

## Retinoblastoma tumor suppressor functions shared by stem cell and cancer cell strategies

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### Abstract

Carcinogenic transformation of somatic cells resembles nuclear reprogramming toward the generation of pluripotent stem cells. These events share eternal escape from cellular senescence, continuous self-renewal in limited but certain population of cells, and refractoriness to terminal differentiation while maintaining the potential to differentiate into cells of one or multiple lineages. As represented by several oncogenes those appeared to be first keys to pluripotency, carcinogenesis and nuclear reprogramming seem to share a number of core mechanisms. The retinoblastoma tumor suppressor product retinoblastoma (RB) seems to be critically involved in both events in highly complicated manners. However, disentangling such complicated interactions has enabled us to better understand how stem cell strategies are shared by cancer cells. This review covers recent findings on RB functions related to stem cells and stem cell-like behaviors of cancer cells.

**Key words:** Stem cells; Cancer; Retinoblastoma; Cancer stem cells

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**Core tip:** Carcinogenic transformation of somatic cells resembles nuclear reprogramming toward the generation of pluripotent stem cells. The retinoblastoma tumor suppressor product retinoblastoma (RB) seems to be critically involved in both events in highly complicated manners. This review covers recent findings on RB functions related to stem cells and stem cell-like behaviors of cancer cells.

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## INTRODUCTION

The cell-of-origin in various types of cancers has been one of the most important areas of research in modern cancer biology, because deep understanding of this can help design of future cancer therapies<sup>[1-4]</sup>. Many studies have indicated that distinct cells-of-origin give rise to distinct features of cancers, and can often predict the prognosis of patients<sup>[5,6]</sup>. However, the debate on cell-of-origin of each cancer is often controversial, because cancer phenotypes do not stereotypically reflect phenotypes of their true cells-of-origin. This is at least partially due to the high developmental plasticity that is acquired after tumor initiation and during tumor progression.

The cancer stem cell hypothesis proposes a model in which like in normal tissues, cancer cells obey the hierarchy of development where stem cell-like cancer cells are placed at the top. This hypothesis does not always explain the cell-of-origin of a specific cancer; however, it has led us to the idea that cancer cells prefer to employ stem cell strategies in order to maintain tumor-initiating clones<sup>[7,8]</sup>. This theory has been reinforced by many findings, including the switchable cell fates of cancer cells, the inseparable relationship between pluripotency and teratogenicity, the requirement for oncogenic elements for the generation of induced pluripotent stem (iPS) cells, and the oncogenic activities of many embryonic genes<sup>[9,10]</sup>.

Sophisticated gene-engineered and tumor-grafted mouse models have been used to trace the cells-of-origin for specific cancers, for instance in case of prostate adenocarcinomas, to basal or luminal cells<sup>[2]</sup>. During these investigations, the existence of unexpectedly high levels of developmental plasticity became apparent, when comparing the cell-of-origin to its resultant tumor. In addition, researchers found switchable cell fates in cultured cancer cell lines that were induced by artificially altering the status of genes, such as tumor suppressor genes.

Although the significance of epigenetic alterations is still unclear, carcinogenesis results from the step-wise accumulation of readable lesions in the genome<sup>[11]</sup>. Given the gain of developmental plasticity, even if it is transient, is essential for carcinogenesis, then the next question should be as follows: Which genes are mechanistically involved in this gain of plasticity?

This question is not satisfactorily answered yet, but a part of answer may be informed by the switchable cell fate of cancer cells. The retinoblastoma tumor suppressor gene (RB) is closely implicated in the change in developmental phenotypes of many types of cancers, including lung cancer, breast cancer, prostate cancer, osteosarcoma, and soft tissue sarcoma. This phenomenon has been attributed to the physical or genetic interaction between the RB gene product (RB) and tissue-specific transcription factors<sup>[12]</sup>. However, emerging evidence indicates that inactivation of RB in particular genetic backgrounds or in certain contexts can lead cells to an undifferentiated state that resembles

that of most immature cells, such as embryonic stem cells<sup>[13-15]</sup>.

We also know that the targeted inactivation of RB, in combination with p53, provides strong experimental tools to determine the cell-of-origin of various types of cancers<sup>[16-18]</sup>. Indeed, these two tumor suppressor pathways are the most commonly inactivated in human cancers, and simultaneous inactivation is sufficient to induce cancers from various types of somatic cells<sup>[19]</sup>. Therefore, one of the optimal ways to understand RB function in the context of full carcinogenesis would be to determine RB functions in a p53-deficient genetic background.

This review briefly summarizes the well-established functions of RB in mammalian cells, presents cross-species evidence for the possible link between RB function and the control of stem cell activities, and describes findings that may explain the molecular mechanisms underlying this link. The RB locus was identified more than a quarter century ago; however, researchers are still providing new wineskins to new wines.

## CELL CYCLE-DEPENDENT AND INDEPENDENT FUNCTIONS OF RB

### *Cell cycle control by RB*

The RB gene was first identified as a tumor suppressor in the childhood malignancies retinoblastoma and osteosarcoma<sup>[20]</sup>. Somatic RB loss typically causes unilateral retinoblastoma with no obvious risk for other types of malignancies. However, germline RB mutation often results in bilateral retinoblastoma, and carriers are at very high risk of various types of cancer over their lifetimes<sup>[21]</sup>. Therefore, researchers proposed that RB might be involved in the core mechanisms of tumorigenesis. Indeed, unveiling the functions of RB in controlling cell cycle progression provided a big breakthrough to the field of cancer research<sup>[22]</sup>.

A primary RB function in cell cycle control is exerted at the G<sub>1</sub>/S transition. RB undergoes dephosphorylation at the end of the M phase with the aid of protein phosphatases (PPs) and resumes its phosphorylated state during the G<sub>1</sub> phase by the action of cyclin D/cyclin-dependent kinase (CDK) 4 or 6 complexes<sup>[23]</sup>. Most of cellular mitogenic signals converge on the transcriptional upregulation of D-type cyclins. This could be one reason that cells in the G<sub>1</sub> phase are most vulnerable to extracellular growth stimuli<sup>[23,24]</sup>.

