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著者	Kitajima Shunsuke, Takahashi Chiaki
著者別表示	北嶋 俊輔, 橋 智聡
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Solicited Review Article

Intersection of retinoblastoma tumor suppressor function, stem cells, metabolism, and inflammation

Shunsuke Kitajima¹ and Chiaki Takahashi² ¹Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA; ²Division of Oncology and Molecular Biology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa, Japan

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Correspondence

Chiaki Takahashi, Division of Oncology and Molecular Biology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa, 920-1192, Japan.

Tel.: +81-76-264-6750; Fax: +81-76-234-4521;

E-mail: chtakaha@staff.kanazawa-u.ac.jp

and

Shunsuke Kitajima, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, 02215, USA.

Tel.: +1-617-632-6049; Fax: +1-617-632-5786;

E-mail: shunsuke_kitajima@dfci.harvard.edu

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The RB tumor suppressor protein forms a transcriptional repression complex with the E2F family of transcription factors and various chromatin modifiers, and thereby negatively regulates G₁/S transition during the cell cycle through the suppression of E2F target genes such as *CCNA1* and *CCNE1*. In response to growth stimuli, the cyclin D–CDK4/6 complex phosphorylates RB, releasing E2F transcription factors and facilitating the G₁/S transition. As almost all human cancer cells carry aberrations in components of the RB pathway, including *INK4A*, *CCND*, *CDK4/6*, *RB*, and *E2Fs*, genetic or functional inactivation of the RB pathway seems to be indispensable for dysregulated proliferation in cancer cells.^(1,2)

Retinoblastoma mutations are found in retinoblastoma, osteosarcoma, and SCLC at a high frequency, but less frequently in more common types of cancer.^(2–4) Mutations in *INK4A* are frequently detected in pancreatic cancer and NSCLC. *CCND* amplification is often seen in breast cancer cells.^(2–4) Although, in part, this may be related to different mutational mechanisms in distinct tissue types, these observations also suggest that the RB pathway is not strictly linear,

The Retinoblastoma (RB) tumor suppressor regulates G₁/S transition during cell cycle progression by modulating the activity of E2F transcription factors. The RB pathway plays a central role in the suppression of most cancers, and RB mutation was initially discovered by virtue of its role in tumor initiation. However, as cancer genome sequencing has evolved to profile more advanced and treatment-resistant cancers, it has become increasingly clear that, in the majority of cancers, somatic RB inactivation occurs during tumor progression. Furthermore, despite the presence of deregulation of cell cycle control due to an *INK4A* deletion, additional *CCND* amplification and/or other mutations in the RB pathway, mutation or deletion of the *RB* gene is often observed during cancer progression. Of note, RB inactivation during cancer progression not only facilitates G₁/S transition but also enhances some characteristics of malignancy, including altered drug sensitivity and a return to the undifferentiated state. Recently, we reported that RB inactivation enhances pro-inflammatory signaling through stimulation of the interleukin-6/STAT3 pathway, which directly promotes various malignant features of cancer cells. In this review, we highlight the consequences of RB inactivation during cancer progression, and discuss the biological and pathological significance of the interaction between RB and pro-inflammatory signaling.

and that loss of function due to genetic ablation of the *RB* gene and loss of E2F binding activity due to hyperphosphorylation of the RB protein are not completely synonymous.

The RB protein shows E2F-independent functions through binding to other nuclear or extra-nuclear partners. For example, RB cooperates with transcription factors such as MYOD or RUNX2 to regulate cell differentiation in an E2F-independent manner.⁽⁵⁾ The RB protein suppresses the degradation of p27 by SKP2 through direct binding to SKP2; this allows RB to attenuate cell cycle progression in an E2F-independent manner.^(6,7) The RB protein is located in the mitochondrial fraction, where it promotes BAX-dependent apoptosis.^(8,9) Hyperphosphorylated RB is not simply inactivated, but rather contributes to suppression of the mTORC2-AKT pathway, leading to enhanced sensitivity to chemotherapy.⁽¹⁰⁾ In addition to genes involved in cell cycle control, the RB–E2F complex suppresses a number of genes involved in pluripotency, cellular metabolism, innate immunity, and cytokine signaling (Fig. 1).^(11–15) The RB–E2F complex colocalizes with EZH2 at intronic and intergenic regions in the genome, and mediates silencing of repetitive DNA sequences.⁽¹⁶⁾ Conversely, in

