather than modulation of Skp2 mRNA expression. In 2004, two independent studies demonstrated that destabilisation of Skp2 in M and early G_1 phases is mediated by a second ubiquitin-ligase termed anaphase promoting complex/cyclosome (APC/C) in association with its activator cdh1 (56 , 57). APC^{cdh1} mediated degradation of Skp2 in M and early G_1 phase is probably important for efficient M phase progression and the timing of S phase entry. In this model, increased levels of Skp2 in mid-G1 required inactivation of APC^{cdh}, which is thought to come about by phosphorylation of Cdh1 and interaction with the inhibitory protein Emi1, which accumulates in G_1 (58, 59). In this way, inactivation of APC^{cdh1} controls the timing of S-phase entry by regulating the accumulation of Skp2. Whether suppression of APC^{cdh1} mediate degradation is also an important step in exit from quiescence (G_0) and entry into G_1 is not clear. This may be mediated by yet another ubiquitin-ligase based on a Cul1 core (56). For example, interference with Cul1 function by antisense oligonucleotides results in stabilisation of Skp2 in quiescent cells, whereas a recombinant Cul1 based ligase can catalyse Skp2 ubiquitination *in vitro*. This suggests that Cul1 ligases may be involved in suppression of Skp2 in quiescent G_0 cells as well as collaborating with Skp2 in late G_1 to promote degradation of Skp2 substrates, thus allowing S phase entry (56).

Changes in Skp2 mRNA and protein expression, which appear largely independent of cell-cycle periodicity, are also important mechanisms regulating cell growth during physiological and pathological conditions. Perhaps the most evident are the examples of elevated expression of Skp2 mRNA and protein observed in numerous human tumours and the associated decrease in $p27^{Kip1}$ levels (60-63). Skp2 mRNA and protein expression is also dramatically elevated during physiological transitions for quiescence to cell proliferation, including after vascular injury (27). Modulation of Skp2 expression is therefore an important mechanism controlling cell proliferation during both physiological and pathological processes.

8.2. Growth Factors

Numerous mitogenic and anti-mitogenic signalling pathways have been shown to modulate Skp2 expression (64). Growth factors clearly play a role in driving increased Skp2 levels in mid-G1 phase (36, 53, 54). In most cell types growth this occurs through stabilization of Skp2 protein rather than changes in Skp2 transcription or mRNA expression (65). This is mediated, at least in part, through activation of the PI3-kinase and ERK signalling pathways (64, 66, 67) (see Figure 4). Treatment of cells with pharmacological inhibitors of these kinases leads to G_1 growth arrest associated with increased levels of $p27^{Kip1}$ and reduced levels of Skp2. How growth factor stimulation controls Skp2 is only just beginning to be understood. A recent study demonstrated that phosphorylation of Skp2 on residue Ser 72 in response to activation of the PI3-

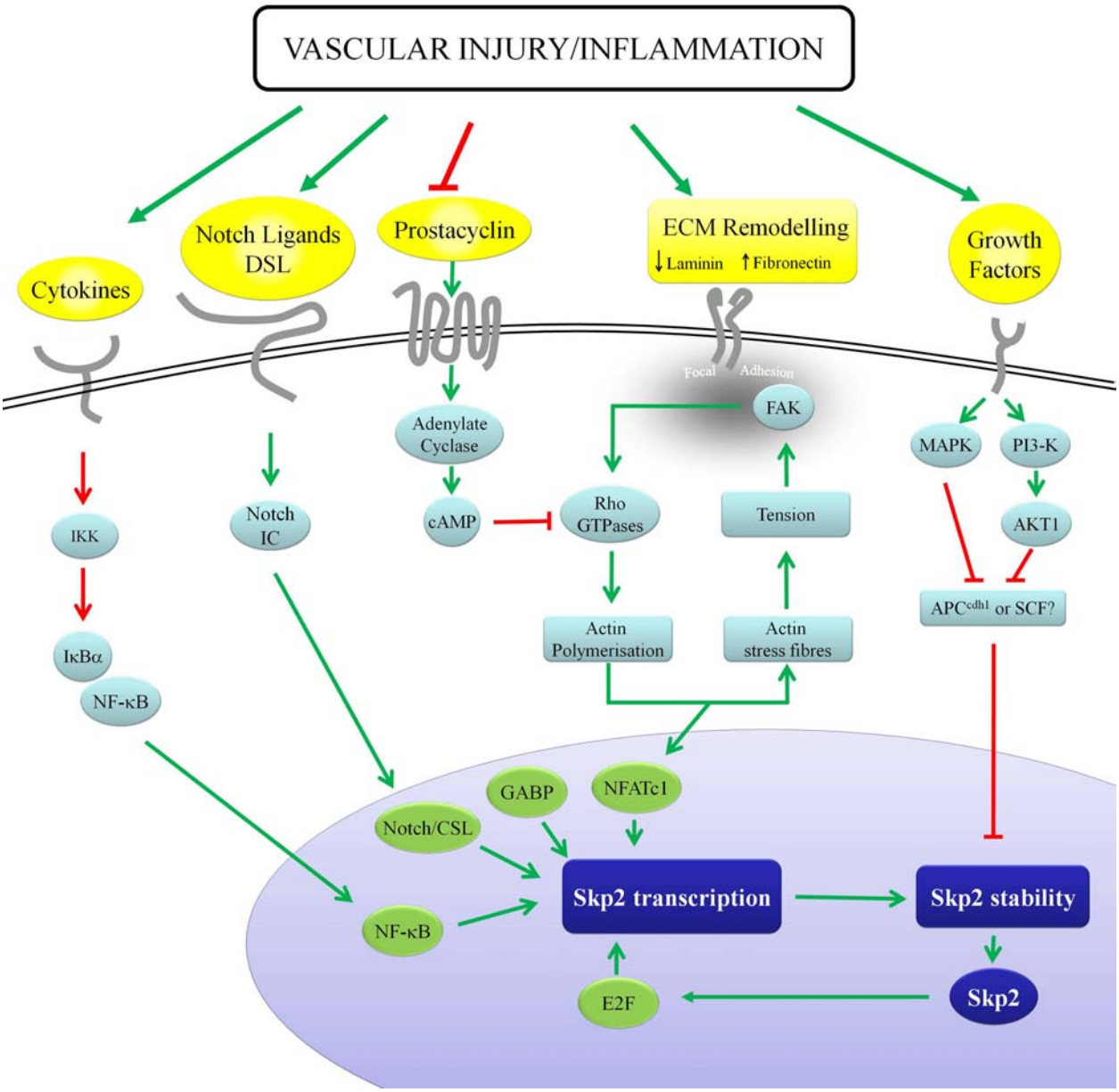

Figure 4. Multiple signals control Skp2 expression. Low expression of Skp2 mRNA and protein contributes towards VSMC quiescence in healthy vessels. Growth factors increase the stability of Skp2 protein by inhibiting the activity of APC^{cdh1} and Cul1 based E3 ubiquitin-ligases that target Skp2 for ubiquitination and degradation. Additional stimuli that promote Skp2 gene transcription are also required before Skp2 protein levels are elevated. Vascular injury and/or inflammation promote remodelling of the vascular ECM composition and compliance. This activates ECM-dependent signalling pathways that result in increased Rho GTPase activity and actin stress-fibre formation, both of which are essential for Skp2 gene transcription and G_1 progression. ECM-dependent Skp2 transcription is mediated at least in part by the NFATc1 transcription factor. Endothelial-derived prostacyclin and its second messenger cAMP inhibit VSMC Skp2 expression by inhibiting Rho GTPase-mediated actin polymerisation. Inflammatory cytokines and activation of Notch signalling also promotes Skp2 transcription by activating NF-kB and Notch/CSL transcription factors that bind and activate the Skp2 promoter. In some cell types Skp2 transcription is elevated in late-G_1. This is mediated by the GA-binding protein (GABP) transcription factor and via a Skp2 auto-induction loop where Skp2 itself promotes activation of E2F1 transcription factor (via cyclin:cdk complex activation and Rb hyper-phosphorylation) which can bind and activate the Skp2 promoter.

Kinase/Akt1 pathway blocks the interaction of APC^{cdh1} with Skp2, thereby preventing Skp2 degradation (68). This provides a direct mechanism linking growth factor stimulation to Skp2 stabilisation in late G_1. Other less

direct mechanisms have also been suggested to play a role. Mitogenic and ERK-dependent stimulation of Skp2 levels has been reported to be, at least partially, a secondary event to up regulation of Cyclin D_1 (64). Silencing of cyclin D_1 induction in embryonic fibroblasts limits the increase in Skp2, while ectopic expression of cyclin D_1 restores Skp2 mRNA and protein expression in ERK inhibited cells, suggesting that activation of cyclin D_1:cdk 4 plays a role (64). Whether this mechanism contributes to Skp2 up regulation in smooth muscle cells is less clear. Mitogenic stimulation of intact arteries fails to increase Skp2 levels, despite robust ERK activation and induction of cyclin D_1 (16) implying that additional signals are also required. The lack of Skp2 mRNA expression in healthy arteries probably explains the refractory nature of the resident VSMC to mitogen stimulation. Without Skp2 mRNA to translate there is no Skp2 protein to be stabilised in response to growth factor stimulation.

