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RB1 and p53 at the crossroad of EMT and triple negative breast cancer

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Triple negative breast cancer (TNBC) is a heterogeneous disease that includes Basal-like and Claudin-low tumors. The Claudin-low tumors are enriched for features associated with epithelial-to-mesenchymal transition (EMT) and possibly for tumor initiating cells. Primary TNBCs respond relatively well to conventional chemotherapy; however, metastatic disease is virtually incurable. Thus, there is a great interest in identifying specific therapeutic targets for TNBC. The tumor suppressor RB1 is frequently lost in Basal-like breast cancer. To test for a causative role of RB1 gene loss in BC and for its effect on specific subtypes, we deleted mouse Rb in mammary stem/bipotent progenitor cells. This led to diverse mammary tumors including TNBC, with a subset of the latter containing p53 mutations and exhibiting features of Basal-like BC or EMT. Combined mutation of Rb and p53 in mammary stem/bipotent progenitors induced EMT type tumors. Here, we review our findings and those of others, which connect Rb and p53 to EMT in TNBC. Furthermore, we discuss how by understanding this circuit and its vulnerabilities, we may identify novel therapy for TNBC.

Key words: retinoblastoma, p53, AP-2, basal-like breast cancer, claudin low, EMT

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Triple Negative Breast Cancer (TNBC)

Breast cancer (BC) is a heterogeneous disease that can be classified by immunohistochemistry (IHC) into Estrogen Receptor alpha (ERα)-positive, HER2/ERBB2/NEU-positive and Triple Negative (TN) tumors, the latter of which do not express hormone receptors or HER2.[1-7] TNBC affects 15–30% of patients. By IHC it can be further divided into Basal-like breast cancer and non-basal tumors, some of which exhibit features of EMT.[8] Basal-like BCs express the basal cytokeratins (CK) CK5/6, CK14, CK17, and/or epidermal growth factor receptor (EGFR), whereas non-basal TNBCs do not express these markers (reviewed in ref. 8–10).

Transcriptional profiling identified five BC subtypes that overlap but are not necessarily identical to the IHC-based classification. This includes ERα$^+$ luminal A and luminal B, HER2$^+$, Basal-like and Claudin-low BC.[7,9,11-16] The latter exhibits low expression of epithelial junction proteins such as Claudin 3, 4 and 7, as well as low levels of E-Cadherin and high levels of EMT markers, including Zeb1, Twist and Snail. Basal-like tumors and cell lines (referred to as Basal-A) express low levels of the tumor suppressor pRb and high levels of the tumor suppressor p53, indicative of stabilizing mutations.[17,18] The status of RB1 in Claudin-low BC and cell lines (Basal-B) is yet to be established.

Breast tumors that form in Brca1 carriers constitute a third group of TNBC, which, like basal-like BC express high level of basal-cytokeratins and EGFR and display high incidence of p53 mutation. Brca1 tumors, however, do not typically show loss of pRb.[8] Thus, inactivation of BRCA1 or RB1 may divide TNBC into two major non-overlapping subclasses, both with p53 mutations. Notably, pRb

and BRCA1 interact with each other,[19] and this interaction has been recently confirmed in a non-biased genome-wide mammalian protein-protein interaction analysis.[20] BRCA1-induced growth arrest is pRb-dependent.[21] It is therefore possible that these tumor suppressors act on a common genetic pathway linking genomic stability with cell cycle progression; inactivation of either gene may suffice to disrupt this checkpoint and induce tumorigenicity. Although Brca1 is not mutated in sporadic Basal-like BC, the gene is often downregulated due to LOH or promoter methylation, or the protein is inactivated by post-translation modifications.[8,9,22] Thus, in carriers of germ-line mutations, Brca1 is completely inactivated and this may be sufficient to drive cancer independent of RB1, whereas in sporadic Basal-like BC, both RB1 and Brca1 may be partially inactivated, each contributing to the high proliferation rate of these tumors. However, the frequency in which RB1 and BRCA1 are co-inactivated, albeit partially, in basal-like BC is yet to be determined.