Phosphorylation of RB alters its three dimensional (3D) structure. This results primarily in the loss of binding affinity to E2F family transcription factors<sup>[25,26]</sup>. Among nine identified E2F family members (E2F1, 2, 3A, 3B, 4-8), RB was shown to bind to at least E2F1, 2, and 3A. Each of these three family members is able to positively transactivate genes, including cyclin E<sup>[27]</sup>. Upregulation of cyclin E in cooperation with CDK2 further promotes RB phosphorylation. This enables cells to cross the boundary between G<sub>1</sub> and S. Further, with the aid of cyclin A, RB attains the maximal level of phosphorylation before cells enter the M phase<sup>[23]</sup>. In addition, when bound to

hypophosphorylated RB, E2Fs form a transcriptional repressor complex that recruits histone deacetylase (HDAC) to epigenetically silence gene transcription<sup>[28]</sup>. Therefore, the phosphorylation status of RB dramatically changes the expression of E2F-targeted genes. The function of RB in restricting the G<sub>1</sub>/S transition is also mediated by its binding to SKP2, which destabilizes p27<sup>KIP1</sup> by enhancing the ubiquitin-proteasome system when freed from phosphorylated RB<sup>[29,30]</sup>. This represents one of E2F-independent functions of RB in the control of cell cycle progression.

RB plays pivotal roles also in M phase, which is most typically represented by the impact of RB inactivation on the chromosomal instability (CIN). E2Fs target a number of M phase genes including MAD2 which functions by inhibiting the anaphase promoting complex/cyclosome (APC/C)-cell division cycle 20 (CDC20) complex. This complex regulates spindle assembly<sup>[31]</sup>. RB also controls the M phase by directly binding to cohesin and condensin II, two critical regulators of centromeric functions<sup>[32]</sup>.

"How many total RB functions are cell cycle-dependent?" is an intriguing question. RB mutants found in partially penetrant retinoblastomas (low grade retinoblastomas with limited genetic inheritance) or retinomas that failed to inhibit the cell cycle but retained the ability to promote terminal differentiation suggested that RB functions in cell cycle control and differentiation might be distinct<sup>[33]</sup>. In addition, phenotypic analyses of *Rb*-deficient mice simultaneously lacking an *E2F* family member allowed at least partial discrimination of the E2F-dependent function from the E2F-independent function<sup>[34]</sup>. However, since E2Fs target both cell cycle-related and cell cycle-unrelated genes, discrimination of cell cycle-dependent functions from cell cycle-independent functions of RB based on the E2F-dependency is difficult.

Artificial and acute alteration of RB status in a wild type genetic background often greatly affects the cell cycle. For instance, it induces cell cycle exit (quiescence or cellular senescence), or inversely cell cycle re-entry<sup>[35,36]</sup>. This change in the cell cycle control is highly drastic, thus can mask cell cycle-independent phenotypes associated with altered RB activity. However, based on the experience of analyzing *Rb*-deficient mice, our group discovered several genetic backgrounds that allow mice or cells to exhibit cell cycle-independent phenotypes following RB inactivation<sup>[14,15,37,38]</sup>. The cell cycle-independent phenotypes include gain of undifferentiated phenotypes and altered chemo-resistance<sup>[15]</sup>. Therefore, we thought that control of the undifferentiated state of cells might represent at least a part of the cell cycle-independent functions of RB.

### Cell cycle-independent functions of RB

Whole genome sequencing studies have revealed that the *RB* loci undergoes the fourth most frequent loss-of-heterozygosity (LOH) found in whole human tumors, following *CDK42A*, *PTEN*, and *SMAD4*. Thus, *RB* mutations definitely can be "driver mutations"<sup>[39]</sup>. However, the type

of tumors in which *RB* mutations occur at their initiation is highly limited, including only retinoblastomas, small cell lung cancers (SCLC), osteosarcomas, and familial melanoma. In other types, in the vast majority of tumors, RB functions are largely maintained during initiation but these functions typically collapse while tumors undergo malignant progression<sup>[40]</sup>.

The question of why RB mutation is rare in the majority of cancers has yielded many interesting answers. Lack of RB in many cell types has already been linked to apoptosis through E2Fs, ARF, and p53<sup>[41]</sup>. In addition, RB residing in mitochondria directly interacts with Bax, and thus regulates apoptosis in a completely E2F-independent and cell cycle-independent manner<sup>[42,43]</sup>. These pathways represent a disadvantage in carcinogenesis upon RB inactivation when it occurs at early steps. The 3T3 cells lacking *Rb* are less susceptible to Ras-transformation, indicating that preservation of RB functions in the interaction of mitogenic signals and the cell cycle might be important for tumor initiation<sup>[44]</sup>.

Our group demonstrated that *Rb*-heterozygous mice generate adenomas or low-grade adenocarcinomas derived from calcitonin-producing cells (C cells) of neuro endocrine origin that exhibit whole evidence of DNA damage response and cellular senescence. However, the genetic background of a homozygous lack of *N-ras* allowed these *Rb*-deficient C cell tumors to progress to highly invasive and metastatic adenocarcinomas<sup>[14,37]</sup>. RB appeared to regulate isoprenylation of the N-Ras protein. Isoprenylation (farnesyl moiety-transfer and geranylgeranyl moiety-transfer) is the chemical reaction that is essential for the initial maturation of this protein. RB loss causes intermediate level upregulation of N-Ras under particular culture conditions, which induces a DNA damage response and subsequently cellular senescence, thus antagonizing full carcinogenesis in a manner similar to oncogene-induced senescence (OIS). The mechanism whereby RB controls N-Ras isoprenylation involves E2F-dependent regulation of sterol regulatory element binding protein (SREBP) transcription factors and direct drive by E2Fs in some of mevalonate (MVA) pathway genes<sup>[14]</sup>. This study indicated a case that RB influences intra-cellular signaling primarily by controlling metabolic pathways. Many reports, including ours, have directly implicated RB in the control of cellular metabolism<sup>[36,45,46]</sup>. This may indicate that RB is simultaneously involved in the control of the cell cycle and metabolic regulation. This may further explain why cell cycle progression and cellular metabolism are tightly coupled. Cells never diminish their volume after rounds of cell division. This is because cells very strictly double biomass up to the time of mitosis, just as they strictly conserve genome size. From this point of view, cellular metabolism for biomass synthesis would not be passively controlled by the demands of cell cycle progression; rather, they are both actively and presumably simultaneously regulated by a common mechanism.

The outcomes of RB inactivation during malignant progression entail not only facilitated G<sub>1</sub>/S transition, but

also many events critical for malignant cell behaviors. These include increased cell motility, angiogenesis, inflammatory response, metabolic rewiring, gain of undifferentiated developmental features, lineage change and altered drug resistance<sup>[36,45]</sup>. Since acquisition of these abilities is not always associated with cell cycle progression, many of these, except G<sub>1</sub>/S control, may more or less represent cell cycle-independent functions of RB. Particularly, gain of undifferentiated developmental features following RB inactivation and lineage changes presumably related to the developmental plasticity of tumors are central interests of this review article.