8.3. Cyclic nucleotides

The endothelium secretes several small molecules involved in the regulation of vessel tone and homeostasis. Prominent amongst these is the prostanoid, prostacyclin (PGI_2), the major product of cyclooxygenase (COX) catalysed metabolism of arachidonic acid. PGI_2 maintains vessel homeostasis by inhibiting platelet activation, promoting vessel relaxation, inhibiting VSMC proliferation and promoting a differentiated non proliferative smooth muscle cell phenotype (69). These effects are mediated by binding of PGI_2 to the seven trans-membrane prostacyclin receptor (IP), which primarily couples to adenylate-cyclase, leading to increased synthesis of the second messenger, 3'-5'-cyclic adenosine monophosphate (cAMP). Reduced synthesis of prostacyclin by injured or dysfunctional endothelium is thought to promote injury-induced smooth muscle cell proliferation since genetic deletion of the prostacyclin receptor in mice leads to increased smooth muscle cell proliferation and neointima formation in response to vessel injury (70). Intracellular levels of cAMP rapidly decrease in response to mitogen stimulation (71) while agents that elevate cAMP levels inhibit smooth muscle cell proliferation both *in vitro* (72, 73) in response to mitogens and *in vivo* after vascular injury (74). Interestingly, these anti-mitogenic effects of cAMP are highly cell-type specific. Paradoxically, numerous studies demonstrate pro-proliferative effects of elevated cAMP in other cell-types (75, 76), including endothelial cells (77). In smooth muscle cells and fibroblasts, both PGI_2-mimetics and elevated cAMP levels block G1 to S phase progression, associated with an increase in $p27^{Kip1}$ levels and reduced activity of cyclin E:cdk2 complex (54, 78, 79). Inhibition of cyclin D_1 expression may also play a role in some cell types (73, 79, 80) but not in others (78). However, deletion of the $p27^{Kip1}$ gene completely abrogates the inhibitory effects of the PGI_2 mimetic cicaprost in both MEFs and vascular smooth muscle cells, indicating that $p27^{Kip1}$ represents a major downstream target mediating PGI_2-induced growth arrest (78). Signals for cAMP-elevation result in increased $p27^{Kip1}$ levels largely due to potent inhibition of Skp2 transcription and protein stability in both cell types (54, 78). Inhibition of this Skp2-dependent degradation of $p27^{Kip1}$ appears to be a major mechanism underlying the anti-mitogenic effects of prostacyclin mimetics and other cAMP-elevating stimuli since ectopic expression of Skp2 prevents the increase in $p27^{Kip1}$ and restores normal cell-cycle progression (54, 78). Consistent with this, treatment of injured vessels *in vivo* with the adenylate cyclase activator, Forskolin, inhibited expression of Skp2, elevated $p27^{Kip1}$ levels, inhibited smooth muscle cell proliferation and blocked neointimal lesion development (54).

The mechanisms underlying cAMP-dependent regulation of Skp2 expression are only partially understood. In smooth muscle cells, cAMP-elevating agents induced a stellate-morphology associated with a dramatic loss of actin stress fibres, reorganisation of the actin cytoskeleton and dissolution of focal adhesions (81, 82). Similar morphological changes and actin cytoskeleton reorganisations have been observed in several other cell types in response to elevated cAMP (83), suggesting that cytoskeleton reorganisation may play a functional role in cAMP anti-mitogenesis. Many studies have demonstrated a critical role for cytoskeleton organisation for G_1 to S phase progression (84) and members of the Rho GTPase have been implicated in this (85, 86). Expression of constitutively active Rho GTPase mutants promotes S-phase entry in Swiss 3T3 cells whereas dominant-negative mutants block it (85). In smooth muscle cells, RhoA and Rac_1 activity are stimulated by mitogens *in vitro* and Rac_1 is activated by vessel injury *in vivo* (81, 82), while inhibition of RhoA or Rac_1 induces G_1 arrest, associated with elevated levels of $p27^{Kip1}$, reduced expression of Skp2 and G_1 cyclins (82, 85, 87-89). Elevated cAMP levels also inhibit the activity of both RhoA and Rac_1 in smooth muscle cells (81, 82) (see Figure 4) and ectopic expression of constitutively active RhoA or Rac_1 mutants completely prevents cAMP-induced cytoskeleton reorganisation, inhibition of Skp2 and growth arrest. The downstream targets of these Rho GTPases involved in the regulation of Skp2 are not clear. Several studies have implicated signaling via CRIB-domain effector proteins such as PAK in Rac_1-dependent cell-cycle progression, possibly via regulation of cyclin D_1 (90, 91) However, Rac_1 mutations that selectively disrupt signalling to these downstream CRIB-domain effectors have no effect on Skp2 expression (82), while effectively blocking the expression of Cyclin D_1. Importantly, these mutations also do not dramatically affect Rac_1-dependent actin polymerisation (91-93). Interestingly, Skp2 appears to be dependent on a different subset of Rac_1-effector proteins that are directly involved in actin-polymerisation (82). This suggests that Rho GTPases control G_1 progression by controlling expression of Skp2 and Cyclin D_1 via divergent downstream pathway, with Skp2 being dependent on actin-polymerisation.

8.4. The extracellular matrix

Interactions with the native vascular extracellular matrix contribute towards the maintenance of vascular smooth muscle cell quiescence (16) (94, 95). Increased proliferation in response to injury or inflammation occurs concomitantly with phenotypic modulation of smooth muscle cells from a contractile differentiated state into a synthetic phenotype, both of

which are dependent on the composition of the ECM (96). Several components of the vascular ECM, including laminin and polymerised collagen, promote a differentiated smooth muscle phenotype and suppress, or at least do not support, proliferation (97) (98). Cells grown on these matrices *in vitro* display reduced S-phase entry associated with an increase in $p27^{Kip1}$ levels and a decrease in Skp2 mRNA and protein expression (15) (53), suggesting that the native vascular ECM may suppresses proliferation by inhibiting Skp2 expression. Consistent with this, smooth muscle cells liberated from these suppressive effects by *ex vivo* enzymatic digestion with collagenase rapidly acquire the ability to express Skp2, degrade $p27^{Kip1}$ and proliferate in response to mitogen stimulation (53) (16). Vascular injury or inflammation induces expression of endogenous matrix degrading metalloproteinases (MMPs) capable of degrading of these non-permissive ECM components (99) (100) (101). At the same time, smooth muscle cells increase secretion of new growth-permissive ECM components, such as vitronectin and fibronectin (96) (102). Expression of fibronectin is increased in atherosclerosis lesions (103) (104) restenosis after angioplasty (105) and transplant arteriopathy (106). Together, these processes remodel the vascular ECM so that it is supports rather than inhibits proliferation. For example, smooth muscle cells cultured on a fibronectin rich matrix display enhanced Skp2 expression and cycle entry (102) (53). Cells deprived of these permissive ECM interactions by suspension culture do not transcribe the Skp2 gene, resulting in elevated $p27^{Kip1}$ levels and G_1 arrest (65) (53). Importantly, ectopic expression of Skp2 rescues $p27^{Kip1}$ degradation and cell-cycle progression in these cells, implying that Skp2 is a major target for adhesion-dependent mitogenic signalling (65).

These pro-mitogenic effects of ECM components such as fibronectin are mediated via integrin receptors which activate signal transduction pathways involved in cell growth. Smooth muscle cells interact with fibronectin and vitronectin via alpha 5 beta 1 and alpha v beta 3 integrins, respectively. An important signalling pathway for these integrin receptors appears to be the ability to activate focal adhesion kinase (FAK) (107), which has been implicated in adhesion-dependent growth control (108). Early work using dominant-negative mutants of FAK indicated that this was mediated by FAK-dependent regulation of Cyclin D_1 transcription (109), although more recent studies show that FAK activation also promotes Skp2 protein stabilisation (53) (110). Consistent with this, FAK activation appears to be associated with increased Skp2 expression, being low in healthy arteries but increased after vessel injury *in vivo* or by culture of smooth muscle cells *in vitro* (53). How FAK regulates Skp2 expression is not clear. Several key studies have now established that FAK promotes cell growth by permitting sustained ERK activation in response to mitogens (108). Cyclin D_1 expression and G_1-S phase progression are both dependent on sustained ERK activity (111). Whether this mechanism contributes towards Skp2 expression is not clear. In embryonic fibroblasts, the reduction in Skp2 expression after ERK inhibition is completely reversed by ectopic Cyclin D_1 expression (64), suggesting that ERK-dependent Skp2 regulation is not direct but secondary to Cyclin D_1 regulation. However, this is likely to be only part of the mechanism. In these cells, Cyclin D_1 modulates Skp2 mRNA expression while in smooth muscle cells FAK only appears to regulate Skp2 protein stability, with no effect on Skp2 transcription. Furthermore, Skp2 transcription is clearly adhesion-dependent in several cell types, implying a role for additional adhesion-dependent but FAK-independent pathways.