The CSC-EMT-Claudin-low Link

Many cancers are organized in a hierarchy whereby relatively rare Tumor Initiating Cells (TICs; also referred to as Cancer Stem Cells, CSC) can initiate and maintain primary tumor growth. These cells, like normal stem cells, can self-renew and differentiate to generate cells that have lost their tumorigenic potential.[23-29] A breast cancer tumorigenicity signature based on differential gene expression in TIC versus non-TIC fractions has been developed.[30-32] Claudin-low BCs exhibit high similarity to the TIC signature—whereas Basal-like, ERα$^+$ and HER2$^+$ tumors share only marginal overlap.[12,31,33]

EMT is an embryonic program by which epithelial cells lose cell-cell contact and polarity.[32,34,35] EMT also involves cytoskeletal changes that increase motility and invasiveness. During EMT, mesenchymal proteins such as N-Cadherin, Vimentin, Fibronectin as well as matrix degrading enzymes are induced. In contrast, epithelial junction proteins like E-Cadherin, and Claudins 3, 4 and 7 are suppressed. EMT is induced by TGFβ as well as by transcription factors such as Snail1/2(Slug), Zeb1/2, Twist1/2 and Ladybird homeobox 1 (*LBX1*).[32,36-38] Activation of the EMT program enables transformed epithelial cells to invade locally, to disseminate via blood or lymphatic vessels and to establish micrometastasis.[34,35] Thus, EMT may account for major steps in the metastatic cascade, with the exception of growth at the secondary site.

Although most BCs do not appear mesenchymal, some not only express EMT inducers but also depend on their expression for continuous growth. For example, MMTV-neu mammary tumor cells, which are not mesenchymal, express Twist, and knockdown of this transcription factor causes cell senescence.[39,40] Expression of EMT-inducers in these tumors may prime them for EMT in response to stromal or other, yet to be defined, signals at tumor margins. Such tumors may not undergo full EMT but rather a limited conversion that allows for detachment of cells from the tumor mass, increasing their motility and invasion. Interestingly, in pancreatic cancer, metastatic initiating cells (MICs) represent a subset of TICs.[41] Thus, the relationship between EMT, TICs and MICs in BC needs to be further explored.

In 2008, Mani et al. showed that breast TICs exhibit spindle morphology and express EMT markers when cultured in vitro.[36] Moreover, expression of EMT inducers increased the breast TIC fraction in a heterogeneous tumor cell population, rendering them highly tumorigenic and resistant to conventional chemotherapy.[32,36] Indeed, following therapy, residual BC cells display mesenchymal and TIC features.[33] An EMT signature has recently been generated; when compared to BC subtypes, the signature most closely resembled Claudin-low BC.[37] Thus, Claudin-low breast tumors highly express genes associated with the breast cancer tumorigenicity and EMT signatures.

RB1 Status in Breast Cancer

Early studies revealed gene rearrangement at the RB1 locus in ~10% of primary breast carcinomas of undefined subtypes, and in ~20–25% of TNBC-derived cell lines.[18,42-48] Some RB1-deficient BC lines (e.g., MDA-MB468) were classified as Basal-like/Basal-A, whereas other (BT549, MDA-MB436) as mesenchymal/Claudin-low/Basal-B.[16,18] Low RB1 gene expression and loss of heterozygosity (LOH) at the RB1 locus were subsequently identified in a high percentage of luminal-B (61.5%) and Basal-like (72%) subtypes.[49,50] LOH does not always correlate with loss of immuno-reactivity. The basis for this is not known, and direct sequencing of RB1 in these tumors is needed to clarify the issue. In a more recent study, loss of pRb protein expression coupled with high expression of p53, indicative of a stabilized mutant form, were demonstrated in most basal-like TNBCs.[17] RB1 LOH, with or without loss of protein expression, was typically associated with increased p16^{ink4a} expression. This CDK4/6 inhibitor is a direct transcriptional target of pRb-E2F, which suppresses its expression by inducing histone H3K27-methylation and thereby recruitment of BMI1 repression complexes to the p16^{ink4a} promoter.[51] Thus, p16^{ink4a} expression serves as a surrogate for pRb loss in TNBC. Furthermore, in pre-neoplastic lesions (DCIS), high p16^{ink4a} expression combined with high Ki67 index is a predictor for tumor progression.[49] In contrast to basal-like BC, less is known about the status of RB1 (protein, mRNA, mutation and LOH) in Claudin-low BC. This gap is likely to be filled soon.