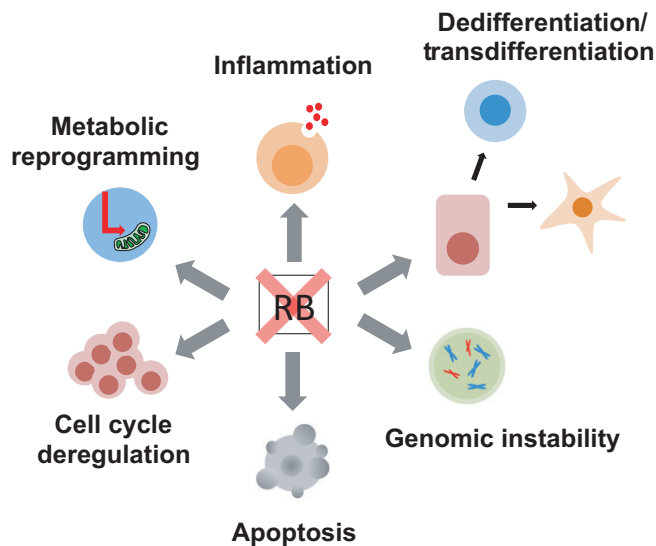


Fig. 1. Inactivation of RB during cancer progression results in multiple malignant phenotypes.

particular contexts, the RB–E2F complex positively regulates gene transcription by forming a complex with transcriptional activators.⁽³⁾ To date, more than 300 proteins have been identified as possible binding partners of RB. The variability in these binding partners could explain the multifunctional aspects of the RB protein.

Stem cell-like features induced by RB inactivation. In the past decade, it has been proposed that tissue stem/progenitor cells with multipotency and self-renewing activity could be the cells of origin for various cancers.⁽¹³⁾ To maintain a normal balance between differentiation and self-renewal in tissue stem cells, RB family proteins, including p107 and p130, strictly regulate the G₁/S transition. In general, depletion of all RB family proteins in tissue stem/progenitor cells causes defects in differentiation potency and promotes self-renewing activity, leading to stem cell expansion and tumor initiation.⁽¹³⁾ However, post-mitotic cells, that is, those that have completed terminal differentiation, could also be cells of origin for cancer. Depletion of RB family proteins in post-mitotic cells induces cell cycle re-entry and dedifferentiation, and even leads to tumor initiation in some contexts. For example, MEFs in which all RB family proteins are inactivated become resistant to G₁ arrest, and acquire cell characteristics similar to those of stem cells, such as increased sphere-forming activity and expression of pluripotent genes.^(17–19) In addition, RB depletion on an *ARF* null background induces cell cycle re-entry and dedifferentiation in post-mitotic muscle cells.⁽²⁰⁾ Moreover, an RB family triple KO in post-mitotic horizontal interneurons of the retina induces metastatic retinoblastoma.⁽²¹⁾ As expected from these findings, RB inactivation contributes to the generation of inducible pluripotent stem cells from human fibroblasts.⁽²²⁾ The RB–E2F1 complex directly suppresses the expression of pluripotent factors such as *POU5F1* and *SOX2* by binding directly to their regulatory regions with recruiting repressive chromatin modifiers, which consequently antagonizes inducible pluripotent stem cell induction.⁽²³⁾ Thus, in addition to deregulation of the cell cycle, RB inactivation in tissue stem/progenitor cells and post-mitotic cells contributes to tumor formation by promoting self-renewal activity and dedifferentiation.