It is now well recognised that Rho family GTPases play a role in adhesion-dependent cell proliferation (85, 88, 89, 91, 112). Just as Rho GTPase members mediate actin cytoskeleton organisation in response to growth factor stimulation, recent studies also implicate them in formation of specific actin structures in response to adhesion and spreading on an ECM. For example, Rho GTPases have been implicated in adhesion-dependent formation of actin-stress fibres, focal complexes, lamellipodia and activation of downstream effector proteins such as PAK (113) (114) (115) (116). Rho GTPase mediated actin-polymerisation appears to be an important event in adhesion-dependent growth control. Dominant-negative mutants of Rho GTPases inhibit Skp2 expression (82, 117) and G_1-S phase progression while constitutively active mutants are able to rescue Skp2 expression and in cells forced into suspension (82, 117). Consistent with this, direct disruption of the actin cytoskeleton integrity or organisation with agents such as cytochalasin D or by preventing cell spreading also inhibits Skp2 expression and induces G1 arrest (82, 117). Clearly, the cytoskeleton is a critical regulator of the cell cycle but how the cytoskeleton controls Skp2 is unknown. Recent studies suggest that generation of cytoskeleton tension may be the critical factor. The level of mechanical tension within the cytoskeleton is determined by multiple factors: (i) the fractional force generated by the actin-myosin II contractile apparatus (118, 119) (ii) the rigidity or compliance of the ECM, and (iii) any tensional forces transmitted through the tissue or substratum to the cell (120-123). Several studies using a variety of different experimental models have now demonstrated that generation of cytoskeleton tension is a prerequisite for G_1-S phase progression. For example, cells grown on low-density ECM molecular coating densities or on micro-fabricated ECM-coated adhesive islands that restrict cell spreading, a pre-requisite for generation of cytoskeleton tension, fail to enter S-phase despite activation of ERK signalling (124). Growth arrest in these cells occurs due to an inability to up regulate cyclin D_1 and Skp2 expression (117). In contrast, cells grown of ECM-densities or on adhesive islands that permit spreading readily enter the cell-cycle in response to mitogens (121, 124). Pharmacological inhibition of acto-mysosin II-generated tension results in potent G1 arrest (125, 126). Lastly, experiments in which cells are cultured on matrices of different rigidities or compliance demonstrate that S-phase entry is enhanced by more rigid, less compliant substrata (127-129). Quiescence induced by a these soft compliant matrices is also associated with increased $p27^{Kip1}$ levels and reduced expression of Skp2 (130). This indicates that Skp2 expression is not only adhesion-dependent but also determined by the composition and the local tension present in the ECM.

Taken together these studies establish a central role for the cytoskeleton and intracellular tension in G_1-S phase control, at least in part by controlling the expression of Skp2. The cytoskeleton appears to act biosensor of the cells readiness to proliferate, integrating multiple singles such as ECM composition and tension, growth factor signals, which can increase intracellular tension by directly activating Rho GTPases, and signals from cyclic-nucleotides, which antagonise formation of a tensed cytoskeleton.

8.5. Transcriptional regulation

Analysis of the Skp2 upstream regulatory sequences reveal the presence of a TATA-less promoter with several cross-species conserved transcription factor binding elements implicated in the regulation of the Skp2 gene in various cell types. The first transcription factor show to positively regulate the Skp2 promoter was GA-Binding protein (GABP), which binds to a consensus element in the proximal promoter in a cell-cycle dependent manner, enhancing Skp2 transcription in S phase (131). Later, others (132, 133) showed that E2F, which is activated in late G1 phase in response to CDK hyper phosphorylation and inactivation of Rb, also contributes towards increased S-phase Skp2 transcription (133). Activation of Skp2 expression in this way indicates the existence of an auto induction loop that has the potential to regulate S phase by promoting $p27^{kip1}$ degradation and, thereby, the activation of cyclin E–Cdk2, phosphorylation of Rb and release of E2Fs, ultimately leading to further stimulation of Skp2 transcription. However, the contribution of this auto-induction loop to increased Skp2 expression in vascular smooth muscle cells is not clear, since S-phase entry, and hence E2F activation, in these cells is not associated with a significant increase in Skp2 mRNA levels, in contrast to that observed in MEFs or NIH 3T3 cells (53, 131, 133). In smooth muscle cells and several other cell types, Skp2 transcriptional regulation is ECM-dependent (53) (65) (117). The mechanisms underlying this regulation are only just beginning to be elucidated. Recent work by Jiang and colleagues demonstrated that ECM tension-dependent Skp2 mRNA expression in bladder and vascular smooth muscle cells is at least in part due to up regulation of the transcription factor NFATc1 and its binding to a consensus element in the proximal Skp2 promoter. Cells grown on relaxed collagen gels fail to express NFATc1 and Skp2 and arrest in G_1 in contrast to cells cultured on tensed collagen gels. Tension dependent expression of Skp2 and induction of smooth muscle cell proliferation also occur *in vivo* in response increased bladder wall tension after bladder ligation (130). Whether similar mechano-regulatory mechanisms control Skp2 expression during in stent restenosis, arterialisation of vein grafts and graft failure, hypertension or atherosclerosis is currently unknown but seem likely. Skp2 transcription in VSMC is also associated with a modulation into a synthetic proliferative phenotype (53). Since smooth muscle cell differentiation status is largely dependent on ECM composition and tension, a common mechanism may underlie this pattern of regulation. Recent evidence suggests that serum response factor (SRF) may play a role in coupling Skp2 expression to VSMC phenotypic modulation. SRF is a widely expressed transcription factor involved in the regulation of smooth muscle cell differentiation marker genes such as smooth muscle α-actin, calponin-1 and transgelin, but also the expression of immediate early genes such as c-fos and Egr-1 and cellular proliferation (134-136). Since SRF is ubiquitously expressed, cell-type specific and signal specific gene expression patterns are determined by several SRF co-factors. Knock-down of SRF in mice impairs vascular recruitment and function of smooth muscle cells (137) but also induces G_1 cell cycle arrest associated with reduced Skp2 expression (138). This suggests the existence of a mechanistic link coupling smooth muscle cell phenotypic modulation to increased proliferative capacity via increased Skp2 expression. How this is achieved is not yet clear but may again be mediated by modulation of the actin cytoskeleton since SRF silencing results in loss of actin-stress fibres (138)

Notch receptors (Notch receptors: 1 to 4) and ligands (Delta, Serrate and Jagged) are transmembrane proteins that have also been implicated in regulation of VSMC phenotypic modulation and proliferation during vascular disease (139, 140). Following cleavage by presenilin, Notch intracellular domains translocate to the nucleus where they interact with the CSL (CBF1, Suppressor of Hairless, Lag-1) family of transcription factors to modulate the expression of Notch target genes that regulate cell fate decisions. For example, levels of active Notch 1 and 3 are inversely related to expression of smooth muscle differentiation marker genes while ectopic expression of constitutively active Notch 1 and 3 inhibits expression of these genes, thus promoting phenotypic modulation (139). Ectopic expression of active Notch 1 and 3 also promotes smooth muscle cell proliferation (140). Although there is limited data on regulation of Skp2 by Notch signalling in smooth muscle cells, this regulation has been observed in several other cell types. For example, Notch activation in 3T3 fibroblasts enhanced Skp2 transcription by binding of Notch/CSL complexes to a consensus element in the proximal Skp2 promoter (141). This is increase in Skp2 transcription underlies the pro-proliferative effects of Notch activation since Skp2 silencing prevents Notch-dependent G_1-S phase progression (141). Similar effects have also been observed in lymphoblastic leukaemia cell lines where Notch 1 activation promotes Skp2 transcription and enhanced S phase entry (142). This suggests that Notch activation may also play a key role in coupling Skp2 transcription smooth muscle cell phenotypic modulation.

The identification of several nuclear factor-kappa B (NF-kB) elements in the proximal Skp2 promoter region suggests that Skp2 may represent an important target for pro-inflammatory mitogenic signals. NF-kB transcription factors play an important role in the transcription of inflammation related genes in response to a variety of stimuli (e.g. interleukin 1, tumour necrosis factor, and lipopolysaccharides) (143). NF-kB has also been implicated in the control of VSMC cell growth (144) (145) by directly controlling transcription of cell-cycle regulatory genes (144, 146). Activation of NF-kB occurs rapidly after

vessel injury or in response to vessel inflammation and has been directly implicated in promoting VSMC proliferation and lesion development (147). For example, gene transfer of I kappa B alpha, the endogenous inhibitor of NF-kB, results in G_1 arrest associated with elevated levels of $p27^{Kip1}$ and inhibition of neointimal lesion development *in vivo* (145, 148). Recent data indicates that these pro-mitogenic effects of NF-kB may be mediated by direct binding of NF-kB subunits to the Skp2 proximal promoter (146). In U2OS osteosarcoma cells, binding of p52 NF-kB subunits to the Skp2 promoter can be detected by chromatin-immunoprecipitation assays while siRNA-mediated silencing of these NF-kB subunits inhibits Skp2 expression (146) (149). Although regulation of Skp2 by NF-kB has not yet been demonstrated in VSMC, the link with NF-kB suggests that Skp2 may represent an important mediator of cell proliferation in response to vessel inflammation.