We need to be very careful in defining what is cell cycle-independent function of RB and what is not. E2F targets contain not only cell cycle-related genes. In addition, many LxCxE proteins that bind to RB are chromatin modifiers; hence, their roles in control of the cell cycle and differentiation are barely discernible. We later discuss the experimental system by which we addressed cell cycle-independent functions of RB in the context of regulation of undifferentiated cell states. In the next paragraph, we introduce accumulating biological evidence implicating RB in multiple stem cell systems. Later, we discuss possible cell cycle-dependent and independent mechanisms underlying RB functions in stem cells.

## CROSS-SPECIES EVIDENCE THAT LINK RB TO STEM CELLS

Figure 1 shows a phylogenetic tree of genes in the RB family (Figure 1). RB gene orthologues do not exist in the genome of many of unicellular organisms, including yeasts, but appear in almost all multicellular organisms<sup>[47]</sup>. In addition, the component including RB alone and/or E2F transcription factors and DP protein(s) are well conserved from plants to animals<sup>[48]</sup>. This indicates that RB might be involved not only in cell-autonomous proliferation control but also in some machineries unique to multicellular organisms. Stem cell conservation attained by asymmetric cell division is apparently unique to multicellular organisms. Therefore, we present cross-species evidence that may link RB to stem cells.

### Mammalian cells

Analysis of RB functions in embryonic development and embryonic stem cells provided a substantial amount of information regarding its potential roles in stem cells. The role of RB in various adult tissue stem cells was once thoroughly summarized by Sage<sup>[19]</sup>. Our current review focuses on the cells-of-origins in which RB inactivation likely gives the first cue for clonal expansion of stem or progenitor cells during carcinogenesis.

Retinoblastoma develops from the retina composed of multiple lineages of cells. The cell-of-origin of retinoblastoma is quite controversial because of the complexity of retinal development and tumor phenotype. More in concrete, findings that contribute to the controversy include the

reported appearance of differentiated and undifferentiated developmental markers of mixed lineages within the same tumor derived from retinoblastoma patients and Rb-deficient mice<sup>[49]</sup>. Human retinoblastomas show typically differentiated features<sup>[50]</sup>, and post-mitotic retinal cells can be the cell-of-origin in mouse models<sup>[51]</sup>. These findings suggest that promoting dedifferentiation and increasing flexibility of fate determination are initially attained by RB inactivation in retinal cells. In other words, RB inactivation in retinal somatic cells endows them with the ability to differentiate to multiple types of cells. The dispute over cell-of-origin might be resolved, since cone precursors have been shown to be specifically vulnerable to Rb-deficiency-induced clonal expansion<sup>[52]</sup>. However, given that the cell-of-origin has been truly traced to one lineage in retinoblastoma development, the findings on mixed and normally inconsistent developmental features co-existing in retinoblastoma tumor cells imply that RB-deficiency not only initiates tumors of a particular cell-of-origin, but also increases plasticity in lineage specification and probably induces dedifferentiation so that tumor cells employ multiple stem or progenitor cell strategies to fit to the retinal microenvironment.

There are several other types of cancers in which RB loss occurs prevalently at initiation: Osteosarcoma and SCLC. In these malignancies, discussions of the cell-of-origin and the role of RB in tumor initiation are inseparable similarly as in case of retinoblastoma. Mesenchymal stem cells or osteo-progenitors can be the cell-of-origin of osteosarcoma. The direct interaction between RB and osteoblast transcription factor Runx2 might at least partially explain the ability of RB to suppress tumor initiation in a lineage-specific manner. Rb deficiency in these cells often cooperates with p53 loss-of-function to generate osteosarcomas<sup>[53]</sup>. p53-deficiency is in some case sufficient to induce osteosarcoma, and p53-deficient osteosarcomas can be converted to brown fat tumors (hibernomas) with subsequent RB inactivation<sup>[12]</sup>. This has been attributed to increased expression of PPAR $\gamma$  which is governed by E2Fs. However, this fate change might also be mechanistically supported by function of RB to influence on cell fate decision that may occur prior to the decision of commitment to terminal differentiation. Hence, in osteosarcoma, like in retinoblastoma, RB might function in mesenchymal stem cells or osteo-progenitors to contribute to suppress the plasticity in lineage specification.

There are also debates on the cell-of-origin of SCLC. Again, simultaneous inactivation of RB and p53 is sufficient to induce SCLC in mouse lungs<sup>[3]</sup>. A study of cell type-specific deletion of RB and p53 indicated that neuroendocrine cells more often gave rise to SCLC than alveolar type II cells<sup>[54,55]</sup>. From this and other evidence, neuroendocrine cells are believed to be the predominant cell-of-origin of SCLC. The differential roles of RB and p53 in the formation of SCLC will be discussed later (see below).

The role of RB in controlling pluripotency was first addressed by testing whether tumor suppressor depletion facilitates iPS induction<sup>[56]</sup>. This study identified p53 but not RB to be an influential molecule in iPS induction.

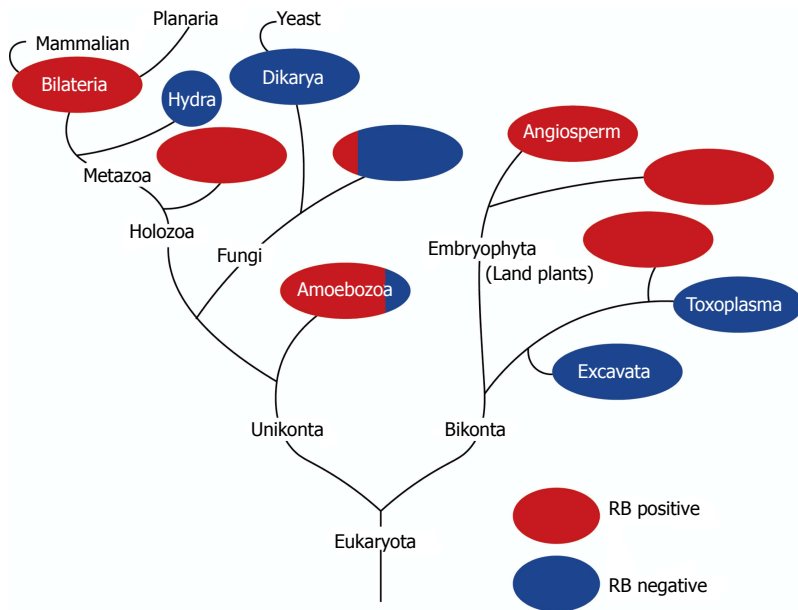


Figure 1 Evolution of the retinoblastoma gene in eukaryotic super-groups. Modified from Desvoyes *et al.*<sup>[67]</sup>. RB: Retinoblastoma.