It should be noted that studies using genetically engineered mouse models have shown that a single *RB* gene deletion in

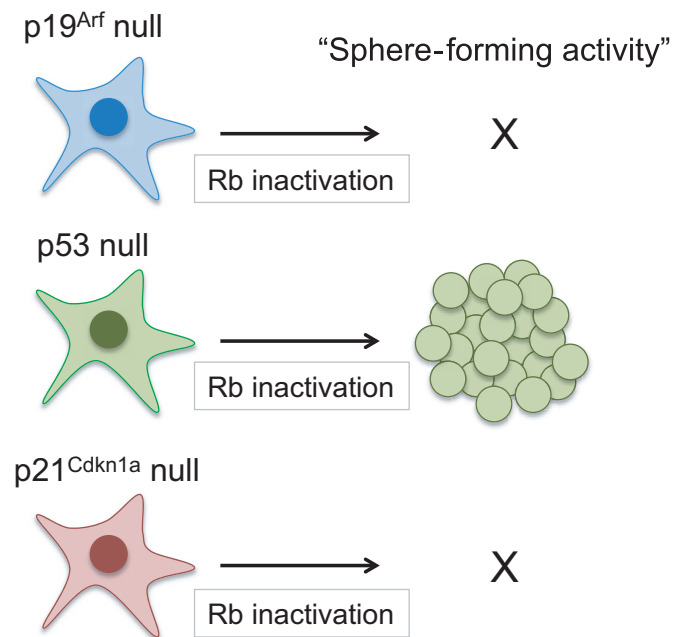


Fig. 2. Inactivation of Rb increases the frequency of sphere-forming activity in *p53*-null background but not in *Arf* or *Cdkn1a*-null backgrounds.

stem/progenitor cells and post-mitotic cells is not sufficient to initiate tumor formation. One of the underlying mechanisms is the induction of apoptosis and/or cell cycle arrest through feedback activation of the p53 pathway, which could be an E2F-dependent or -independent function. For example, suppression of RB function by an SV40 T antigen mutant (T₁₂₁) induces p53-dependent cell death and cell cycle arrest in human embryonic stem cells, even though RB is strongly phosphorylated in these cells.⁽²⁴⁾ Another possible mechanism is functional compensation by p107 and p130. In Rb heterozygous mice, p130-dependent cellular senescence is induced in C cells following *Rb* loss of heterozygosity.^(25,26)

Simultaneous mutation in the *p53* and *RB* genes is frequently observed in human cancer cells. A number of studies using genetically engineered mouse models have reported that RB inactivation in a *p53*-null background results in tumors that are less differentiated compared to a WT *p53* background.^(27,28) *Rb* heterozygous mice develop low-grade thyroid medullary cancer derived from C cells as a consequence of biallelic loss of *Rb*. The *p53*-null background allowed this C cell tumor to develop dedifferentiated phenotypes depicted by lower expression of calcitonin. This was not seen in backgrounds lacking other p53 pathway genes such as *Arf* and *Cdkn1a*, even though thyroid tumors from all of these mice showed similar levels of Ki67 positivity.⁽²⁹⁾ Similarly, RB depletion in *p53* KO MEFs, but not in *Arf* and *Cdkn1a* KO MEFs, induced sphere-forming activity in 3-D culture supplemented with limited growth factors, an indicator of stem-like activity, despite the fact that these cells showed nearly identical proliferation rates in 2-D culture (Fig. 2).⁽²⁹⁾ Depletion of RB in *p53* KO MEFs, however, did not induce tumorigenicity in immunodeficient mice, suggesting that the acquisition of stem-like activity caused by Rb/p53 double inactivation is independent of the transformation from normal to cancer cells. Taken together, these findings show that the genetic interaction between Rb and p53 is a critical determinant of the undifferentiated state in both normal and tumor cells.