9. SCF^{SKP2} AS A DRUG TARGET IN CARDIOVASCULAR DISEASE

The ubiquitin-proteasome system (UPS) plays a critical role in the regulation of normal cell growth by controlling the degradation of numerous cell-cycle regulatory proteins. Because of this, the UPS has emerged as an attractive of pharmacological target for the development of novel anti-proliferative therapies, despite its apparent lack of specificity. In 2003, the proteasome inhibitor bortezomib (Velcade ^{TM} Millennium Pharmaceuticals, Inc) was the first in a new class of drugs approved by the Food and Drug Administration for treating multiple myeloma, demonstrating the efficacy of UPS targeting drug therapies. Several studies have also demonstrated positive effects of proteasome inhibition in animal models of cardiovascular disease. For example, potent anti-inflammatory effects of low dose proteasome inhibition have been observed *in vitro* and *in vivo*, possibly via inhibition of NF-kB activation and anti-oxidative effects (150, 151). Other beneficial effects include inhibition of intimal smooth muscle cell hyperplasia, reduced macrophage infiltration, increased apoptosis (152) and inhibition of endothelial dysfunction (153). However, it is not all good news. ApoE-deficient mice treated with Velcade develop a more rupture-prone plaque phenotype (154). Numerous undesirable 'side-effects' have also been reported in patients receiving proteasome-inhibition therapy, including reduced left ventricular ejection fraction (155), anaemia, neutropenia, thrombocytopenia, neuropathy, nausea, diarrhoea and fever (156). These adverse effects may result from inhibition non-proteasome targets but are just as likely to be due to specific inhibition of the wide-ranging functions of the UPS, in which case new structurally diverse proteasome inhibitors would not be expected to be any better. One approach to overcome these problems may be to target specific components of the UPS, such as SCF^{Skp2} E3 ligases, which only target a limited number of proteins for degradation, may be more attractive targets. In theory, these E3-ligase inhibitors should be highly specific drugs, allowing finer control over specific cellular processes without the undesirable 'side-effects' of global proteasome inhibition. However, no E3 inhibitor has yet reached the clinic. This may be because targeting E3 ligases is more difficult than targeting classical enzymes with discreet active sites. SCF^{skp2} inhibitors would need to target protein-protein interactions, which are traditionally thought to be much harder than active-site inhibition. This scepticism may come from early unsuccessful attempts to find inhibitors of SH2 domain protein interactions. These difficulties probably relate to the nature of the protein:protein interaction interface. In SH2 domain surface is flat, with no pocket or protein cavity into which a small molecule can be fitted. However, this is not the case for all protein interaction interfaces. Protein interactions interfaces which have a protein cavity of suitable size, housing a key residue that is essential for the interaction and with which a small molecule can interact may well be susceptible to these types of inhibitors. Using high throughput screening, several studies have now successfully identified small molecule inhibitors of protein protein interactions (157, 158). In 2004 Vassilev and co workers from Hoffmann-La Roche, Inc based at Nutley, New Jersey, USA described a new class of drugs termed the Nutlins that could block the interaction between the Mdm2 ubiquitin-ligase and its substrate p53 (159). This landmark study demonstrated for the first time that small molecules that disrupt protein protein interactions can inhibit ubiquitin-ligases. More recently, high throughput screening has identified a small molecular inhibitor (termed Compound A or CpdA) that blocks the recruitment of Skp2 to the SCF ligase (158). CpdA blocks $p27^{Kip1}$ ubiquitination and degradation *in vitro* and induces cell-cycle arrest in multiple myeloma cell lines, indicating that Skp2 inhibition is a real possibility.

Only with more research will it become clear if SCF^{Skp2} targeting molecules will be effective therapies for treating restenosis or even vein graft failure, and more importantly if they would be better than existing therapies such as rapamycin or paclitaxel eluting stents. One concern may be that SCF^{Skp2} inhibition would suffer from the same problems of existing anti-proliferative drugs, namely inhibition of proliferation in most cell types, including re-growing endothelial cells. One alternative strategy may be to target components of signalling pathways that control Skp2 expression, and hence cell proliferation, in a cell-type specific manner (160). The prostacyclin-cAMP-Rho GTPase pathway is probably a good candidate since it negatively controls Skp2 and proliferation in VSMC but not in endothelial cells. Furthermore, activation of this pathway using a prostacyclin analogue cicaprost blocked VSMC proliferation in a $p27^{kip1}$-dependent manner, while rapamycin remains anti-mitogenic even in $p27^{kip1}$-null cells, suggesting that this pathway highly specifically blocks VSMC proliferation in a $p27^{kip1}$ dependent manner (161).

Inhibition of SCF^{Skp} in these ways will unleash the powerful growth suppressive activity of $p27^{Kip1}$ and is likely to represent a promising strategy for treating vascular proliferative diseases, such as in stent restenosis and late vein graft failure. More research is now needed to fully realise the true therapeutic potential of ubiquitin-ligase and Skp2 inhibition based therapies for the treatment of cardiovascular disease.

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11. REFERENCES

1. R. Ross and J. A. Glomset: Atherosclerosis and arterial smooth muscle cell *Science*, 180(4093), 1332-1339 (1973)

2. R. Ross, J. Glomset and L. Harker: Response to Injury and Atherogenesis. *American Journal of Pathology*, 86(3), 675-684 (1977)

3. G. K. Owens, M. S. Kumar and B. R. Wamhoff: Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiological Reviews*, 84(3), 767-801 (2004)

4. M. H. Hoofnagle, J. A. Thomas, B. R. Wamhoff and G. K. Owens: Origin of neointimal smooth muscle - We've come full circle. *Arteriosclerosis Thrombosis and Vascular Biology*, 26(12), 2579-2581 (2006)

5. R. J. Dilley, J. K. McGeachie and F. J. Prendergast: A Review of the Histologic-Changes in Vein-to-Artery Grafts, with Particular Reference to Intimal Hyperplasia. *Archives of Surgery*, 123(6), 691-696 (1988)

6. G. D. Angelini and A. C. Newby: The Future of Saphenous-Vein as a Coronary-Artery Bypass Conduit. *European Heart Journal*, 10(3), 273-280 (1989)

7. J. Y. Jeremy, P. Gadsdon, N. Shukla, V. Vijayan, M. Wyatt, A. C. Newby and G. D. Angelim: On the biology of saphenous vein grafts fitted with external synthetic sheaths and stents. *Biomaterials*, 28(6), 895-908 (2007)

8. J. Pickering, L. Weir, J. Jekanowski, M. Kearney and J. Isner: Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularisation. *J. Clin. Invest.*, 91, 1469-1480 (1993)

9. E. R. Obrien, C. E. Alpers, D. K. Stewart, M. Ferguson, N. Tran, D. Gordon, E. P. Benditt, T. Hinohara, J. B. Simpson and S. M. Schwartz: Proliferation in primary and restenotic coronary atherectomy tissue - Implications for antiproliferative therpay. *Circulation Research*, 73(2), 223-231 (1993)

10. G. W. Stone, S. G. Ellis, D. A. Cox, J. Hermiller, C. O'Shaughnessy, J. T. Mann, M. Turco, R. Caputo, P. Bergin, J. Greenberg, J. J. Popma, M. E. Russell and T.-I. Investigators: A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. *New England Journal of Medicine*, 350(3), 221-231 (2004)

11. J. W. Moses, M. B. Leon, J. J. Popma, P. J. Fitzgerald, D. R. Holmes, C. O'Shaughnessy, R. P. Caputo, D. J. Kereiakes, D. O. Williams, P. S. Teirstein, J. L. Jaeger, R. E. Kuntz and S. Investigators: Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *New England Journal of Medicine*, 349(14), 1315-1323 (2003)

12. D. Henderson and B. Gunalingam: Very Late Stent Thrombosis of a Sirolimus-Eluting Stent. *Catheterization and Cardiovascular Interventions*, 68, 406-408 (2006)

13. I. Iakovou, T. Schmidt, E. Bonizzoni, L. Ge, G. M. Sangiorgi, G. Stankovic, F. Airoldi, A. Chieffo, M. Montorfano, M. Carlino, I. Michev, N. Corvaja, C. Briguori, U. Gerckens, E. Grube and A. Colombo: Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *Jama-Journal of the American Medical Association*, 293(17), 2126-2130 (2005)

14. B. Lagerqvist, S. James, U. Stenestrand, J. Lindback, T. Nilsson and L. Wallentin: Long-Term Outcomes with Drug-Eluting Stents versus Bare-Metal Stents in Sweden. *The New England Journal of Medicine*, 356, 1009-1019 (2007)

15. H. Koyama, E. W. Raines, K. E. Bornfeldt, J. M. Roberts and R. Ross: Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell*, 87(6), 1069-1078 (1996)

16. T. Izzard, C. Taylor, S. Birkett, C. Jackson and A. Newby: Mechanisms underlying maintenance of smooth muscle cell quiescence in rat aorta: role of the cyclin dependent kinases and their inhibitors. *Cardiovasc Res*, 52, 242-252 (2002)