Interestingly, a recent survey of RB1 status in metastatic breast cancer revealed two cases with duplication of the entire gene.[52] This may be related to a phenomena observed in colorectal carcinoma where high expression of pRb was shown, paradoxically, to protect from E2F-induced apoptosis.[53,54] In line with this, expression of constitutively active phospho-mutant Rb transgenes in mouse mammary epithelium induces adenocarcinoma.[55] Thus, like overexpression and loss-of-function mutations in its major target, E2F1, both activation and inactivation of pRb can be oncogenic in the mammary gland. These observations serve as a cautionary note to chronic activation of the pRb pathway as part of preventive regimens.

pRb controls cell proliferation, survival and differentiation by functioning primarily as a transcriptional co-repressor.[56,57] It binds transcription factors such

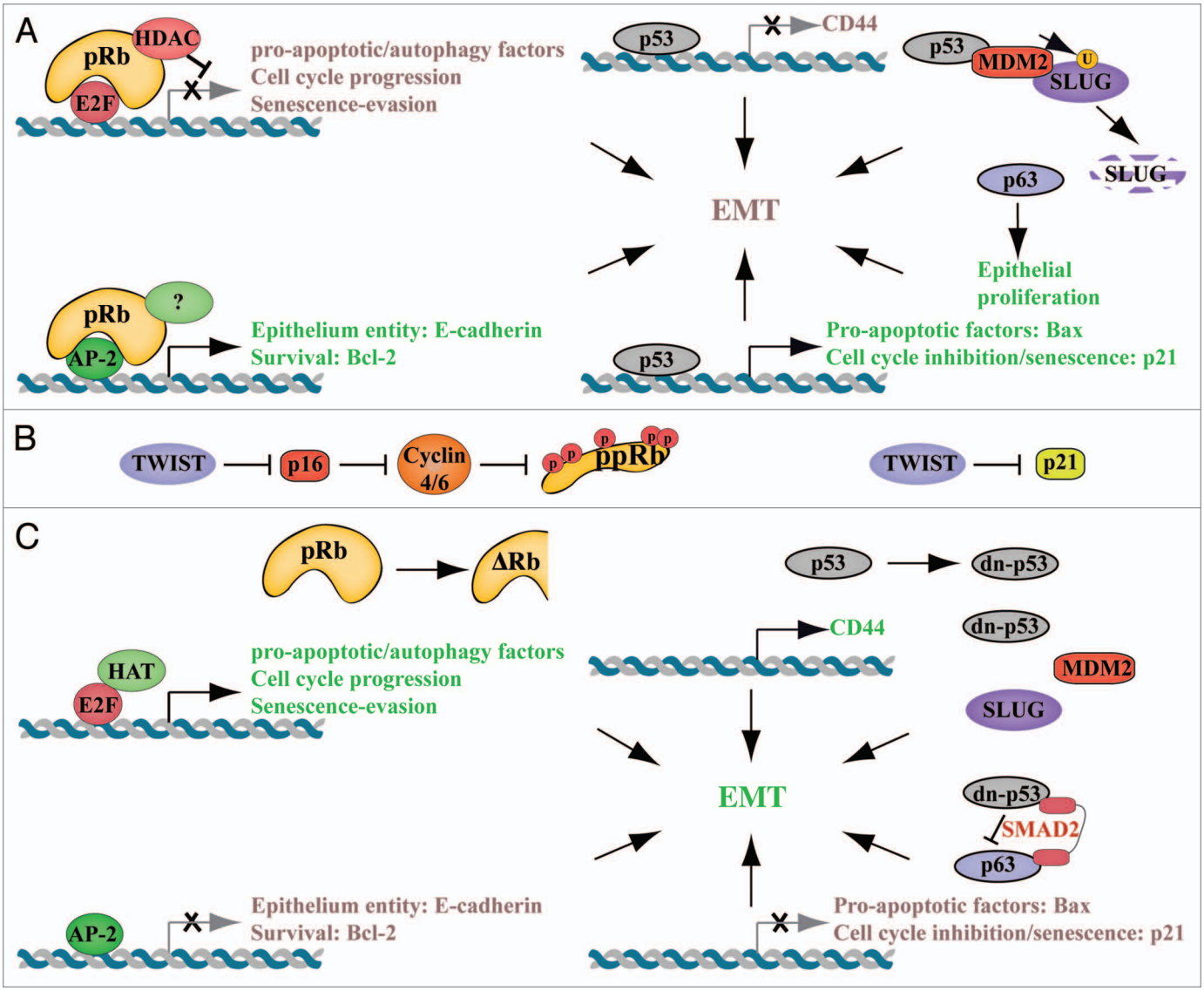

Figure 1. The RB1-p53-EMT connection. (A) In normal cells, pRb interacts with E2F to inhibit pro-apoptotic and pro-autophagy genes such as PUMA and BNIP3, respectively, as well as cell cycle progression and senescence genes such as cyclinE and MCM7. pRb also stimulates AP-2 to induce BCl-2 and E-Cadherin expression thereby maintaining survival and epithelial identity. In response to genotoxic stress p53 may inhibit cell proliferation by inducing $p21^{Cip1}$, thereby allowing DNA repair to occur, or if DNA damage is unrepairable, it may induce cell death by activating apoptotic genes such as Bax. p53 also inhibits the CSC gene CD44, and forms a ternary complex with MDM2 and SLUG to induce SLUG degradation via the proteosome. EMT-epithelial-to-mesenchymal transition; P-phosphate group; ub, ubiquitin. (B) In luminal tumors like $HER2^+$ BC, various signals