Inactivation of RB promotes pro-inflammatory signaling. It is becoming increasingly clear that in lung, prostate, and breast cancer the acquisition of therapy resistance associated with dedifferentiation, transdifferentiation, and hormone-independent growth is tightly correlated with aberration of the *RB* gene during cancer progression. For example, acquisition of drug resistance to EGFR-targeted therapy in EGFR mutant NSCLC is achieved by transdifferentiation to SCLC, which occurred due to loss of the *RB* gene.⁽³⁰⁾ In prostate cancer, simultaneous inactivation of RB and p53 in luminal-type cancer cells leads to increased lineage plasticity and induces the formation of AR-independent basal-like cells.^(31,32) Another study indicated that E2F1 activation following RB inactivation directly enhances AR expression, thereby promoting hormone-independent growth.⁽³³⁾ In breast cancer, RB inactivation induces epithelial–mesenchymal transition and hormone-independent growth in ER-positive luminal-type cancer cells.^(34,35) We have reported that RB inactivation in luminal-type cancer cells induces stem cell-like features associated with an enhanced secretion of pro-inflammatory cytokines, including IL-6, resulting in hormone-independent growth.⁽³⁶⁾

Estrogen receptor-negative breast cancers generally carry *RB* mutations at a higher frequency, contain a stem cell-like fraction, and show a higher secretion of pro-inflammatory cytokines compared to other subtypes of breast cancer.^(37–39) Previous studies have shown that pro-inflammatory cytokines, and the major downstream STAT3 and nuclear factor- κ B signaling pathways in breast cancer, are essential for emergence and maintenance of the stem cell-like fraction, and for acquisition of drug resistance. For example, single supplementation of IL-6 or IL-8 in luminal-type breast cancer cells induces mammosphere-forming activity, which is often used as a marker activity of breast cancer stem cells.^(40–43) Similarly, another study indicated that activation of the IL-6/STAT3 pathway in luminal-type breast cancer cells induces hormone-independent growth.⁽⁴⁴⁾ Activation of the IL-6/STAT3 pathway contributes to the acquisition of drug resistance in various other types of cancer, such as lung and liver, in addition to breast cancer.^(45–47)

It has been proposed that RB directly regulates pro-inflammatory signaling. Activation of E2F following RB inactivation directly induces prostaglandin-endoperoxide synthase 2/COX2 expression in basal-like breast cancer cells.⁽⁴⁸⁾ In addition, RB depletion induces IL-6 secretion in the *Cdkn1a*-deficient epidermis.⁽⁴⁹⁾ Indeed, E2F binding sites have been reported to be localized in the gene regulatory region of some cytokines and their receptors, and the RB–E2F complex directly suppresses cytokine expression, including chemokine (C-X-C motif) ligand 1 and 2 and IL-8.⁽⁵⁰⁾ In contrast, we recently reported an inverse correlation between RB and IL-6 expression in breast cancer, as mentioned above,⁽³⁶⁾ but this is not due to direct E2F binding to the IL-6 promoter (discussed below).

Based on these findings, it has been assumed that activation of pro-inflammatory signaling by RB inactivation directly contributes to the promotion of self-renewal and the undifferentiated state. Indeed, we showed that hormone-independent growth, sphere-forming activity, and tumor initiation activity induced by RB depletion in MCF-7 cells, a luminal-type breast cancer cell line, is antagonized by suppression of IL-6 or STAT3.⁽³⁶⁾ These results indicate that RB inactivation contributes to the acquisition of these malignant features, not only through “cell intrinsic mechanisms” such as defects in cell cycle control, but also through “cell extrinsic mechanisms” such as hypersecretion of pro-inflammatory cytokines. As the humanized anti-IL-6 receptor antibody, tocilizumab, shows a

specific therapeutic effect on RB-depleted MCF-7 cells, RB inactivation-induced IL-6 hypersecretion may be a potential therapeutic target.