17. E. Hulleman and J. Boonstra: Regulation of G1 phase progression by growth factors and the extracellular matrix. *Cellular and Molecular Life Sciences*, 58(1), 80-93 (2001)

18. C. J. Sherr and J. M. Roberts: Inhibitors of mammalian G(1) cyclin-dependent kinases. *Genes & Development*, 9(10), 1149-1163 (1995)

19. A. Jawien, V. Lindner, D. F. Bowen-Pope, S. M. Schwartz, M. A. Reidy and A. W. Clowes: Platelet-derived growth factor PDGF stimulates arterial smooth muscle cell proliferation *in vivo*. *FASEB Journal*, 4(3), A342 (1990)

20. A. Schmidt, B. Levkau, A. Skaletzrorowski, G. Breithardt and E. Buddecke: Arterial smooth muscle cell proliferation is induced by endogenous bFGF and inhibited by vFGF-specific antisense oligonucleotides *Circulation*, 90(4), 463-463 (1994)

21. R. Barshavit, M. Benezra, A. Eldor, E. Hyam, J. W. Fenton, G. D. Wilner and I. Vlodavsky: Thrombin immobilized to extracellular matrix is a potent mitogen for vascular smooth muscle cells - nonenzymatic mode of action. *Cell Regulation*, 1(6), 453-463 (1990)

22. J. Fingerle and T. Kraft: The induction of smooth muscle cell proliferation *in vitro* using an organ culture system. *Inter. Angio.*, 6, 65-72 (1987)

23. G. D. Angelini, A. A. Soyombo and A. C. Newby: Smooth muscle cell proliferation in response to injury in an organ culture of human saphenous vein. *Eur. J. Vasc. Surg.*, 5, 5-12 (1991)

24. J. Fingerle, Y. Au, A. Clowes and M. Reidy: Intimal lesion formation in rat carotid arteries after endothelial denudation in absence of medial injury. *Arteriosclerosis*, 10, 1082-1087 (1990)

25. F. Tanner, Z. Yang, E. Duckers, D. Gordon, G. Nabel and E. G. Nabel: Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circulation Research*, 82(3), 396-403 (1998)

26. F. Tanner, M. Boehm, Levent, M. Akyurek, H. San, Z. Yang, J. Tashiro, G. Nabel and E. G. Nabel: Differential effects of the cyclin-dependent kinase inhibitors p27(kip1), p21(Cip1)m and p16(ink4) on vascular smooth muscle cell proliferation. *Circulation*, 101(7), 2022-2025 (2000)

27. Y. J. Wu, G. B. Sala-Newby, K. T. Shu, H. I. Yeh, K. I. Nakayama, K. Nakayama, A. C. Newby and M. Bond: S-phase kinase-associated protein-2 (Skp2) promotes vascular smooth muscle cell proliferation and neointima formation *in vivo*. *Journal of Vascular Surgery*, 50(5), 1135-1142 (2009)

28. M. L. Fero, M. Rivkin, M. Tasch, P. Porter, C. E. Carow, E. Firpo, K. Polyak, L. H. Tsai, V. Broudy, R. M. Perlmutter, K. Kaushansky and J. M. Roberts: A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell*, 85(5), 733-744 (1996)

29. H. Kiyokawa, R. D. Kineman, K. O. ManovaTodorova, V. C. Soares, E. S. Hoffman, M. Ono, D. Khanam, A. C. Hayday, L. A. Frohman and A. Koff: Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell*, 85(5), 721-732 (1996)

30. K. Nakayama, N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Hori, D. Y. Loh and K. Nakayama: Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell*, 85(5), 707-720 (1996)

31. M. Pagano, S. Tam, A. Theodoras, P. Beerromero, G. Delsal, V. Chau, P. Yew, G. Draetta and M. Rolfe: Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*, 269, 682-685 (1995)

32. K. Won and S. Reed: Activation of cyclin E-CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO Journal*, 15, 4182-4193 (1996)

33. J. Vlach, S. Hennecke and B. Amati: Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27(Kip1). *Embo Journal*, 16(17), 5334-5344 (1997)

34. H. Zhang, R. LKobayashi, K. Galaktionov and D. Beach: p19Skp-1 and p45Skp-2 are essential elements of the cyclin A-Cdk2 S phase kinase. *Cell*, 82, 915-925 (1995)

35. C. Bai, P. Sen, K. Hofmann, L. Ma, M. Goebl, J. W. Harper and S. J. Elledge: SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*, 86(2), 263-274 (1996)

36. A. Carrano, E. Eytan, A. Hershko and M. Pagano: SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nature Cell Biology*, 1, 193-199 (1999)

37. J. P. Jin, T. Cardozo, R. C. Lovering, S. J. Elledge, M. Pagano and J. W. Harper: Systematic analysis and nomenclature of mammalian F-box proteins. *Genes & Development*, 18(21), 2573-2580 (2004)

38. L. Busino, F. Bassermann, A. Maiolica, C. Lee, P. M. Nolan, S. I. H. Godinho, G. F. Draetta and M. Pagano: SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science*, 316(5826), 900-904 (2007)

39. N. Zhang, J. Liu, X. Ding, F. Aikhionbare, C. J. Jin and X. B. Yao: FBXL5 interacts with p150(Glued) and regulates its ubiquitination. *Biochemical and Biophysical Research Communications*, 359(1), 34-39 (2007)

40. M. G. Roukens, M. Alloul-Ramdhani, S. Moghadasi, M. O. den Brouw and D. A. Baker: Downregulation of vertebrate Tel (ETV6) and Drosophila Yan is facilitated by an evolutionarily conserved mechanism of F-box-mediated ubiquitination. *Molecular and Cellular Biology*, 28(13), 4394-4406 (2008)

41. E. Latres, D. S. Chiaur and M. Pagano: The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. *Oncogene*, 18(4), 849-854 (1999)

42. K. Masuda, Y. Ishikawa, I. Onoyama, M. Unno, I. M. de Alboran, K. I. Nakayama and K. Nakayama: Complex regulation of cell-cycle inhibitors by Fbxw7 in mouse embryonic fibroblasts. *Oncogene*, 29(12), 1798-1809

43. K. Klotz, D. Cepeda, Y. M. Tan, D. H. Sun, O. Sangfelt and C. Spruck: SCFFbxw7/hCdc4 targets cyclin E2 for ubiquitin-dependent proteolysis. *Experimental Cell Research*, 315(11), 1832-1839 (2009)

44. X. Xu, A. Sarikas, D. Dias-Santagata, G. Dolios, P. Lafontant, S. Tsai, W. Zhu, H. Nakajima, H. Nakajuima, L. Field, R. Wang and Z. Pan: The Cul7 E3 ubiquitin ligase targes insulin receptor substrate 1 for ubiquitin-dependent degradation. *Molecular Cell*, 30, 403-414 (2008)

45. T. H. Lee, K. Perrem, J. W. Harper, K. P. Lu and X. Z. Zhou: The F-box protein FBX4 targets PIN2/TRF1 for ubiquitin-mediated degradation and regulates telomere maintenance. *Journal of Biological Chemistry*, 281(2), 759-768 (2006)

46. D. I. Lin, O. Barbash, K. G. S. Kumar, J. D. Weber, J. W. Harper, A. J. P. Klein-Szanto, A. Rustgi, S. Y. Fuchs and J. A. Diehl: Phosphorylation-dependent ubiquitination of cyclin D1 by the SCFFBX4-alpha B crystallin complex. *Molecular Cell*, 24(3), 355-366 (2006)

47. J. M. Hsu, Y. C. G. Lee, C. T. R. Yu and C. Y. F. Huang: Fbx7 functions in the SCF complex regulating Cdk1-cyclin B-phosphorylated hepatoma up-regulated protein (HURP) proteolysis by a proline-rich region. *Journal of Biological Chemistry*, 279(31), 32592-32602 (2004)

48. U. Kossatz, N. Dietrich, L. Zender, J. Buer, M. Manns and P. Nisar: Skp2-dependent degradation of p27Kip1 is essential for cell cycle progression. *Genes and Development*, 18, 2602-2607 (2004)

49. K. Nakayama, H. Nagahama, Y. A. Minamishima, S. Miyake, N. Ishida, S. Hatakeyama, M. Kitagawa, S. Iemura, T. Natsume and K. I. Nakayama: Skp2-mediated degradation of p27 regulates progression into mitosis. *Developmental Cell*, 6(5), 661-672 (2004)

50. D. Frescas and M. Pagano: Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nature Reviews Cancer*, 8(6), 438-449 (2008)

51. S. Y. Kim, A. Herbst, K. A. Tworkowski, S. E. Salghetti and W. P. Tansey: Skp2 regulates Myc protein stability and activity. *Molecular Cell*, 11(5), 1177-1188 (2003)

52. A. Marti, C. Wirbelauer, M. Scheffner and W. Krek: Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. *Nature Cell Biology*, 1(1), 14-19 (1999)