such as NFκB activation stimulate EMT-inducing transcription factors like TWIST that bypass senescence by suppressing $p16^{ink4a}$ and $p21^{Cip1}$ (thereby inhibiting pRb and bypassing p53) and cooperate with oncogenes such as RAS to induce features of EMT. (C) In some TNBCs, RB1 and p53 are inactivated. RB1 loss bypasses oncogene induced senescence and promotes deregulated cell proliferation. In addition, loss of pRb may alter cell fate by abrogating E-Cadherin expression. Dominant-negative p53 induces CD44, stabilizes SLUG and blocks p63, the latter in collaboration with activated RAS and SMAD2. Thus, the combined loss of pRb and p53 bypasses senescence, increases cell proliferation and, in certain contexts, induces EMT.

as members of the E2F protein family and recruits chromatin-modifying enzymes like HDAC1 to silence gene expression (**Fig. 1**). Many E2F responsive promoters regulate genes required for escape from senescence, for DNA replication and for controlling cell death pathways.[58-60] Apoptosis downstream of pRb loss is typically mediated by the p53 tumor suppressor.[61,62] Accordingly, pRb and p53 are commonly inactivated together in cancer, and some DNA tumor viruses express oncoproteins, such as SV40 large T antigen (Tag), which sequester Rb (and its relatives p107 and p130) together with p53.[63,64] Mammary gland-specific transgenic expression of Tag, or a truncated form, T_{121}, which binds pRb family but not p53, induces mammary tumors.[65-67] In contrast, mammary placodes from $Rb^{-/-}$ embryos develop normally when transplanted into recipient mammary glands and do not form tumors (see below).[68] Thus, the long-term consequences of Rb inactivation alone on mammary epithelial transformation and breast cancer subtype were until recently unknown.