Regulatory feedback mechanisms for fine-tuning intracellular ROS in RB-inactivated cells. Many groups have reported that RB and E2Fs control DNA replication and mitosis as well as the G₁/S transition during the cell cycle. Inactivation of RB therefore induces genomic instability. For example, RB–E2Fs form a complex with condensin II, which is essential for normal chromosome condensation and subsequent DNA replication.^(51,52) Inactivation of RB therefore leads to abnormal chromosome segregation. In addition, excessive expression of Mad2, a spindle-assembly checkpoint protein, which is directly induced by E2F, causes chromosome instability.⁽⁵³⁾ Furthermore, accelerated cell cycle progression by RB inactivation results in over-consumption of nucleotides, leading to DNA damage.⁽⁵⁴⁾ In fact, according to a comprehensive genomic analysis of human tumors, genomic instability is thought to be higher in tumors with mutations in the RB pathway.^(51,55)

Inactivation of RB is also known to cause oxidative stress by increasing ROS production. This could be related to aberrant autophagy in RB-inactivated cells,⁽⁵⁶⁾ because proper degradation of mitochondria by autophagy (i.e. mitophagy) prevents the accumulation of dysfunctional mitochondria, one of the main sources of intracellular ROS production. In myoblast cells, defects in autophagy induced by RB inactivation lead to an accumulation of dysfunctional mitochondria, which in turn inhibits the differentiation of myoblasts to myotubes.⁽⁵⁷⁾ In contrast, mitochondrial abnormalities in myoblast cells due to RB inactivation have been shown to depend on deregulation of KDM5A, a binding partner of RB.⁽⁵⁸⁾ Comprehensive proteomic analysis of RB-inactivated cells identified functional abnormalities in the mitochondria.⁽⁵⁹⁾ In senescent cells, RB directly promotes mitochondrial oxidative phosphorylation through activation of metabolic genes.⁽⁶⁰⁾ From these findings, it is assumed that RB-inactivated cells produce high levels of mitochondrial superoxide due to abnormal electron transfer to oxygen as a result of mitochondrial dysfunction. Conversely, enhanced mitochondrial activity also increases ROS production associated with elevated oxygen consumption. The RB–E2F complex is known to directly suppress the expression of oxidative metabolism-related enzymes and mitochondrial protein translation genes.^(61,62) Disruption of this complex therefore seems to provoke oxidative metabolism, such as the tricarboxylic acid cycle and FAO. Indeed, we reported that RB depletion in MCF-7 cells promotes mitochondrial superoxide production with elevated oxygen consumption and FAO.⁽³⁶⁾ Taken together, these results suggest that RB-inactivated cancer cells are exposed to more DNA damage and oxidative stress than RB-intact cancer cells, even though cancer cells in general already show higher levels of DNA damage and ROS production compared to normal cells.

Accumulation of DNA damage and oxidative stress induces apoptosis and cellular senescence, although lower levels of ROS production act as a growth signal. Thus, RB-inactivated cancer cells tend to undergo cell death in response to DNA-damaging agents and excessive ROS production.^(35,63) In order for RB-inactivated cancer cells to display a high proliferation rate in the presence of high ROS production, activation of antioxidant pathways seems to be essential to maintain redox balance in cells at a non-toxic level. A number of reports have indicated that activation of antioxidative pathways during cancer progression contributes to the acquisition of stem-like activity, including drug resistance and tumor-initiating activity.

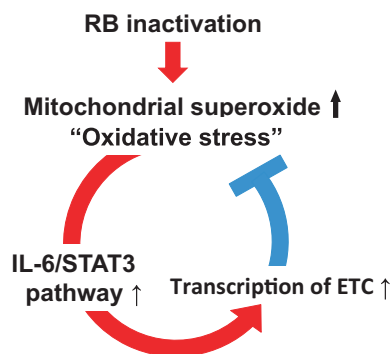


Fig. 3. Regulatory feedback loop between interleukin-6 (IL-6) secretion and mitochondrial superoxide production for fine-tuning intracellular reactive oxygen species (ROS) to non-toxic levels in RB-inactivated cells. Inactivation of RB induces oxidative stress through enhanced mitochondrial ROS production, leading to hypersecretion of IL-6. In contrast, IL-6/signal transducer and activator of transcription 3 (STAT3) activation enhances the function of respiratory chain complex in mitochondria and prevents deregulated ROS production.