53. M. Bond, G. Sala-Newby and A. Newby: Focal Adhesion Kinase (FAK)-dependent regulation of S-phase Kinase-associated Protein-2 (Skp-2) Stability. *Journal of Biological Chemistry*, 279, 37304-37310 (2004)

54. Y. Wu, M. Bond, G. Sala-Newby and A. Newby: Altered S-phase kinase-associated protein-2 levels are a major mediator of cyclic nucleotide-induced inhibition of vascular smooth muscle cell proliferation. *Circulation Research*, 98(9), 1141-1150 (2006)

55. H. Sutterluty, E. Chatelain, A. Marti, C. Wirbelauer, M. Senften, U. Muller and W. Krek: $p45^{skp2}$ promotes $p27^{kip1}$ degradation and induces S phase in quiescent cells. *Nature Cell Biology*, 1, 207-214 (1999)

56. C. Wirbelauer, H. Sutterluty, M. Blonel, M. Gstaiger, M. Peter, F. Reymond and W. Krek: The F-box protein Skp2 is a ubiquitinylation target of Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. *EMBO J*, 19(20), 5361-5375 (2000)

57. T. Bashir, N. Dorrello, V. Amador, D. Guardavaccaro and P. M: Control of the SCFSkp2-Cks1 ubiquitin ligase bt the APC/C-Cdh1 ubiquitin ligase. *Nature*, 428(6979), 190-193 (2004)

58. T. Bashir and M. Pagano: Don't skip the G(1) phase how APC/C-Cdh1 keeps SCFSkp2 in check. *Cell Cycle*, 3(7), 850-852 (2004)

59. J. Y. Hsu, J. D. R. Reimann, C. S. Sorensen, J. Lukas and P. K. Jackson: E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). *Nature Cell Biology*, 4(5), 358-366 (2002)

60. M. Gstaiger, R. Jordan, M. Lim, C. Catzavelos, J. Mestan, J. Slingerland and W. Krek: Skp2 is oncogenic and overexpressed in human cancers. *Proceedings of the National Academy of Sciences of the United States of America*, 98(9), 5043-5048 (2001)

61. J. W. Harper, G. Yang, W. Tan, A. Frolov, T. Wheeler, T. Thompson and G. Ayala: Elevated Skp2 protein expression in human prostate cancer is associated with reduced recurrence-free survival. *Modern Pathology*, 15(1), 678 (2002)

62. D. Hershko, G. Bornstein, O. Ben-Izhak, A. Carrano, M. Pagano, M. M. Krausz and A. Hershko: Inverse relation between levels of p27(Kip1) and of its ubiquitin ligase subunit Skp2 in colorectal carcinomas. *Cancer*, 91(9), 1745-1751 (2001)

63. S. Signoretti, F. Monti, B. Isaac, D. Waltregny, J. Dilks, R. Gelman, M. Pagano and M. Loda: The p27 ubiquitin ligase Skp2 is overexpressed in breast cancer. *Laboratory Investigation*, 81(1), 197 (2001)

64. J. Villanueva, Y. Yung, J. L. Walker and R. K. Assoian: ERK activity and G1 phase progression: Identifying dispensable versus essential activities and primary versus secondary targets. *Molecular Biology of the Cell*, 18(4), 1457-1463 (2007)

65. A. C. Carrano and M. Pagano: Role of the F-Box protein Skp2 in adhesion-dependent cell cycle progression. *Journal of Cellular Biology*, 153(7), 1381-1389 (2001)

66. M. Bond, Y. Wu, G. Sala-Newby and A. Newby: Biphasic effect of $p21^{Cip1}$ on vascular smooth muscle cell proliferation: Role of PI3-kinase signalling and Skp2-mediated degradation. *Cardiovascular Research*, 69, 198-206 (2005)

67. F. Pene, Y. E. Claessens, O. Muller, F. Viguie, P. Mayeux, F. Dreyfus, C. Lacombe and D. Bouscary: Role of the phosphatidylinositol 3-kinase/Akt and mTOR/P70S6-kinase pathways in the proliferation and apoptosis in multiple myeloma. *Oncogene*, 21(43), 6587-6597 (2002)

68. D. M. Gao, H. Inuzuka, A. Tseng, R. Y. Chin, A. Toker and W. Y. Wei: Phosphorylation by Akt1 promotes cytoplasmic localization of Skp2 and impairs APC-Cdh1-mediated Skp2 destruction. *Nature Cell Biology*, 11(4), 397-U92 (2009)

69. A. N. Fink, W. H. Frishman, M. Azizad and Y. Agarwal: Use of prostacyclin and its analogues in the treatment of cardiovascular disease. *Heart Dis*, 1(1), 29-40 (1999)

70. Y. Cheng, S. Austin, B. Rocca, B. Koller, T. Coffman, T. Grosser, J. Lawson and G. FitzGerald: Role of Prostacyclin in the Cardiovascular Response to Thromboxance A2. *Science*(296), 539-541 (2002)

71. J. Froehlich and M. Rachmeler: Effect of adenosine 3' - 5' -cyclic monophosphate on cell proliferation. *Journal of Cell Biology*, 55, 19-31 (1972)

72. J. Assender, K. Southgate, M. Hallett and A. Newby: Inhibition of proliferation, but not of Ca2+ mobilization by cyclic AMP and GMP in rabbit aortic smooth-muscle cells. *Biochemical Journal*, 288, 527-532 (1992)

73. N. Kronemann, W. Nockher, R. Busse and V. Schini-Kerth: Growth-inhibitory effect of cyclic GMP- and cyclic AMP-dependent vasodilators on rat vascular smooth muscle cells: effect on cell cycle and cyclin expression. *British Journal of Pharmacology*, 126, 349-357 (1999)

74. C. Indolfi, E. V. Avvedimento, E. DiLorenzo, G. Esposito, A. Rapacciuolo, P. Giuliano, D. Grieco, L. Cavuto, A. M. Stingone, I. Ciullo, G. Condorelli and M. Chiarello: Activation of cAMP-PKA signaling *in vivo* inhibits smooth muscle cell proliferation induced by vascular injury. *Nature Medicine*, 3(7), 775-779 (1997)

75. I. Pastan, G. Johnson and W. Anderson: Role of cyclic nucleotides in growth control. *Annual Review of Biochemistry*, 44, 491-522 (1975)

76. D. Hochbaum, K. Hong, G. Barila, F. Ribeiro-Neto and D. L. Altschuler: Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis. *Journal of Biological Chemistry*, 283(8), 4464-4468 (2008)

77. C. Gillis, B. Jonzon and A. Heagerstrand: Effects of sera, basic fibroblast growth factors, heparin and cyclic AMP-stimulation of proliferation of human vascular endothelial cells. *Cellular and Molecular Biology*, 41, 1131-1138 (1995)

78. S. A. Stewart, D. Kothapalli, Y. V. Yung and R. K. Assoian: Antimitogenesis linked to regulation of Skp2 gene expression. *Journal of Biological Chemistry*, 279(28), 29109-29113 (2004)

79. S. Fukumoto, H. Koyama, M. Hosoi, K. Yamakawa, S. Tanaka, H. Morii and Y. Nishizawa: Distinct role of cAMP and cGMP in the cell cycle control of vascular smooth muscle cells - cGMP delays cell cycle transition through suppression of cyclin D1 and cyclin-dependent kinase 4 activation. *Circ. Res.*, 85(11), 985-991 (1999)

80. G. LAllemain, J. Lavoie, N. Rivard, V. Baldin and J. Pouyssegur: Cyclin D1 expression is a major target of the cAMP-induced inhibition of cell cycle entry in fibroblasts. *Oncogene*, 14(16), 1981-1990 (1997)

81. S. Pelletier, C. Julien, M. Popoff, N. Lamarche-Vane and S. Meloche: Cyclic AMP induces morphological changes of vascular smooth muscle cells by inhibiting a Rac-dependent signaling pathway. *J. Cell. Physiol.*, 204, 412-422 (2005)

82. M. Bond, Y. J. Wu, G. B. Sala-Newby and A. C. Newby: Rho GTPase, Rac_1, regulates Skp2 levels, vascular smooth muscle cell proliferation, and intima formation *in vitro* and *in vivo*. *Cardiovascular Research*, 80(2), 290-298 (2008)

83. A. E. Christensen, F. Selheim, J. de Rooij, S. Dremier, F. Schwede, K. K. Dao, A. Martinez, C. Maenhaut, J. L. Bos, H. G. Genieser and S. O. Doskeland: cAMP analog mapping of Epac1 and cAMP kinase - Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *Journal of Biological Chemistry*, 278(37), 35394-35402 (2003)

84. S. Huang and D. Ingber: A discrete cell cycle checkpoint in late G1 that is cytoskeleton-dependent and MAP Kinase (ERK)-independent. *Experimental Cell Research*, 275, 255-264 (2002)

85. M. Olson, A. Ashworth and A. Hall: An Essential Role of Rho, Rac and Cdc42 GTPases in Cell Cycle Progression Through G1. *Science*, 269, 1270-1272 (1995)

86. C. Welsh and R. Assoian: A growing role for Rho family GTPases as intermediaries in growth factor- and adhesion-dependent cell cycle progression. *Biochimica et Biophyisica Acta*, 1471, M21-M29 (2000)