However, two later studies indicated that RB suppresses iPS induction from fibroblasts. A screen for short hairpin RNAs (shRNAs) that enhance iPS induction efficiency identified RB<sup>[57]</sup>. Another study indicated that RB cleavage by caspase 3/8 is critical for iPS induction<sup>[58]</sup>. More recently, RB was directly implicated in the transcriptional control of *Oct4* and *Sox2*<sup>[59]</sup>. These findings indicate that RB inactivation leads to a state favorable for iPS induction by facilitating the induction of embryonic genes that induce a pluripotent state. This function of pRB may partly explain how stem cell functions can be shared by cancer cells. We indeed often observed increased expression of *Oct4* and *Sox2* in spheres induced by RB inactivation<sup>[59]</sup>. The effect of RB inactivation on the cell cycle control is also contributable to iPS cell induction. We will discuss later many of the signals required for iPS induction are possibly controlled by pRB (see below).

MEFs lacking all members of the RB family can form embryoid body-like 3D structures in suspended culture conditions that express higher levels of embryonic genes and can form teratoma-like tumors when inoculated into immune-deficient mice<sup>[13]</sup>. Since mostly tumor-derived spheres express higher levels of embryonic genes than when cultured under 2D conditions, this observation might result from carcinogenic change in MEFs.

Kareta *et al.*<sup>[59]</sup> (our study) demonstrated that RB inactivation in *p53*<sup>-/-</sup> MEFs give rise to sphere formation without carcinogenic conversion. Sphere formation in the absence of serum and in the presence of limited growth factors (bFGF and EGF) is thought to represent increased self-renewal/symmetric cell division. Preceding this observation, we analyzed C cell adenocarcinomas developed in *Rb*-heterozygous mice that simultaneously lacked *p53*, *Ink4a*, *Arf* or *Cdkn1a* (*p21*). *Rb*-heterozygous mice typically develop low-grade C cell adenocarcinomas. Simultaneous lack of an additional gene allowed *Rb*-deficient C cells to develop full brown tumors at seemingly similar levels. However, as compared to other

genotypes of C cell tumors that were all calcitonin-positive, thyroid tumors that developed in *Rb*<sup>+/-</sup> *p53*<sup>-/-</sup> mice showed virtually no expression of calcitonin. However, earlier neuroendocrine lineage markers, including synaptophysin, were expressed. These findings indicated that simultaneous inactivation of *Rb* and *p53* induced a highly undifferentiated status in neuroendocrine cells that were originally destined to develop into C cells. We then analyzed *Rb*<sup>-/-</sup>; *p53*<sup>-/-</sup> MEFs in comparison with *Rb*<sup>+/+</sup>; *p53*<sup>-/-</sup> MEFs. They showed insignificant differences in cell cycle progression; however, *Rb*<sup>-/-</sup>; *p53*<sup>-/-</sup> MEFs showed significantly higher self-renewal activity and increased expression of embryonic genes. This may represent a cell cycle-independent function of RB in controlling stem cell-like features. Lastly, we screened an FDA-approved drug library and found that some drugs reported to be effective as cancer stem cell therapies were also effective in cells lacking *Rb* and *p53*<sup>[15]</sup>.

### Tumor cell fate

Here, we focus on four tumor types (SCLC, breast cancer, prostate cancer, and soft tissue sarcoma) in which RB inactivation during tumor progression presumably contributes to the increased developmental plasticity of tumor cells.

*p53* is the primary gene mutated in SCLC (75%-90%)<sup>[60]</sup>. *p53* mutations are found also in normal bronchioles of patients, suggesting that this mutation most likely occurs at tumor initiation<sup>[61]</sup>. The second most frequent mutation occurs at *RB* loci. Recent comprehensive genomic profiling of SCLC revealed that bi-allelic losses at *p53* and *RB* loci were 100% and 93%, respectively<sup>[62]</sup>. This signifies that the simultaneous inactivation in *p53* and *RB* occurs in almost 90% of all cases. This study showed a previously unexpected high frequency of *RB* inactivation in SCLC. In terms of determining *RB* function in SCLC development, other gene mutations relevant to *RB* loss in *RB*-intact SCLC (7% of *p53*-mutated cases) should be noted. Semenova *et al.*<sup>[3]</sup> discussed possible *RB* functions in *p53*-mutated SCLC cells.

They pointed out that pluripotency genes such as *OCT4* and *SOX2* are frequently amplified in SCLC<sup>[63]</sup> and that enhancers of zesta 2 (*EZH2*), which is implicated in the neural stem cell maintenance, are very often upregulated in SCLC following RB inactivation<sup>[64,65]</sup>. Finally, they concluded that "RB loss (in SCLC) is associated with an increase in cell plasticity"<sup>[63]</sup>. Although more evidence is needed to finally accept this notion, it is definitely an attractive hypothesis.

Recent publication from Engelman group demonstrated that gain of resistance to tyrosine kinase inhibitors in non-small-cell lung cancers (NSCLCs) harboring epidermal growth factor receptor (EGFR) mutation is associated with fundamental histological transformation from NSCLC to SCLC at a certain frequency (5%-15%). Surprisingly, in such subset of resistant cancers, RB was lost at 100% frequency<sup>[66]</sup>. This report indicates that the developmental plasticity enhanced by RB inactivation is coupled to gain of drug resistance. The mechanism of drug resistance associated with NSCLC-to-SCLC conversion is currently unknown, but it would be definitely attractive to investigate RB functions in this context.

Breast cancers are frequently characterized by RB pathway inactivation, and low RB expression is a hallmark of basal-like breast cancers<sup>[67-69]</sup>. RB inactivation in luminal type breast cancer induces tamoxifen resistance<sup>[70]</sup>, possibly owing to a gain-of-undifferentiated status following RB inactivation. Simultaneous inactivation of p53 and RB is prevalent in basal-like cancers<sup>[71]</sup>. Basal-like and luminal type breast cancers have been recently suggested to stem from common luminal progenitor cells<sup>[72]</sup>. Because p53 mutations are common in most breast cancer types<sup>[73]</sup>, RB status might be one of the determinants of basal-like or luminal type cancers. If so, RB could be implicated in determining the fate of breast cancers.