For example, antioxidative pathways are highly activated in the stem cell-like fraction of cancer cells compared to other non-tumorigenic fractions, leading to lower levels of ROS and resistance to various cellular stresses.⁽⁶⁴⁾ Moreover, CD44 and NRF2 directly induce cancer stem-like activity through activation of antioxidative and detoxification signaling pathways.^(65,66)

Interleukin-6 hypersecretion induced by RB inactivation provides cells with an antioxidative function that neutralizes mitochondrial superoxide (Fig. 3). It has been reported that the promoter region of the *IL-6* gene contains an antioxidant responsive element, which is a binding sequence for NRF2, a master regulator of the transcriptional response to oxidative stress, which itself can upregulate IL-6 secretion.^(67,68) We showed that treatment of RB-depleted MCF-7 cells with a neutralizing antibody to IL-6 induced further mitochondrial superoxide production and subsequent cell death.⁽³⁶⁾ In contrast, treatment with recombinant IL-6 antagonized mitochondrial superoxide production, and promoted sphere-forming activity and hormone-independent growth.⁽³⁶⁾ These findings suggest

that the induction of stem cell-like activity induced by RB inactivation as described above depends, at least in part, on the antioxidative function of IL-6.

Metabolic reprogramming associated with RB inactivation is thought to promote intracellular antioxidant activity. For example, RB-inactivated cells show an increased dependency on glutamine consumption, which is an amino acid essential for glutathione synthesis, a representative intracellular antioxidant.^(69,70) Fatty acid oxidation also provides an antioxidative metabolic pathway by promoting NADPH production, which is another intracellular antioxidant.⁽⁷¹⁾ In fact, it has been shown that FAO is essential for maintaining self-renewal activity in both hematopoietic stem cells and cancer stem-like cells, and FAO inhibition results in a profound decrease in NADPH levels, an accumulation of ROS, and subsequent cell death.^(72,73) Similarly, we reported that RB inactivation in MCF-7 cells activates FAO, and treatment with an FAO inhibitor increases ROS accumulation.⁽³⁶⁾ Collectively, IL-6 hypersecretion and metabolic reprogramming, including FAO activation, associated with RB inactivation play key roles in the regulation of redox balance, which leads to cancer progression.

Transcriptional and post-transcriptional regulation of pro-inflammatory cytokines by RB. While RB cooperates with various binding partners to directly regulate the expression of various targets as mentioned above, the expression of some pro-inflammatory cytokines, including IL-6, is induced by cellular stresses such as oxidative stress and DNA damage associated with RB inactivation. We showed that increased mitochondrial superoxide production due to RB inactivation promotes IL-6 secretion through activation of the JNK pathway in MCF-7 cells (Fig. 4).⁽³⁶⁾ Given that IL-6 possesses an antioxidative function, hypersecretion of IL-6 induced by RB inactivation can be considered part of a feedback mechanism to maintain the intracellular redox balance within a non-toxic range.