Consequences of Rb Deletion in the Mammary Gland

To test for a causative role of RB1 loss in BC and ultimately to develop new therapies for TNBC, we used a floxed Rb mutant allele (Rb^f),[69] to delete Rb in mouse mammary epithelium. Deletion of Rb via two different MMTV-Cre lines induced focal acinar hyperplasia with squamous metaplasia.[70] These lesions progressed into histologically diverse mammary tumors. RNA microarray analysis revealed that the $Rb^{-/-}$ tumors clustered with mouse models of luminal-B or basal-like BC. The basal-like $Rb^{-/-}$ tumors resembled Tag or DMBA-induced tumors and exhibited proliferation and EMT markers. Additional $Rb^{-/-}$ tumors exhibited spindle-shape morphology and features of EMT. The EMT and Tag-like, but not the DMBA or luminal-B-like tumors, expressed dominant negative (dn) mutant forms of the tumor suppressor p53, as revealed by IHC and verified by sequencing. To test for consequences of combined Rb and p53 deletion, we generated MMTV-Cre:$Rb^{f/f}$:$p53^{f/f}$ double mutant mice. These mice succumbed to lymphoma at a young age. Therefore, we transplanted Rb/p53-deficient mammary epithelium from mutant mice into the mammary fat pads of recipient mice. This resulted in the development of EMT tumors that were histologically indistinguishable from EMT tumors in MMTV-Cre:$Rb^{f/f}$ mice with p53 mutations.[70] Together, these results demonstrate a causal role for Rb in breast cancer and show that cooperating oncogenic events, such as p53 mutation, dictate tumor subtype after Rb loss.

The Rb-p53 Circuit

The importance of combined RB1 and p53 inactivation for neoplastic transformation was first revealed by the fact that several DNA tumor viruses express oncoproteins to target both proteins. Indeed, combined mutation of these tumor suppressors accelerate cancer formation, and increase (and alter) the spectrum of tumors found in individual knockout mice.[70-72] The best defined mechanism by which RB1 and p53 cooperate is at the level of senescence escape and cell proliferation.[58] In addition, apoptosis induced in differentiating cells lacking Rb is often, though not always, p53 dependent.[61,62,72,74]

RB1 and p53 may interact at additional levels. Loss of Rb has recently been shown to induce a catastrophic collapse of the mitochondrial network, increased autophagy flux and reduced ATP level during cellular differentiation; autophagy inhibitors and hypoxia could rescue this defect.[75-77] The effect of hypoxia was glycolysis- and HIF1α-dependent, suggesting that differentiating Rb-deficient cells survive due to a shift in ATP production from oxidative phosphorylation to glycolysis.[75] Given that p53 regulates both autophagy and glycolysis,[78,79] mutation in p53 may allow differentiating Rb deficient cells to survive by opposing apoptotic or autophagic cell death as well as by inducing a metabolic shift toward glycolysis (Warburg effect). In this regards, it is interesting that TNBC and in particularly Claudin-low tumors are more hypoxic and glycolytic than other BC subtypes.[7,80,81] Thus, mutations in Rb and p53 may cooperate to promote cell survival and proliferation under oncogenic stress or hypoxic conditions. The following section summarizes evidence that mutation of both genes may also cooperate in the induction of EMT.

The Rb-p53-EMT Connection

Although much attention has been given to the role of pRb as a transcriptional co-repressor when bound to E2F-DP, pRb can act as a transcriptional activator when bound to other transcription factors.[56,72] Importantly, pRb binds the AP-2 transcription factor and thereby induces transcription of Bcl-2 and E-Cadherin.[82,83] Depletion of RB in MCF7 breast tumor cells, which express wild-type (wt) RB1 and p53 genes, was subsequently shown to induce EMT by reducing E-Cadherin expression, as well as by inducing Slug and Zeb-1 expression.[84] Thus, Rb keeps the EMT program in check at multiple levels.