RB was also implicated in epithelial-to-mesenchymal transition (EMT) in breast cancer cells. Taya group demonstrated that RB depletion in a luminal type MCF-7 breast cancer cells induced EMT and overexpression of RB inhibited the EMT in MCF10A non-tumorigenic breast mammary epithelial cells<sup>[74]</sup>. They also demonstrated that RB controls transcription of *SLUG* and *ZEB-1* in cooperation with the transcription factor activator protein 2 $\alpha$ .

RB is deeply implicated in prostate cancer development, especially during its progression. Although RB inactivation is observed in only 5% of primary prostate cancers, its rate rises to 40% in metastatic tumors. Furthermore, the RB signaling pathway is altered in 34% and 74% of primary tumors and metastatic loci, respectively<sup>[75]</sup>. One study demonstrated a high rate of RB loci deletion and DNA methylation in the RB promoter in metastatic castration-resistant prostate cancers (CRPC)<sup>[76]</sup>. RB depletion in hormone-dependent human prostate cancer cells induces androgen-independent cell growth through upregulation of androgen receptor (AR) in an E2F1-dependent manner<sup>[77]</sup>. RB and p130 are involved in the regulation of *EZH2* transcription in prostate cancer cells, whose upregulation is often observed during prostate cancer progression<sup>[78]</sup>. Loss of RB in prostate cancer

cells increases the expression of nucleolar and spindle-associated protein 1 (*NUSAP1*), which is associated with a poor prognosis in prostate cancer<sup>[79]</sup>. Recently, we observed that RB depletion in androgen-dependent prostate cancer cells induces several lipid metabolism-related genes and some typical malignant features, including tumor spheroid formation. These observations indicate that inactivation of RB strongly promotes prostate cancer progression.

Simultaneous mutations in RB and p53 are frequently found in human soft tissue sarcomas<sup>[80]</sup>. Conditional inactivation of both tumor suppressors by subcutaneous injection of AdCMVC into *p53<sup>flox/flox</sup>; Rb<sup>flox/flox</sup>* mice induced undifferentiated high-grade pleomorphic type sarcomas from locally resident cells. Inactivation of p53 but not RB is sufficient to induce well-differentiated sarcomas, such as rhabdomyosarcoma and leiomyosarcoma, but typically not sufficient to induce undifferentiated types of tumors<sup>[81]</sup>. These findings indicate that RB inactivation does not directly contribute to the initiation of sarcoma development, but rather does contribute to converting well-differentiated types of tumors to undifferentiated types in a p53-deficient background. We recapitulated this finding in an *in vitro* culture system. We first developed poorly spherogenic p53-null soft tissue sarcoma cell lines from soft tissue sarcomas that subcutaneously developed in approximately 10% of *p53<sup>-/-</sup>* mice with a C57BL/6 background. Additional depletion of Rb successfully induced less differentiated highly spherogenic and less differentiated sarcomas from "poorly spherogenic" p53-null soft tissue sarcoma cell lines. These findings indicate a possibility that in soft tissue sarcomas, RB directs both the self-renewal and plasticity of developmental features.

### Planarians

Relying on a large population of pluripotent adult stem cells, planarians exhibit extraordinary high regenerative capacities. Zhu and Pearson presented a comprehensive study on the RB system in these organisms<sup>[82]</sup>. Planarians possess unexpectedly few RB system components: A single Rb family member, single E2F (*E2F4-1*), and single DP. They are primarily expressed in planarian stem cells, and knockdown of any of these components significantly phenocopied the stem cell loss induced by irradiation or RNAi against stem cell-specific genes. The RB system was found to be indispensable for planarian stem cell self-renewal and survival; however, it was dispensable for late differentiation. Interestingly, planarians have 20 homologs to cyclin genes and none of them is homologous to cyclin E. An HDAC1 and a cyclin D homolog are expressed specifically in planarian stem cells, and knockdown of either of them induced deficiencies in stem cell functions. The simplicity of the RB system and ease of visualizing stem cell behaviors in planarians make this a valuable system for the RB research field, especially with regard to stem cell functions. Additionally, the molecular mechanism whereby the RB system contributes to the extraordinarily high regenerative capacity of this creature is great of interest.

**Plant cells**

RB family proteins and their binding partners had existed before multicellular organisms appeared on the earth, and are shared by plants and animals<sup>[82]</sup>. *Arabidopsis thaliana* has one ortholog of an RB family protein, which was named retinoblastoma-related protein (RBR)<sup>[83]</sup>. Inactivation of RBR led to the expansion of root stem cells without affecting the ability of progenitor cells (descendants) to self-renew and differentiate<sup>[48]</sup>. The mechanism whereby RBR controls maintenance of root stem cells seems to involve two transcription factors shortroot (SHR) and scarecrow (SCR). SCR interacts with RBR through an LxCxE motif. Thus, surprisingly, the role of LxCxE motifs is well conserved between animals and plants. More surprisingly, the RBR status affects the function of the SHR/SCR complex to spatiotemporally control the expression of a plant homolog of D-type cyclin (CYC D6;1). CYC D6;1, in cooperation with its corresponding cyclin-dependent kinase (CDK), promotes phosphorylation of RBR<sup>[83]</sup>. These findings indicate that in plants, the RB ortholog exerts the function of controlling stem cells through the regulation of well-conserved cell cycle machinery. Further investigation in this field might unveil many unexpected aspects of RB functions in the control of stem cells.

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## MECHANISTIC ASPECT OF RB FUNCTIONS IN CONTROLLING STEM CELL ACTIVITIES IN MAMMALIAN CELLS

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**Cell cycle**

Analysis of the cell cycle status in embryonic stem (ES) cells provided valuable information on how its alteration might contribute to the acquisition of increased self-renewal and pluripotency. Conklin and Sage<sup>[84]</sup> provided a concise perspective on the possible roles of RB in maintaining the ES cell functions. ES cells have a rapid cell cycle. Because of the prolonged or continuous expression of cyclin E and A family members and lower or no expression of many CDK inhibitors, human and mouse ES cells maintain high levels of activity in multiple CDKs. pRB is consequently hyperphosphorylated for longer periods in ES cells than in normally cycling cells. No obvious cell cycle phenotype in mouse ES cells following inactivation of all RB family members might be consistent with this view<sup>[85,86]</sup>.

An extraordinarily shorter G<sub>1</sub> phase as compared to the relatively prolonged S phase in ES cells would be beneficial to lessen the susceptibility to differentiate upon receiving stimuli to lender cells to do so. As G<sub>1</sub> as well is the period that is most vulnerable to mitogenic signals that directs cells to decide to proliferate, arrest or senesce, this phase could be the most critical one in the decision to differentiate upon various stimuli. Keeping the G<sub>1</sub> phase shorter could be the primary role of RB hyperphosphorylation in ES cells. Consistent with this view, overexpression of the constitutively active

(non-phosphorylatable) form of RB (RB<sup>7LP</sup>) in human ES cells induced cell cycle arrest, followed by spontaneous differentiation and p53-dependent cell death<sup>[87]</sup>.

