Review Article

Induction of cell senescence by targeting to Cullin-RING Ligases (CRLs) for effective cancer therapy

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Abstract: Cullin-RING ligases (CRLs) are the biggest family of multiunit ubiquitin E3 ligases, controlling many biological processes by promoting the degradation of a broad spectrum of proteins associated with cell cycle, signal transduction and cell growth. The dysfunction of CRLs causes a lot of diseases including cancer, which meanwhile offers us a promising approach to cancer therapy by targeting to CRLs. Recent studies have demonstrated that genetic or pharmaceutical inactivation of CRLs often leads to cancer cell death by activating multiple cell-killing pathways including senescence, an emerging anticancer mechanism of therapeutic agents. Here, we summarize the induction of cellular senescence and its mechanism of action, triggered by targeting to specific subunits of CRLs via multiple approaches including siRNA silencing, genetic knockout as well as small molecule inhibitor, exhibiting anticancer effect in vitro and in vivo.

Keywords: CRLs, senescence, RBX1/ROC1, Skp2, cullin neddylation, MLN4924

Introduction

Cullin-RING Ligases (CRLs) as anticancer targets

In eukaryote, intracellular short-lived protein turnover is primarily regulated by ubiquitin-proteasome system (UPS) [1], which consists of two main steps: the attachment of ubiquitin molecule to specific protein (ubiquitination) and the proteolysis of ubiquitinated protein by 26S proteasome. Ubiquitination is mediated by E1-E2-E3 enzyme cascade, in which ubiquitin is first activated by ubiquitin-activating enzymes (E1) in a ATP-dependent manner, then transferred to ubiquitin-conjugating enzymes (E2), and finally conjugated to substrate by ubiquitin-ligase enzymes (E3) in a poly-ubiquitin chain. After that, ubiquitinated protein is recognized by 26S proteasome and degraded to small peptides (Figure 1) [2-4].

Normally, modulation of UPS facilitates physiological processes, while its aberrant regulation may trigger many diseases including cancer, which provides us a rational approach to cancer therapy by targeting to UPS. Bortezomib, as a specific inhibitor of 26S proteasome, has been approved by FDA for the treatment of progressive multiple myeloma [5]. However, side effects of the drug are widely reported due to the inhibition of the whole UPS-mediated protein degradation by Bortezomib [6-9]. The findings indicate that molecules targeting to other UPS components, particularly to one or a subset of E3s, may alleviate or overcome the harmful symptoms of bortezomib, since the specificity of substrate degradation largely depends on E3s, which mount over 600 compared to a proteasome, 2 E1s and ~40 E2s in human [10].

As the biggest multiunit family of ubiquitin E3 ligases, Cullin-RING ligases (CRLs) consist of two core subunits: cullin (cullin 1, 2/5, 3, 4A/4B and 7) and RING-finger protein RBX/ROC [11]. Structural analysis revealed that C-terminus of cullin interacts with RING domain of RBX/ROC which recruits E2 conjugated ubiquitin to the complex, while N-terminus of cullin recruits substrates for degradation via substrate recep...
Cell senescence targeting to CRLs for cancer therapy

Figure 1. Schematic of protein degradation by ubiquitin proteasome system (UPS). Protein degradation through UPS can be divided into two successive processes, ubiquitylation and degradation. Firstly, protein is ubiquitylated through E1-E2-E3 cascade reaction. Then ubiquitylated proteins are recognized and degraded to small peptides by 26S proteasome.

Figure 2. The schematic structure and functions of Cullin-RING ligase (CRLs). CRLs are composed of core catalytic subunit, cullin and RING-finger protein RBX/ROC, which assemble as a multi-subunit complex by recruiting ubiquitin transfer enzyme E2 to RBX/ROC and substrates to the N-terminal of cullin through the motif recognized by substrate receptor (SR) with/without the assistance of an adaptor protein. The activation of CRLs requires the modification of NEDD8 through neddylation. As a complex, CRLs regulate a set of biological processes including cell cycle, transcription signal transduction and survival.