87. U. Laufs, D. Marra, K. Node and J. K. Liao: 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing Rho GTPase-induced down-regulation of $p27^{Kip1}$. *Journal of Biological Chemistry*, 274(31), 21926-21931 (1999)

88. C. F. Welsh: Rho GTPases as key transducers of proliferative signals in G1 cell cycle regulation. *Breast Cancer Research and Treatment*, 84, 33-42 (2004)

89. C. F. Welsh and R. K. Assoian: A growing role for Rho family GTPases as intermediaries in growth facto- and adhesion-dependent cell cycle progression. *BBA*, 1471(M21-M29) (2000)

90. O. Gjoerup, J. Lukas, J. Bartek and B. Willumsen: Rac and Cdc42 are potent stimulators of E2F-dependent transcription capable of promoting retinoblastoma

susceptibility gene product hyperphosphorlyation. *J. Biol. Chem.*, 273, 18812-18818 (1998)

91. J. Westwick, Q. Lambert, G. Clark, M. Symons, L. Aelst, R. Pestell and C. Der: Rac regulation of transformation, gene expression, and actin organisation by multiple, PAK-independent pathways. *Mollecular and Cellular Biology*, 17(3), 1324-1335 (1997)

92. M. Bond, A. H. Baker and A. C. Newby: Nuclear factor kappa B activity is essential for matrix metalloproteinase-1 and-3 upregulation in rabbit dermal fibroblasts. *Biochemical and Biophysical Research Communications*, 264(2), 561-567 (1999)

93. M. Lamarche, N. Tapan, L. Stowers, P. Burbelo, P. Aspenstrom, T. Brisges, J. Chant and A. Hall: Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently. *Cell*, 87, 519-529 (1996)

94. A. C. Newby and A. B. Zaltsman: Molecular mechanisms in intimal hyperplasia. *Journal of Pathology*, 190, 300-309 (2000)

95. R. Assoian and E. Marcantonio: Cell Adhesion in Vascular Biology: The Extracellular Matrix as a cell Cycle Control Element in Atherosclerosis and Restenosis. *J. Clin. Invest.*, 98(11), 2436-2439 (1996)

96. J. Thyberg, K. Blomgren, J. Roy, P. Tran and U. Hedin: Phenotypic modulation of smooth muscle cells after arterial injury is associated with changes in the distribution of laminin and fibronectin. *J. Histochemistry and Cytochemistry*, 45(6), 837-846 (1997)

97. U. Hedin, B. Bottger, E. Forsberg, S. Johansson and J. Thyberg: Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. *J. Cell. Biol.*, 107, 307-319 (1988)

98. N. Sakata, K. Kawamura and S. Takebayashi: Effects of collagen matrix on proliferation and differentiation of vascular smooth muscle cells *in vitro*. *Exp. Mol. Pathol.*, 52, 179-91 (1990)

99. Z. S. Galis and J. J. Khatri: Matrix metalloproteinases in vascular remodeling and atherogenesis - The good, the bad, and the ugly. *Circulation Research*, 90(3), 251-262 (2002)

100. A. C. Newby: Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiological Reviews*, 85(1), 1-31 (2005)

101. A. C. Newby: Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. *Cardiovascular Research*, 69(3), 614-624 (2006)

102. J. Roy, P. K. Tran, P. Religa, M. Kazi, B. Henderson, K. Lundmark and U. Hedin: Fibronectin promotes cell cycle entry in smooth muscle cells in primary culture. *Experimental Cell Research*, 273(2), 169-177 (2002)

103. M. Glukhova, M. Frid, B. Shekhonin, T. Vasilevskaya, J. Grunwald, M. Saginati and V. Koteliansky: Expression of extracellular domain A fibronectin sequence in vascular smooth muscle cells is phenotype dependent. *J Cell Biol*, 109, 357-366 (1989)

104. J. Labat-Robert, M. Szendroi, G. Godeau and L. Robert: Comparative distribution patterns of type I and III collagens and fibronectin in human arteriosclerotic aorta. *Pathol Biol (Paris)*, 33, 261-265 (1985)

105. N. Clausell, V. deLima, S. Molossi, P. Liu, E. Turley, A. Gotlieb, A. Adelman and M. Rabinovitch: Expression of tumor necrosis factor {alpha} and accumulation of fibronectin in coronary artery restenotic lesions retrieved by atherectomy (19935)

106. N. Clausell, S. Molossi and M. Rabinovitch: Increased interleukin-1 beta and fibronectin expression are early features of the development of the postcardiac transplant coronary arteriopathy in piglets. *Am. J. Pathol.*, 142, 1772-1786 (1993)

107. J. Parsons: Focal adhesion kinase: the first ten years. *J. Cell. Sci.*, 116, 1409-1416 (2003)

108. M. Renshaw, L. Price and M. Schwartz: Focal adhesion kinase mediates the integrin signalling requirements for growth factor activation of MAPK kinase. *J Cell Biology*, 147, 611-618 (1999)

109. J. Zhao, R. Pestell and J. L. Guan: Transcriptional activation of cyclin D1 promoter by FAK contributes to cell cycle progression. *Molecular Biology of the Cell*, 12(12), 4066-4077 (2001)

110. P. Bryant, Q. Zheng and K. Pumiglia: Focal adhesion kinase controls cellular levels of $p27^{Kip1}$ and $p21^{Cip1}$ through Skp-2-dependent and -independent mechanisms. *Molecular and Cellular Biology*, 26(11), 4201-4213 (2006)

111. K. Roovers, G. Davey, X. Zhu, E. Bottazzi and R. Assoian: a5b1 integrin controlls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Molecular Biology of the Cell*, 10, 3197-3204 (1999)

112. C. F. Welsh, K. rpoovers, J. Villanueva, Y. Liu and M. A. A. Schwartz, R K: Timing of cyclin D1 expression within G1 phase is controlled by Rho. *Nature Cell Biology*, 3, 950-957 (2001)

113. M. del Pozo, L. Price, N. Alderson, X.-D. Ren and M. Schwartz: Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. *EMBO J*, 19(9), 2008-2014 (2000)

114. K. Rottner, A. Hall and J. V. Small: Interplay between Rac and Rho in the control of substrate contact dynamics. *Current Biology*, 9(12), 640-648 (1999)

115. A. Hall, N. Lamarche, D. Mackay, K. Nagata, A. Puls and N. Tapon: Co-ordinated regulation of tee actin cytoskeleton and of gene transcription by the Rho GTPase family. *Molecular Biology of the Cell*, 8, 1354-1354 (1997)

116. N. Lamarche, N. Tapon, L. Stowers, P. D. Burbelo, P. Aspenstrom, T. Bridges, J. Chant and A. Hall: Rac and CDC42 induce actin polymerization and DNA synthesis independently of p65PAK and the JNK cascade. *Molecular Biology of the Cell*, 7, 1336-1336 (1996)

117. A. Mammoto, S. Huang, K. Moore, P. Oh and D. Ingber: Role of RhoA, mDia, and ROCK in Cell Shape-dependent Control of the Skp2-p27Kip1 Pathway amd the G1/S Transition. *Journal of Biological Chemistry*, 279, 26323-26330 (2004)

118. K. Burridge, M. ChrzanowskaWodnicka and C. L. Zhong: Focal adhesion assembly. *Trends in Cell Biology*, 7(9), 342-347 (1997)

119. D. M. Helfman, E. T. Levy, C. Berthier, M. Shtutman, D. Riveline, I. Grosheva, A. Lachish-Zalait, M. Elbaum and A. D. Bershadsky: Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. *Molecular Biology of the Cell*, 10(10), 3097-3112 (1999)

120. A. Mammoto and D. Ingber: Cytoskeletal control of growth and cell fate switching. *Current Opinion in Cell Biology*, 21, 864–870 (2009)

121. C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides and D. E. Ingber: Geometric control of cell life and death. *Science*, 276 (1997)

122. D. E. Ingber: Tensegrity I. Cell structure and hierarchical systems biology. *Journal of Cell Science*, 116(7), 1157-1173 (2003)

123. D. E. Ingber: Tensegrity II. How structural networks influence cellular information processing networks. *Journal of Cell Science*, 116(8), 1397-1408 (2003)

124. S. Huang, C. Chen and D. Ingber: Control of Cyclin D1, $p27^{kip1}$, and cell cycle progression in humna capillary endothelial cells by cell shape and cytoskeleton tension. *Molecular Biology of the Cell*, 9, 3197-3193 (1998)

125. J. Dhawan and D. Helfman: Modulation of acto-myosin contractility in skeletal muscle myoblasts uncouples growth arrest from differentiation. *Journal of Cell Science*, 117, 3735-3748 (2004)

126. K. Bhadriraju and L. Hansen: Extracellular Matrix- and Cytoskeleton-Dependent Changes in Cell Shape and Stiffness. *Experimental Cell Research*, 278, 92-100 (2002)

127. T. Ulrich, E. de Juan Pardo and D. Kumar: The Mechanical Rigidity of the Extracellular Matrix Regulates the Structure, Motility, and Proliferation of Glioma Cells. *Cancer Research*, 69, 4167-4174 (2009)