The difference in undifferentiated behaviors between *Rb*<sup>+/+</sup>; *p53*<sup>-/-</sup> and *Rb*<sup>-/-</sup>; *p53*<sup>-/-</sup> MEFs predicted that there could be cell cycle-independent functions of RB in controlling stemness. *p53*<sup>-/-</sup> MEFs exhibited similar proliferation phenotypes regardless of Rb genotype; however, *Rb*<sup>-/-</sup>; *p53*<sup>-/-</sup> showed increased sphere forming activity relative to *Rb*<sup>+/+</sup>; *p53*<sup>-/-</sup> MEFs<sup>[15]</sup>. The same phenomenon was observed in mouse soft tissue sarcoma cells and mouse mammary gland epithelial cells. Given that RB-deficiency has no or very little impact on cell proliferation in a *p53*-null genetic background, the mechanism whereby RB-deficiency effects stem cell-like features of multiple types of cells can be separated from that governs cell proliferation.

**Quiescence and apoptosis**

In contrast to mouse ES cells, inactivation of all RB family members in human ES cells exhibited abnormal quiescence, featured by G<sub>2</sub>/M arrest and cell death<sup>[87]</sup>. Cell death depended on the p53-p21 module, similar to RB overexpression. Thus, in human ES cells, both hypo- and hyper-activation of RB are counteracted by cell cycle arrest and p53 pathway activation, indicating a critical role for RB in the homeostatic control of ES cell activities. p53 is typically expressed in human ES cells at a low level<sup>[88]</sup>. This might be beneficial for ES cells so that they are not too sensitive to alterations in RB activity status. Mouse hematopoietic stem cells lacking all RB family proteins exhibit impaired quiescence control and apoptosis in lymphoid progenitor cells<sup>[89]</sup>. In MEFs, the absence of p53 endowed RB-deficient cells significant increase in self-renewal activity when cultured in the presence of limited growth factors<sup>[15]</sup>. However, in a *p53*<sup>-/-</sup> background, the RB status did not impact cell proliferation of MEFs under regular culture conditions. It should be noted again that in mouse ES cells, lack of RB family proteins generated no difference in the cell cycle<sup>[85,86]</sup>. These findings indicate that RB may control stemness beyond its role in cell cycle control.

**Cellular senescence**

Cellular senescence is believed to be strongly inhibited in stem cells, otherwise irreversible growth arrest could lead to total elimination of a stem cell pool from tissues. RB plays pivotal roles in inducing and maintaining cellular senescence, not only by controlling required transcription of genes, but also by being involved in senescence-associated heterochromatic foci (SAHF)<sup>[90]</sup>. Therefore, hyperactivation of RB can be harmful to stem cells, as was shown in a human ES cell study<sup>[87]</sup>.

Surprisingly, loss of RB function, as well, can induce cellular senescence. As mentioned above, thyroid tumors that developed in *Rb*-heterozygous mice were typically low-grade adenocarcinomas or adenomas of

C cell origin; however, the genetic background lacking *N-ras*<sup>[37]</sup> or either *Ink4a*, *Arf*, *Suvh39*<sup>[14]</sup>, *ATM*<sup>[38]</sup> or *p53*<sup>[15]</sup> allow these tumors to develop into highly invasive and metastatic type medullary adenocarcinomas. The immunohistochemical observation of Rb-deficient C cell tumors lacking no other genes revealed whole evidence of cellular senescence, including increased expression of p16<sup>Ink4a</sup> and HP-1, and positive  $\beta$ -galactosidase staining<sup>[14]</sup>. Since a simultaneous lack of genes mediating DNA damage response (*ATM*) and cellular senescence (*Ink4a*) allowed malignant progression of C cell tumors, we concluded that these cellular responses prevented Rb-deficient premalignant cells from developing into malignant cells. Analysis of *Rb-N-ras* DKO mice revealed that the mechanism whereby RB loss induces a DNA damage response involved p130<sup>[14]</sup>. Another system in which RB loss possibly induces cellular senescence under particular culture conditions is MEFs. *Rb* loss alone does not allow MEFs to escape senescence when cultured at low density. However, simultaneous loss of *N-ras* or *Ink4a* allowed MEFs to escape cellular senescence upon low cell density plating<sup>[14]</sup>. The reason why *N-ras* loci are associated with susceptibility to senescence was explained by the E2F-dependent control of Ras isoprenylation<sup>[14]</sup>.

There are three other tumor suppressors whose loss of function can induce cellular senescence in particular contexts. These are PTEN, VHL and NF1<sup>[90-93]</sup>. Somatic cells are protected from carcinogenesis when these tumor suppressor genes are inactivated at early steps of carcinogenesis. This could be the major reason that RB mutations are detected in only limited types of cancer at their initiation.

ES cells, iPS cells, and tissue stem cells seem to confer lower RB activity in order to accelerate self-renewal and to keep undifferentiated state. We do not know whether cellular senescence machineries are simultaneously suppressed during the period that RB function is suppressed in stem cells. Unlike *Rb*<sup>-/-</sup>; *p53*<sup>-/-</sup> MEFs, *Rb*<sup>-/-</sup>; *Ink4a*<sup>-/-</sup> MEFs did not form spheres<sup>[15]</sup>. However, regarding iPS induction, the *Ink4a/Arf* locus appeared to be a barrier to reprogramming<sup>[94]</sup>. Therefore, RB functions in controlling cellular senescence could be intimately involved in the regulation of stemness.

### Chromosomal instability

Chromosomal instability (CIN) might be one of the events that are seemingly not shared by normal stem cells and cancer cells. In tumor cells, RB inactivation, especially when combined with p53 mutation, led cells to accumulate chromosomal aberrations<sup>[95]</sup>. Inactivation of all RB family members in human ES cells causes CIN; however, clonal expansion of these cells was blocked by G2/M arrest and cell death<sup>[87]</sup>. Presumably, then, normal stem cells are much less tolerant of CIN than cancer cells. RB status could influence CIN through its effects on Mad2 transcription and its direct interaction with cohesion and condensin II<sup>[96]</sup>. The rapid cell cycle

in ES cells might increase the risk of accumulating DNA damage due to hyper-replication and nucleotide deficiency<sup>[97]</sup>. How ES cells are protected from these risks and why cancer cells tolerate CIN needs to be clarified.