p53 is often mutated in human cancer leading to aggressive tumors with poor prognosis.[85] This is at least in part because mutant p53 can bind and sequester its relative p63. In cancer cells expressing oncogenic RAS and TGFβ, dn-p53 traps p63 in a non-functional ternary complex with activated SMAD2.[86] This compromises the function of p63, which normally inhibits EMT, migration and invasion by promoting epithelial identify and cell proliferation.[87] Mutation of p53 may induce EMT and metastasis by several other mechanisms. First, wt-p53 forms a ternary complex with MDM2 and SLUG to promote degradation of this EMT-inducer,[88] while p53 mutation enhances SLUG expression by opposing its degradation. Second, wt p53 directly represses hyaluronic acid receptor (CD44) gene expression, whereas mutant p53 overrides this effect.[89] Third, dominant-negative (dn) p53 enhances α5/β1-integrin and EGFR recycling, thereby promoting motility and invasion.[90]

As noted, loss of Rb and p53 mutation bypass oncogene-induced senescence.[58,91] Overexpression of TWIST proteins in BC was shown to rescue RAS-induced cellular senescence by suppressing the $p21^{Cip1}$ and $p16^{ink4a}$ promoters. Inhibition of these CDK inhibitors counteracts p53 and inactivates pRb, respectively.[39,40,92,93] Thus, in tumors with wt pRb and p53, expression of EMT-inducers bypass/shut down these tumor suppressors, thereby evading oncogene-induced senescence (**Fig. 1B**). In TNBC, RB1 and p53 loss/mutation allow cells to escape from senescence and undergo EMT through multiple mechanisms depicted in **Figure 1C**.

Interestingly, in a model of small cell lung cancer based on combined mutations in Rb and p53, the bulk of tumor is neuroendocrine, but a transition to mesenchymal type occurs in a subset of tumor cells in response to Ras activation.[94] The mesenchymal cells promote metastasis of the neuroendocrine cells, highlighting the dynamic interactions within different tumor clones and the indirect effect (in this case) of the mesenchymal component on cancer spread. Furthermore, these results suggest that additional oncogenic events (e.g., RAS activation) are required to promote EMT in Rb/p53-deficient cells.

Stem/Bipotent Cell-Specific Effect of Rb Loss

Mammary tumors developed in multiparous but not nulliparous MMTV-Cre:$Rb^{f/f}$

mice.[70] In contrast, mammary tumors were not formed in multiparous WAP-Cre:$Rb^{f/f}$ or WAP-Cre:$Rb^{f/f}$:$p107^{-/-}$ mice even after a 2-year follow-up.[70] WAP-Cre targets alveolar stem cells/progenitors during pregnancy.[95,96] Indeed, WAP-Cre:$ROSA26^{GFP}$ Cre-reporter mice exhibit GFP expression primarily in $CD24^{+}$ luminal progenitors and to a much lesser extent in $CD24^{+}$:$CD49f^{hi}$ mammary stem/bi-potent cells.[95] Consistent with this, we found that in multiparous WAP-Cre:$Rb^{f/f}$ mice the Rb^{f} allele was deleted in luminal progenitors but not in myoepithelial progenitors or stem/bipotent cells.[70] In contrast, the Rb^{f} allele was deleted in all three fractions from multiparous MMTV-Cre:$Rb^{f/f}$ mice, most efficiently in the stem/bipotent compartment. Thus, the effect of Rb inactivation is critically dependent on the cell within the mammary stem cell hierarchy in which it is deleted. $Rb^{\Delta f}$ alveolar progenitors may be refractory to Rb loss, or conversely, readily die upon differentiation in the absence of Rb. Analysis of double mutants with combined deletion of Rb and p53 via WAP-cre, currently underway, may resolve this issue.

The unique sensitivity of specific mammary cell types to RB1 gene loss has also been documented in a recent report from the Nikitin group.[97] In their study, Rb was deleted using a new MMTV-Cre line, which did not induce tumor formation in nulliparous females. It would be interesting to test the effect of pregnancy on tumorigenicity in these mice, and whether this MMTV-Cre line targets mammary stem/bi-potent progenitors or only luminal progenitors as WAP-Cre. Rb deletion via the Nikitin's MMTV-Cre line did accelerate tumorigenicity and altered the type of tumors that developed in combination with p53 deletion. Importantly, Rb loss in this model substituted for recurrent amplification on chromosome 9A1, which includes the anti-apoptotic genes cIAP1 and cIAP2, and the pro-survival factor Yap1,[98] suggesting that deregulation of these genes may be a prerequisite for tumor formation in cooperation with p53 mutation, hence potential therapeutic targets. The Rb/p53 double mutant tumors were heterogeneous and included but were not exclusively EMT subtype as observed following Rb/p53 deletion with our MMTV-Cre line.