Recent studies have demonstrated that aberrant expression or deregulation of CRLs may favor the development and progression of cancer. For example, as a member of F-box protein, Skp2, functioning as an onco-protein, is often over-expressed in many human primary cancers and correlated with poor prognosis [15-17]. In contrast, another F-box protein, FBW7, acting as a tumor suppressor by degrading several oncoproteins, including MYC, JUN, cyclin E and notch, is often mutant or lost in gastric, pancreatic, colon carcinoma and T-cell acute lymphoblastic leukemia (T-ALL) to facilitate carcinogenesis and progression [18-20]. Recently, we found that RING-finger protein RBX1/ROC1 and RBX2/ROC2/SAG, are overexpressed in a set of human primary tumors, including lung and liver tumors [21, 22]. Moreover, CRLs inactivation induced either by siRNA silencing of essential components of CRLs (such as skp2 and RBX1/ROC1) or by small molecule inhibitors (such as MLN4924), triggers cell death to suppress cancer cell growth both in vitro and in vivo, indicating CRLs are promising anticancer targets for drug discovery [23].

Senescence as an anticancer mechanism

Senescence is an irreversible state of cell growth arrest, which can be triggered by diverse of cellular stresses, including alternation of telomeres, DNA damage, oxidative stress and oncogene activation [24-26]. Although senescent cells lack of the capacity of replication, they are still metabolically active with morpho-
logical changes, such as enlarged and flattened cell shape. Senescent cells can be detected by the expression of senescence-associated beta-galactosidase (SA-β-gal) through biochemical assay [27]. Many senescent cells can also be marked by senescence associated heterochromatin foci (SAHFs) [28] and senescence-associated DNA damage foci (SDFs) [29].

In mechanism, senescence is mainly manipulated through canonical p53/p21 and p16/pRB signaling pathway [30]. Senescence induced by some stimuli, such as DNA damage response (DDR), is primarily mediated through p53/p21 pathway [31]. As DDR occurs, p53 transmits signals to down-stream effector p21, a cell cycle inhibitor, which induces cellular senescence directly or through inactivation of CDK2 and CDK4 indirectly, resulting in hypo-phosphorylation of pRB and subsequently cell cycle arrest during senescence [32, 33]. Apart from p53/p21 pathway, DDR can also trigger senescence through p16/pRB pathway. During the initiation of p16/pRB pathway, cyclin-dependent kinase inhibitor p16 is up-regulated, which inhibits the activity of CDK4 and CDK6, resulting in cell cycle arrest and hypo-phosphorylation of pRB. As a result, hypo-phosphorylation of pRB inhibits transcription of genes regulated by E2F, which up-regulates the expression of ARF facilitating to activation of senescence [26, 32].

Senescence has been well-recognized as an important mechanism for protection against cancer due to its anti-proliferative effect [34, 35]. In vitro, cells undergoing stimuli-induced cellular senescence represent a notable morphological transformation, slow replication rate and cell cycle arrest [25]. In vivo, senescence has been observed in premalignant tumors in the lung of mouse which was transected with K-Ras<sup>G12D</sup>, but absent in lung adenocarcinomas [36]. Oncogenic mutant B RAF<sup>V600E</sup> represents characteristics of senescence in human benign naevi, but rarely in melanoma [37]. Moreover, genetic depletion of tumor suppressor such as PTEN [38], p53 and p16 [39], or disruption of senescence pathway by deletion of histone methyltransferase Suv39H1 [40] results in the development of malignant tumor in mice. Although cancer cells possess the capacity of resisting to apoptosis, diverse of anticancer agents, such as cisplatin [41], have been proved to be capable of inducing senescence as a mechanism of growth suppression. Recent studies from our and other groups showed that inactivation of CRLs also triggers senescence and/or apoptosis in cancer cells. Thus, cellular senescence induced by targeting to CRLs may be a promising strategy for cancer treatment and prevention.

Induction of senescence by targeting to CRLs for effective anticancer therapy

**Targeting ring-finger protein RBX1/ROC1**

As an essential component of CRLs, RBX1/ROC1 contains a RING-H2 finger domain (C3H2C3) at the C-terminus which is required for recruitment of zinc atom and E2 for ubiquitination of substrates, while its N-terminus binds to cullin for recruiting substrate receptors to form functional E3 ligases [42-44]. Previous studies showed that RBX1/ROC1 is critical for proliferation and development in many species, including *Caenorhabditis elegans* [45], *Drosophila* [46] and mouse [47]. However, the role of RBX1/ROC1 in tumorigenesis and progression is rarely reported.