128. E. Hadjipanayi, V. Mudera and R. Brown: Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. *Journal of Tissue Eng Regen Med*, 3, 77-84 (2008)

129. J. Winer, P. Janmey, M. McCormick and M. Funaki: Bond marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Eng Part A*, 15, 1147-154 (2009)

130. X. G. Jiang, P. F. Austin, R. A. Niederhoff, S. R. Manson, J. J. Riehm, B. L. Cook, G. Pengue, K. Chitaley, K. Nakayama, K. I. Nakayama and S. J. Weintraub: Mechanoregulation of Proliferation. *Molecular and Cellular Biology*, 29(18), 5104-5114 (2009)

131. H. Imaki, K. Nakavama, S. Deiehouzee, H. Handa, K. Kitagawa, T. Kamagawa and K. Nakayama: Cell cycle-dependent regulation of the Skp2 promoter by GA-binding protein. *Cancer Research*, 63, 4607-4613 (2003)

132. L. Zhang and C. Wang: F-box protein Skp2: a novel transcriptional target of E2F. *Oncogene*, 25, 2615-2627 (2006)

133. Y. Yung, J. L. Walker, J. M. Roberts and R. K. Assoian: A Skp2 autoinduction loop and restriction point control. *Journal of Cell Biology*, 178, 741-747 (2007)

134. A. Dalton and R. Treisman: Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell*, 68, 597-612 (1992)

135. R. Hipskin, V. Rao, C. Mueller, E. Reddy and A. Nordheim: Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. *Nature*, 354, 531-534 (1991)

136. G. K. Owens, M. Kumar and B. Wamhoff: Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease. *Physiological Reviews*, 84, 767-801 (2003)

137. J. Miano, N. Ramanan, M. Georger, K. de Mesy Bentley, R. Emerson, R. Balza Jr., Q. Xiao, H. Weiler, D. Ginty and R. Misra: Restricted inactivation ofserum response factor to the cardiovascular system. *Proc. Natl. Acad. Sci. USA*, 101, 17132-17137 (2004)

138. D. Werth, G. Grassi, N. Konjer, B. Dapas, R. Farra, C. Giansante, R. Kandolf, G. Guarnieri, A. Nordheim and O. Heidenreich: Proliferation of human primary vascular smooth muscle cells depends on serum response factor. *European Journal of Cell Biology*, 89(2-3), 216-224 (2010)

139. D. Morrow, A. Scheller, Y. Birney, C. Sweeney, S. Guha, P. Cummins, R. Murphy, D. Walls, E. Redmond and P. Cahill: Notch-mediated CBF-1/RBP-Jk-dependent regulation of human vascular smooth muscle cell phenotype *in vitro*. *Am. J. Physiol. Cell. Physiol.*, 289, C1188-C1196 (2005)

140. C. Sweeney, D. Morrow, Y. Birney, S. Coyle, C. Hennessy, A. Scheller, P. Cummins, D. Walls, E. Redmond and P. Cahill: Notch 1 and 3 receptors modulate vascular smooth muscle cell growth, apoptosis and migration via a CBF-1/RBP-Jk dependent pathway. *The FASEB Journal*, 18, 1421-+ (2004)

141. L. Sarmento, H. Huang, A. Limon, W. Gordon, J. Fernandes, M. Tavares and L. Miele: Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and $p27^{Kip1}$ degradation. *Journal of Experimental Medicine*, 202(1), 157-168 (2005)

142. T. Dohda, A. Maljukova, L. Liu, M. Heyman, D. Grander, D. Brodin, O. Sangfelt and U. Lendahl: Notch signaling induces SKP2 expression and promotes reduction of $p27^{Kip1}$ in T-cell acute lymphoblastic leukemia cell lines. *Experimental Cell Research*, 313(14), 3141-3152 (2007)

143. U. Sienbenlist, G. Francesco and K. Brown: Structure regulation and function of NF-kappa B. *Ann. Rev. Cell Biol.*, 10, 405-455 (1994)

144. M. V. Autieri, B. A. Olson, T. L. Yue and G. Z. Feuerstein: Antisense Oligonucleotides to the p65 Subunit of NF-kB Inhibit Human Vascular Smooth-Muscle Cell Adherence, Migration, and Proliferation. *Circulation*, 90(4 Pt2), 511-511 (1994)

145. M. Cejna, J. M. Breuss, H. Bergmeister, R. de Martin, Z. Y. Xu, M. Grgurin, U. Losert, H. Plenk, B. R. Binder and J. Lammer: Inhibition of neointimal formation after stent placement with adenovirus-mediated gene transfer of I kappa B alpha in the hypercholesterolemic rabbit model: Initial results. *Radiology*, 223(3), 702-708 (2002)

146. A. Barre and N. Perkins: A cell cycle regulatory network controlling NF-kB subunit activity and function. *The EMBO Journal*, 26, 4841-4855 (2007)

147. B. Cercek, M. Yamashita, P. Dimayuga, J. Zhu, M. Fishbein, S. Kaul, P. Shah, J. Nilsson and J. Regnstrom: Nuclear factor-kB activity and arterial response to balloon injury. *Atherosclerosis*, 131, 59-66 (1997)

148. B. S. Zuckerbraun, C. A. McCloskey, R. S. Mahidhara, P. K. M. Kim, B. S. Taylor and E. Tzeng: Overexpression of mutated I kappa B alpha inhibits vascular smooth muscle cell proliferation and intimal hyperplasia formation. *Journal of Vascular Surgery*, 38(4), 812-819 (2003)

149. B. Barre and N. D. Perkins: The Skp2 Promoter Integrates Signaling through the NF-kappa B, p53, and Akt/GSK3 beta Pathways to Regulate Autophagy and Apoptosis. *Molecular Cell*, 38(4), 524-538 (2010)

150. A. Ludwig, M. Fechner, N. Wilck, S. Meiners, N. Grimbo, G. Baumann, V. Stangl and K. Stangl: Potent anti-inflammatory effects of low-dose proteasome inhibition in the vascular system. *Journal of Molecular Medicine-Jmm*, 87(8), 793-802 (2009)

151. P. Elliott, T. Zollner and W. Boehncke: Proteasome inhibitions: a new anti-inflammatroy strategy. *J Mol Med*, 81, 235-245 (2003)

152. S. Meiners, M. Laule, W. Rother, C. Guenther, I. Prauka, P. Muschick, G. Baumann, P. Kloetzel and K. Strangl: Ubiquitin-Proteasome pathway as a new target for the prevention of restenosis. *Circulation*, 105, 483-489 (2002)

153. M. Lorenz, N. Wilck, S. Meiners, A. Ludwig, G. Baumann, K. Stangl and V. Stangl: Proteasome inhibition prevents experimentally-induced endothelial dysfunction. *Life Sciences*, 84, 929-934 (2009)

154. J. L. Van Herck, G. R. Y. De Meyer, W. Martinet, H. Bult, C. J. Vrints and A. G. Herman: Proteasome inhibitor bortezomib promotes a rupture-prone plaque phenotype in ApoE-deficient mice. *Basic Research in Cardiology*, 105(1), 39-50 (2010)

155. S. Khan: Cardiac complications may be higher with use of proteasome inhibitors in patients with rheumatoid arthritis. *Medical Hypotheses*, 71(5), 818-818 (2008)

156. A. Palumbo, F. Gay, S. Bringhen, A. Falcone, N. Pescosta, V. Callea, T. Caravita, F. Morabito, V. Magarotto, M. Ruggeri, I. Avonto, P. Musto, N. Cascavilla, B. Bruno and M. Boccadoro: Bortezomib, doxorubicin and dexamethasone in advanced multiple myeloma. *Annals of Oncology*, 19(6), 1160-1165 (2008)

157. T. Berg, S. B. Cohen, J. Desharnais, C. Sonderegger, D. J. Maslyar, J. Goldberg, D. L. Boger and P. K. Vogt: Small-molecule antagonists of Myc/Max dimerization inhibit Myc-induced transformation of chicken embryo fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, 99(6), 3830-3835 (2002)

158. M. Lepourcelet, Y. N. P. Chen, D. S. France, H. S. Wang, P. Crews, F. Petersen, C. Bruseo, A. W. Wood and R. A. Shivdasani: Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. *Cancer Cell*, 5(1), 91-102 (2004)

159. L. T. Vassilev, B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi and E. A. Liu: *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*, 303(5659), 844-848 (2004)

160. Y. J. Wu, H. I. Yehl, C. J. Y. Hou, C. H. Tsai, A. C. Newby and M. Bond: Beyond Oncogenesis: the Role of S-Phase Kinase-Associated Protein-2 (Skp2) in Vascular Restenosis. *International Journal of Gerontology*, 2(4), 158-166 (2008)

161. P. Castagnino, D. Kothapalli, E. Hawthorne, T. Xu and R. K. Assoian: Cell-type and cell-cycle-specific anti-mitogenesis by cicaprost. *Prostaglandins and Other Lipid Mediators*, In Press (2010)

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