In addition, we have shown that RB regulates the expression of many genes, including pro-inflammatory cytokines, in a miRNA-dependent manner. MicroRNAs are non-coding RNAs, consisting of 20–25 bases, that bind to the 3'-untranslated region of mRNA and regulate gene expression through degradation and/or translational repression of mRNA.⁽⁷⁴⁾ From a comprehensive analysis of miRNA expression, we found that

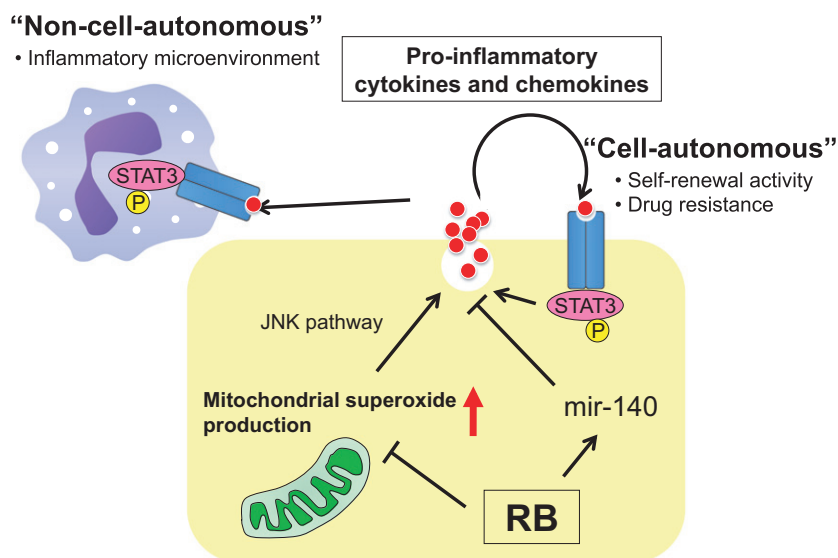


Fig. 4. Schematic presentation of the summary in this review. Inactivation of RB induces mitochondrial reactive oxygen species production and subsequent activation of pro-inflammatory and the signal transducer and activator of transcription 3 (STAT3) loop in a JNK pathway-dependent manner. Hypersecretion of interleukin-6 is also post-transcriptionally promoted by downregulation of microRNA-140 (mir-140) following RB inactivation. Hypersecretion of pro-inflammatory cytokines might contribute to establishment of the inflammatory tumor microenvironment.

RB inactivation significantly reduced mir-140 expression in mouse sarcoma cells and MCF-7 cells.⁽⁷⁵⁾ MicroRNA-140 has been implicated in the suppression of hepatocellular carcinoma, NSCLC, colon cancer, breast cancer, and ovarian cancer through inhibition of growth factor signaling.^(76–80) Although the molecular mechanism that underlies the regulation of mir-140 by RB is still unclear, mir-140 downregulation following RB inactivation upregulates the expression of multiple pro-inflammatory cytokines, including IL-6, and other growth and angiogenic factors that have a mir-140 binding sequence in the 3'-untranslated region (Fig. 4).⁽⁷⁵⁾

Conclusion

Inactivation of RB is frequently observed during cancer progression. Cell cycle deregulation is one of the most important features associated with RB inactivation, however, RB is a multifunction protein interacting with wide varieties of binding partners and has cell cycle-independent functions. Therefore, RB inactivation during cancer progression induces various malignant phenotypes, including undifferentiated state, drug resistance, and cytokine hypersecretion, in addition to cell cycle deregulation. In this review, we especially focused on stem-like features induced by RB inactivation associated with hypersecretion of pro-inflammatory cytokines such as IL-6. This phenomenon is context-dependent to some extent, and the molecular mechanisms underlying the control of their expression and its impact on the tumor microenvironment remains incompletely characterized. By clarifying these unsolved

questions, we could propose not only novel aspects of RB function but also a new window for development of targeted therapy to RB-inactivated cancer cells.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AR	androgen receptor
C cell	thyroid calcitonin-producing cell
E2F	E2 factor
ER	estrogen receptor
FAO	fatty acid oxidation
IL	interleukin
MEF	mouse embryonic fibroblast
miRNA	microRNA
NRF2	nuclear factor erythroid-related factor 2
NSCLC	non-small-cell lung cancer
RB	retinoblastoma
ROS	reactive oxygen species
SCLC	small-cell lung cancer
SKP2	S-phase kinase-associated protein 2
STAT	signal transducer and activator of transcription

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