Recently, we found that RBX1/ROC1 is overexpressed in a set of primary human cancer tissues, such as lung and liver cancer [21, 48], suggesting that RBX1/ROC1 plays a critical role in tumor formation and progression. Moreover, siRNA silencing of RBX1/ROC1 significantly inhibits the growth of several cancer cell lines both in vitro and in vivo, indicating that RBX1/ROC1 is required for cancer cell growth. Further analysis showed that down-regulation of RBX1/ROC1 induces senescence in tested cancer cell lines, including human colon cancer HCT116 cells, lung cancer H1299 cells and glioma U87 cells (Figure 3). Mechanically, RBX1/ROC1 knockdown-induced senescence in these cells is not associated with p53/p21 and p16/pRB pathways.

Interestingly, our recent studies have revealed that RBX1/ROC1 may induce senescence in a p53/pRB-independent, but p21-dependent manner in liver cancer cell lines [45]. Unlike senescence induced by RBX1/ROC1 knockdown in H1299 and U87 cells in which p21 was not involved, we found that ROC1 knockdown-induced senescence is largely dependent on p21 in HepG2 and Huh7 liver cancer cells, since...
Cell senescence targeting to CRLs for cancer therapy

a) p21 is significant accumulated in response to RBX1/ROC1 knockdown and b) simultaneous knockdown of p21 remarkably abrogates RBX1/ROC1 silencing-induced senescence in these hepatic cancer cells [48]. This apparent discrepancy regarding the potential role of p21 in senescence induction is likely due to cell line-dependent accumulation of CRL/SCF substrates in response to RBX1/ROC1 knockdown.

Targeting oncogenic F-box protein Skp2

The selective degradation of proteins by Skp1-Cullin-F-box (SCF) E3 ligase, one of well-characterized family member of CRLs, is determined by F-box proteins as substrate receptors [49]. As a member of F-box proteins, SKP2 (S-phase kinase associated protein 2, also known as Fbx11) regulates the proteolysis of cyclin-dependent kinase inhibitors (CDKIs) and tumor suppressor proteins [50], and thus plays an oncogenic role in tumor formation and progression. Overexpression of Skp2 is frequently observed and associated with poor prognosis in many aggressive cancers, including acute myelogenous leukemia [51], non-small cell lung cancer [52], breast cancer [53] and nasopharyngeal carcinoma [54], indicating Skp2 as a prognostic marker and potential anticancer target. Previous studies showed that knockdown of Skp2 leads to accumulation of p27 and cell growth arrest of melanoma in vitro and in vivo [55], whereas enforced expression of Skp2 overcomes barrier of cell cycle arrest in hormone-dependent breast cancer cells and androgen-dependent prostate cancer cells [56].

Skp2 regulates cellular senescence by controlling the turnover of tumor suppressors, such as p27 (Figure 3). Loss of Skp2 accompanied by stabilization of p27 was detected in senescent human fibroblasts, suggesting that down-regulation of Skp2 triggers cellular senescence [57]. Knockdown of EWS-Fli1 (associated with oncogenesis of Ewing family tumors) initiated senescent phenotype, concomitant with the down-regulation of Skp2 and the accumulation of p27 protein, whereas silencing of p27 partially rescued senescence-like phenotype [58]. Moreover, in murine models, Skp2 depletion coupled with aberrant expression of oncogene and inactivation of tumor suppressor triggered senescence and tumor regression by up-regulating tumor suppressors p27, p21 and Atf4, although genetic depletion of Skp2 alone did not induce cellular senescence [59]. Interestingly, it was previously reported that Skp2 knockdown could also trigger apoptosis and/or autophagy [60-62] in cancer cell lines, which suggests potential crosstalk among these cellular responses and renders a new direction for future investigation.