### Epigenetics

Through LxCxE motifs, RB interacts with numerous chromatin modifiers, including DNMT1, SUV39H1, Suv4-20H1, BRN1, BRG1, HDAC, and KDM5A/JARID1A/RBP2<sup>[36]</sup>. Among these, KDM5A might mediate RB function to control stemness. KDM5A demethylates tri- and dimethylated lysine 4 in histone H3. ES cells lacking KDM5A failed to maintain OCT4 and NANOG expression upon stimulation to promote differentiation<sup>[98]</sup>. KDM5A controls RB-dependent myogenic differentiation, at least partially, through the regulation of mitochondrial function<sup>[99]</sup>. Importantly, loss of KDM5A in Rb-heterozygous mice attenuated pituitary tumorigenesis<sup>[98]</sup>. KDM5A was first identified in a screen to find proteins that bind to pRB mutants unable to bind to E2Fs<sup>[100]</sup>. pRB mutants unable to bind to KDM5A failed to control differentiation. The mechanism whereby KDM5A controls mitochondrial function involves PGC-1/PPAR $\gamma$ c1a<sup>[99]</sup>. It is of great interest to determine whether other genes under the influence of RB-KDM5A axis control stemness based on the effect on their epigenetic status.

Our experience with the stem cell-like behaviors exhibited by RB-p53 double deficient cells indicated that some of stem cell-like features are not reversed by RB reconstitution. This suggests that the effect of RB deficiency on stem cell-like behaviors might depend on its role in epigenetic control. Many chromatin modifiers carrying LxCxE motifs may be involved in the epigenetic function of pRB; however, so far, only some of them have been characterized regarding their role in controlling stem cell functions. We demonstrated that Rb in mouse cells exerts its influence on the epigenetic control of *Ink4a*, *Shc*, and *FoxO6* through DNMT1; however, its significance in the control of stemness has not yet been elucidated<sup>[38]</sup>.

### Tissue specific transcription factor

RB plays critical roles in the terminal differentiation of cells owing to its genetic and physical interaction with tissue-specific transcription factors, including MYOD, C/EBP, GR, GATA-1, PU-1, CBFA-1, PDX1, RUNX2, and NF-IL6<sup>[36]</sup>. It is totally unknown whether the interaction between RB and these factors has any role in tissue stem cells. Myoblast regeneration induced by RB and ARF depletion in post-mitotic muscle cells<sup>[101]</sup> may involve the elimination of MYOD functions because there is a physical interaction between RB and MYOD. Additionally, the physical interaction of RB and RUNX2 may explain dedifferentiation in osteoblasts that would occur upon development of osteosarcoma.

The interaction of RB with ID2, KDM5A, and EID1 may govern differentiation in a less tissue-specific manner.



ID2, when overexpressed in Nestin-expressing cells, induced precocious neural stem cell depletion<sup>[102]</sup>. RB is to some extent dispensable in brain development during the embryonic stage since Rb-deficient embryonic brains exhibited almost normal development, except for ectopic cell cycle entry and cell death in cortical neurons<sup>[103,104]</sup>. However, the role of RB in adult neural stem cells has not been sufficiently addressed yet.

### Embryonic gene

There are reports that NANOG and SOX2 induce hyperphosphorylation of pRB through regulation of CDC25A and CDK6<sup>[84]</sup>. OCT4, in cooperation with miR-335, induces hyperphosphorylation of RB by suppressing PP1 through NIP1 and CCNF<sup>[105]</sup>. Following RB hyperphosphorylation, freed E2Fs transactivate OCT4-targeted genes. These findings indicate the general function of RB in orchestrating the embryonic gene network. The most surprising finding regarding the role of RB in the embryonic gene network was recently made by the Wernig and Sage laboratory<sup>[59]</sup>. They discovered that RB is directly involved in the transcriptional control of OCT4 and SOX2. They also demonstrated that Sox2 deletion attenuated pituitary tumorigenesis in Rb-heterozygous mice. These findings clearly revealed the strong influence of RB on the embryonic gene network.

These findings also explain the upregulation of a series of embryonic genes in MEFs lacking all RB family members<sup>[13]</sup> and also in those lacking Rb and p53<sup>[15]</sup> when forming embryoid body or spheres under nutrition-restricted and floating conditions. However, there was no evidence of upregulation of these genes when cells were cultured under 2D culture conditions in the presence of serum. This implies that regulation of OCT4 and SOX2 by RB is influenced by the environment where cells are placed and other genes that play a critical role in allowing RB to control embryonic genes. It would be of great interest to survey such genes.

### Metabolism

There are significant similarities between stem cell metabolism and cancer cell metabolic reprogramming (rewiring)<sup>[106,107]</sup>. RB functions in various metabolic pathways have attracted attention from cancer researchers<sup>[36,45,46,108]</sup>. Given that RB is the central molecule in cell cycle control, it is reasonable that RB also responds to demands for increasing biomass that is coupled to the cell cycle. It is well known that RB controls thymidine kinase 1 (TK1) and dihydrofolate reductase (DHFR), both of which are required for nucleotide synthesis<sup>[109]</sup>. However, the biomass needed for doubling the cell size during the S phase contains not only nucleotides but also amino acids, lipids, and many other carbon metabolites. pRB undergoes post-translational modification by various nutrient signals involving SIRT1 and AMPK and by cyclin/CDK complexes stimulated by various mitogenic signals<sup>[110,111]</sup>. RB regulates the transcription or the activity of wide range of enzymes, signaling molecules, and transcription factors, including OXPHOS genes, MVA pathway genes, UCP-1, SOD2, ASCT2, GLS1, PKA, AKT, mTOR, PDK4, PGC-1, ERR, FOXc2, HIF-1, BNIP3,

SREBP-1,2, PPAR and KDM5A<sup>[12,14,112-124]</sup>. Genes encoding many of these factors are supposed to be driven by E2F transcription factors. Myc is also a well-established downstream molecule of RB, and is implicated in the transcriptional control of GLUT1, HK2, PKM2, and LDH-A<sup>[45]</sup>.

OXPHOS activity is significantly correlated to the efficacy of iPS induction<sup>[125]</sup>. Besides its interaction with OCT4 and SOX2, RB might be implicated in nuclear reprogramming through its influence on OXPHOS.