As noted, embryonic $Rb^{-/-}$ mammary epithelial cells (placodes) do not develop mammary tumors following transplantation into recipient mammary glands.[68] Potentially, in contrast to acute inactivation of Rb in MMTV-Cre:$Rb^{f/f}$ mice, chronic inactivation as in transplantation experiments of $Rb^{-/-}$ embryonic placodes may activate compensatory genes like p107.[91] Inactivation of Rb in all epithelial cells such as in $Rb^{-/-}$ placodes may also deprive potential tumor cells from an epithelial-derived $Rb^{+/+}$ niche. Indeed, analysis of $Rb^{-/-}$:$Rb^{+/+}$ chimeric mice has demonstrated that $Rb^{-/-}$ mutant cells can survive and efficiently contribute to most adult tissues in the presence of wild-type cells.[99,100] Finally, MMTV-Cre:$Rb^{f/f}$ mice developed tumors only following pregnancy. While female mice transplanted with $Rb^{-/-}$ placodes were taken through pregnancy, the transplanted cells are not connected to the nipple and may therefore undergo aberrant lactation and involution that sensitize $Rb^{-/-}$ epithelial cells to apoptosis.

Addiction to Rb Loss

Tumor cells are often addicted to the oncogenic events that induced their transformation.[101,102] In the best studied system, Tet-MYC, it was shown that addiction to this oncogene is tissue/tumor-dependent and associated with escape from senescence, apoptosis, failure in ribosome biogenesis and protein synthesis[103-106] or with glutamine starvation.[107,108] We and others showed that overexpression of wild-type pRb delays proliferation of certain cells with low level of cyclin-dependent kinase activity, whereas other cells are refractory to re-expression of wild-type pRb, but sensitive to constitutively active, phosphomutant pRb.[55,109-112] Transduction of Rb/p53 tumor cells from our conditional Rb BC model with a retrovirus expressing wild-type pRb resulted in strong suppression of growth in vitro and tumor formation in vivo.[70] These tumors are therefore "addicted" to continual loss of pRb, indicating that despite their aggressive properties their tumorigenic phenotype can be inhibited, for example, by drugs that can mimic pRb function.

To understand how to mimic pRb function, it would be important to investigate how reintroduction of Rb blocks tumorigenicity of Rb-deficient tumors. It may simply suppress cell proliferation, but it may also induce cell senescence, cell death (in certain contexts), differentiation, mesenchymal-to-epithelial conversion or altered cell metabolism. Indeed, Rb-transduced Rb/p53 EMT tumor cells appear flattened and larger than control cells. In addition, Rb/p53 EMT tumor cells quickly acidify the medium, suggesting they secret lactic acid, the main byproduct of glycolysis. We therefore expect Rb-deficient EMT tumors to be inherently hypoxic and glycolytic, as is the case for TNBC.[17,80,81] Whether Rb/p53 tumors are hypoxic and glycolytic in vivo, and whether this is due to Rb loss is yet to be determined. Elucidating the basis for addiction to Rb loss may help guide therapeutic interventions to inhibit growth of Rb/p53 mutant TNBC.