Targeting cullin neddylation by NAE inhibitor MLN4924

Post-translational neddylation of cullin is a process of adding ubiquitin-like protein NEDD8 (neuronal precursor cell-expressed developmentally down-regulated protein 8) to cullins of CRLs, which is required for the activation of CRLs. During the process, NEDD8 is firstly activated by an E1 enzyme (Nedd8-activating enzyme, NAE), then transferred to specific E2 enzymes (UBC12 and UBE2), and finally conjugated to cullins by E3s [63-65]. Thus, small molecule inhibitors that specifically target to neddylation pathway, are likely to inactivate CRLs and alleviate potential therapeutic cytotoxicity compared to proteasome inhibitor bortezomib [66, 67].

A selective inhibitor of NAE, MLN4924, was recently discovered by Millennium Pharmaceuticals, Inc. via a high-throughput screening [68]. Structural analysis revealed that MLN4924 mimics to AMP and forms a NEDD8-MLN4924 adduct resembling to NEDD8-adenylate, which competitively blocks the active site of NAE and the conjugation of NEDD8 to CRLs [69]. By blocking cullin neddylation, MLN4924 inactivates CRLs and causes the accumulation of CRL substrates, which subsequently triggers multiple cell death pathways in cancer cells. Because of its remarkable anticancer efficacy and well-tolerance in preclinical studies, MLN4924 has been advanced into phase I clinical trials as a promising investigational anticancer agent.

Previous studies have showed that MLN4924 induces cell cycle disturbance and apoptosis by triggering DNA damage response in HCT116 colon cancer cells [68, 70]. Besides, MLN4924 induces the inhibition of NF-κB pathway in acute myeloid leukemia (AML) [71] and diffuse large B-cell lymphoma [72], triggering apoptosis.
Recent studies from our and other’s groups demonstrated that MLN4924 also triggers cell senescence in diverse cancer cells (such as colon cancer HCT116, lung cancer H1299 and glioma U87 cells), which contributes to MLN4924-induced growth suppression [70, 73] (Figure 3). Further study revealed that MLN4924 induced-senescence is dependent on p21, a known substrate of CRLs, but not p16/pRB and p53 [73]. Furthermore, senescence induced by MLN4924, even at a low dose (0.1μM), is irreversible [73]. These findings reveal a novel mechanism of MLN4924 action and show that MLN4924 could be further developed as an effective anticancer agent by inducing irreversible senescence.

Conclusion and perspectives

Induction of senescence by inactivating CRLs as a mechanism of growth suppression has a significant application potential for cancer therapies. Firstly, senescence response represents a general phenomenon to overall inactivation of CRLs via both genetic (such as by knock-down of RBX1/ROC1) and pharmaceutical (such as by MLN4924) approaches, which may be of more significance to apoptosis-resistant cancer cells. Secondly, senescence induced by targeting CRLs is p53-independent, suggesting that all human cancers can be treated by CRLs inhibitors regardless of p53 status. Thirdly, senescence response induced by CRLs inhibitors at low dose seems to be irreversible, which makes it possible to use low doses of CRL-targeted drugs to achieve a greater therapeutic index.

Further investigation in the following directions should be carried out to further develop senescence as a novel strategy for CRLs-targeted cancer therapy. Firstly, CRLs contain hundreds of subunits of which different combinations can form different functional E3 ligases targeting specific sets of substrates for degradation. It is largely unknown how many CRLs subunits and substrates contribute to carcinogenesis and inhibition of these subunits could trigger cell senescence [74]. By using comprehensive approaches, including siRNA screening [75], proteomics [76, 77], and global protein stability (GPS) combined with SILAC [78], may solve the issue. Moreover, the molecular basis for senescence induction by targeting to different components of CRLs in different cell lines remains to be fully elucidated. In addition, a growing number of studies emerge to demonstrate that inactivation of CRLs may trigger several cellular responses, including senescence, apoptosis and autophagy [48, 79, 80] in cancer cells (Figure 3). The elucidation of cross-talk among these phenotypes and potential relevance of other cellular responses to senescence induction will definitely facilitate the development of senescence as a mechanism of growth suppression.

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Reference

Cell senescence targeting to CRLs for cancer therapy

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Cell senescence targeting to CRLs for cancer therapy