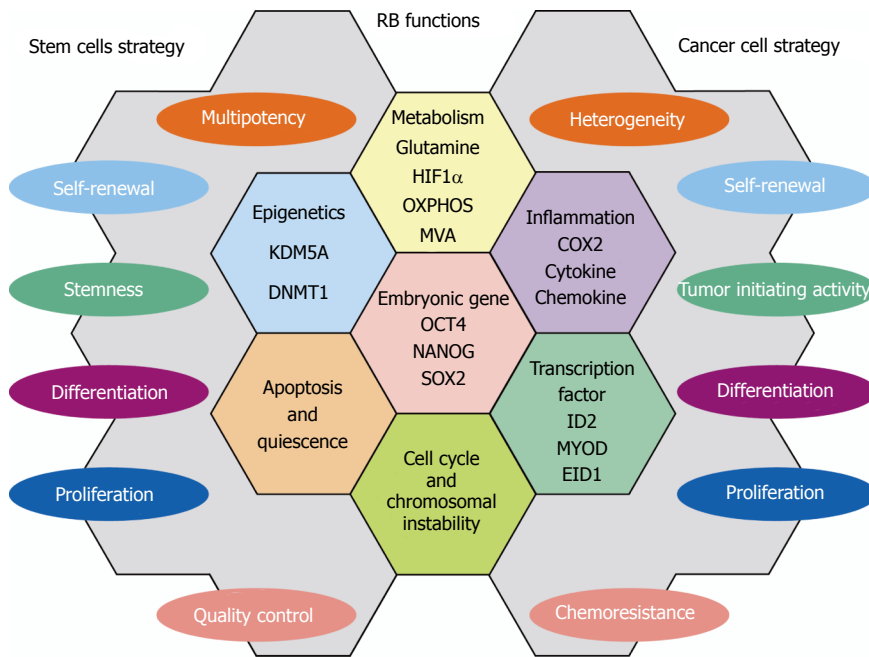
HIF-1 $\alpha$  activation explains why hypoxia facilitates iPS induction and self-renewal of tumor cells<sup>[126,127]</sup>. Additionally, this molecule was implicated in the long-term maintenance of hematopoietic stem cell (LT-HSC) populations<sup>[128]</sup>. Thus, RB status presumably has a big impact on glycolysis and TCA activity through the functional interaction with HIF-1. We have recently identified an HIF-1-independent target of RB in the glycolytic pathway. It is of interest to address how RB and HIF-1 cooperate in the control of the glycolytic pathway.

Recent studies from two independent research groups highlighted the link between RB status and glutaminolysis in *Drosophila* and mammalian cells<sup>[116,129]</sup>. Activation of the glutamine pathway not only fuels the TCA cycle, but also contributes to control of the cellular reactive oxygen species (ROS) level by regulating glutathione synthesis<sup>[130]</sup>. This may give both stem cells and cancer cells an advantage in terms of the maintenance of stemness. Additionally, ASCT2 in cooperation with GLUT1 appears to regulate human hematopoietic stem cell lineage specification<sup>[131]</sup>. Increased dependency on glutamine metabolism is frequently observed in cancers, and has been linked to the emergence of drug resistance<sup>[132,133]</sup>. Glutamine metabolism could be an important link between stem cells and cancer cells.

While addressing the question of why Ras proteins are activated after RB loss, we discovered that RB controls a number of enzymes involved in the MVA pathway. This study has been extended to another study that addresses the role of the MVA pathway in controlling stem cell-like activity in prostate cancer cells. The mechanism that links RB to Ras include SREBP-1,2. The characterization of Rb-Srebf-1 DKO mice revealed that RB status has a big impact on the control of fatty acid quality. We also identified molecules that explain differential self-renewal activity between p53<sup>-/-</sup> cells and Rb-deficient p53<sup>-/-</sup> cells (see above). These include enzymes involved in the glycolytic pathway.

### Inflammation

RB status has been linked to pro-inflammatory phenotypes in breast cancer cells<sup>[68]</sup>. This study highlighted COX2 as a target of the E2F transcription factor. However, our reassessment of these data revealed that many pro-inflammatory cytokines and chemokines, including IL-6, CCL2, and CCL5, are upregulated when breast cancers express lower levels of RB. A similar observation was made in mice lacking Rb in the back skin of p21<sup>-/-</sup> mice<sup>[134]</sup>. The tumors derived from these mice exhibited high levels of pro-inflammatory cytokines and evidence of infiltration by



**Figure 2** A schematic representation of molecular functions of retinoblastoma that may be shared by stem cells and cancer cells. RB: Retinoblastoma; MVA: Mevalonate.

immune cells. Indeed, many cytokines and chemokines, and even their receptors, are often regulated by E2F transcription factors<sup>[135,136]</sup>.

Among cytokines and chemokines, IL-6 and CCL2 are drawing attention as strong inducers of iPS cells. A study demonstrated that IL-6 is more potent than c-Myc in terms of their abilities to induce iPS cells<sup>[137]</sup>. Another study demonstrated that CCL2 plays pivotal role in the maintenance of pluripotency in ES and iPS cells<sup>[138]</sup>. There are many reports indicating that pro-inflammatory status is critical for cancer stem cells to evolve<sup>[139-142]</sup>. Many of pro-inflammatory factors stimulates JAK/STAT3 pathway, thus contribute to enhance self-renewal of stem cells and possibly cancer stem cells. This could be one of core mechanisms that are shared by stem cells and cancer cells. Our recent efforts revealed that the RB status in soft tissue sarcoma, breast and prostate cancer cells significantly alter the pro-inflammatory status of these cells, and this significantly enhances self-renewal activity and chemo-resistance.

RB may control innate immunity as well. The RB-E2F1 complex appears to regulate toll-like receptor 3 (TLR3)<sup>[143]</sup>. In *Drosophila*, RB, in cooperation with dCAP-D3, upregulates innate immunity<sup>[144]</sup>. Several reports demonstrated that RB-deficiency in tumors attenuates the innate immune response, thereby promoting tumor development<sup>[145]</sup>. There is also a report that showed a positive role for innate immunity in inducing iPS cells<sup>[146]</sup>.

## CONCLUSION

In this manuscript, we described a part of numerous RB functions that are employed commonly as strategies to control stem cells and suppress cancer cells. These findings may help readers to understand how stem cell strategies are shared by cancer (stem) cells. Figure 2 demonstrates RB functions that may be shared by

stem cells and cancer cells. Although RB is one of tumor suppressors those have been characterized for long time and by big number of cancer researchers, our curiosity on its hidden roles in various biological events never fades away. In the near future, more number of stem cell and regenerative medicine researchers may stand up by this molecule. Although beyond the scope of this article, RB is implicated in the regeneration of many tissues/organs<sup>[19]</sup>. Discovery of its target in the context of the undifferentiated state of cancer cells or in drug resistance may lead us to develop powerful tools in both cancer therapy and regenerative medicine.

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