Targeting the Rb-p53-EMT Circuit in TNBC

RB1 status dictates tumor response to therapy.[113] Thus, Rb-deficient tumor cells are more sensitive than isogenic Rb-proficient cells to chemotherapy but more resistant to hormonal-therapy. Currently, no specific therapy for Basal-like or Claudin-low BC is available and these patients are treated with combinations of cytotoxic drugs such as Docetaxel-Carboplatin,[114] Idarubicin-Prednimustine[115] and Cyclophosphamide-Methotrexate-fluorouracil 5FU (CMF).[116] Clearly, Basal-like and Claudin-low tumors are distinct subtypes and should be treated with specific regimens. Indeed, whereas Basal-like tumors are responsive to conventional chemotherapy, Claudin-low tumors are relatively resistant.[9,12,117,118] We envision that future treatment of TNBC will first require sub-classification into Basal-like, Claudin-low and other TNBC subtypes (e.g., metaplastic), followed by IHC analysis to assess pRb loss, and high $p16^{ink4a}$ and p53 expression (*dn*-mutation). Depending on results from these tests, specific therapy could be provided. The

following are experimental avenues to be explored in search for such therapies.

Targeting p53. TNBC (basal-like and Claudin-low) with *dn* point mutations in p53 may be highly sensitive to drugs such as PRIMA-1 (p53 reactivation and induction of massive apoptosis), which refold and restore tumor suppressor function to many dn-p53 mutant proteins. The PRIMA-1 analogs, APR-246, is currently assessed in clinical trials.[119-121]

Targeting Rb-deficiency. As the Rb pathway is not druggable; i.e., restoration of RB1 function following mutation or deletion is not feasible, an alternative approach is to mimic its function as discussed in the previous section, or, conversely, to specifically kill Rb-deficient tumor cells. For example, the E2F1 promoter is deregulated in RB1 deficient tumor cells and this may offer a therapeutic window to kill cancer but not normal cells.[122] Indeed, Rb-deficient cells express high levels of pro-apoptotic factors and are prone to apoptosis;[60] therapeutic induction of E2F1, other pro-apoptotic factors or treatment with inducers of apoptosis may tilt the balance in favor of apoptotic cell death.[60,123] Alternatively, CDK inhibitors may be used to suppress cell cycle progression in the absence of pRb through activation of p107, p130 and other targets.[113]

Chemotherapy. The RB1-signature in BC includes proliferation-associated genes that are targets of conventional chemotherapy such as TOP2A (doxorubicin, etoposide), thymidylate synthetase (5-FU), ribonucleotide reductase M2 (hydroxyurea) and CDK1 (flavopiridol, staurosporine). This may explain the increased sensitivity of RB-deficient cells to chemotherapy.[113] Drugs that target Claudin-low BC may be different than those that target Basal-like BC; small-molecule drug screens may identify such subtype-specific cytotoxic drugs.

Targeting cooperating oncogenic and metastatic networks. BC cells typically contain over a dozen independent mutations that cooperate to induce transformation. Identification of oncogenic networks that cooperate with mutations in Rb and p53 would offer new therapeutic targets. Such cooperating mutations can be identified by genomic and transcriptome sequencing, copy number analysis or through functional screens using lentishRNA[124] and transposon-based mutagenesis.[125] For example, a recent RNAi screen for genes that can transform Tag immortalized human mammary epithelial cells identified PTPN12 tyrosine phosphatase as a tumor suppressor of TNBC.[126]

Targeting EMT inducers. Given the connection between EMT and TICs, inhibition of EMT inducers may target the TIC fraction. As noted, knockdown of Twist genes in several mesenchymal and non-mesenchymal tumor types induced cellular senescence,[40] suggesting that targeting this signaling network may be a promising approach.

Targeting hypoxia and glycolysis. Finally, while hypoxia and glycolysis confer metastatic advantage and protection from chemotherapy, they also represent vulnerabilities to drugs that inhibit these processes or their consequences such as Unfolded Protein Response (UPR) and Autophagy. Given the effect of Rb loss on these processes,[127] Claudin-low and Basal-like BC may be particularly sensitive to such inhibitors.

Additional therapeutic strategies including drugs currently evaluated for the treatment of TNBC such as PARP and EGFR inhibitors should also be explored. Combination therapy toward the above targets will increase specificity, lessen side effects and reduce the likelihood that resistant variants could emerge, thereby preventing relapse.

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