p53 in breast cancer: mutation and countermeasures

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1. ABSTRACT

p53 is the primary arbiter of the mammalian cell's response to stress, the governor of life and death. It is the nexus upon which signals converge from an array of sensors that detect damage to DNA or to the mitotic spindle or the cytoskeleton, hypoxia, cell detachment, growth factor deprivation, oncogene expression and other forms of stress. Depending on the type, intensity and duration of the signals, p53 in turn transactivates batteries of genes specifying cell cycle arrest, DNA repair, apoptosis, or other anti-neoplastic functions. At the same time, p53 represses anti-apoptotic and survival functions. The type, intensity and duration of signaling dictate the sequellae. While this response is combinatorial, the frequent perturbation of p53 function in a wide spectrum of cancers attests to its central role in the suppression of neoplasia. As our understanding of regulation by and of p53 has deepened, many possibilities have been suggested for re-establishing p53 or its effectors in tumor cells. This review will briefly summarize the role of p53 mutations in the etiology and treatment of breast cancer and then consider the wide array of strategies being developed to re-establish p53 function in tumor cells.

2. INTRODUCTION

p53 is arguably one of the most important transcription factors in mammals even though it is entirely dispensable for early development and is nearly undetectable in normal cells in the absence of stress (1). Stimuli such as DNA damage or hypoxia cause p53 to accumulate in the nucleus and bind to the promoters of at least 300 different genes regulating such processes as cell cycle arrest, apoptosis, DNA repair and angiogenesis (2-4). The significance of this regulation *in vivo* is illustrated in a mouse p53 knockout model. All mice bearing the deletion perish by one year of age due to a multiplicity of cancers (1).

Signals of cell distress reach p53 primarily through post-translational modifications that influence its activity. The protein contains a central DNA binding domain sandwiched between N-terminal transactivation and C-terminal tetramerization domains (5). Its transcriptional activity is regulated by a host of stress-responsive and cell cycle-regulatory protein kinases, most of which phosphorylate the transcriptional activation domain (6, 7). Tetramerization, nuclear localization, and
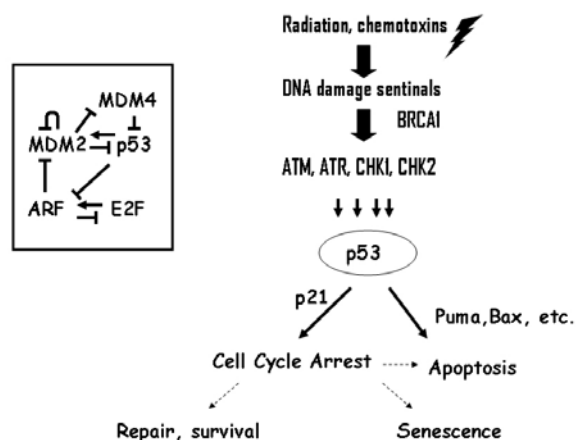
Figure 1. p53 signaling. A greatly simplified schematic of p53 DNA damage response. Lesions attract a complex of proteins including BRCA1 that initiate a protein kinase cascade (ATM etc.) leading to phosphorylation of p53 at multiple sites. The stabilized and activated p53 then binds to promoters of cell cycle-arrest effector genes like p21. Depending on circumstances, arrest may allow DNA repair and cell survival or induction of apoptosis or senescence. If the DNA damage is severe, apoptosis effectors such as PUMA, Bax and NOXA may be activated immediately. Inset, core feedback regulation of p53. Oncogene activation triggers E2F overexpression followed by ARF. ARF blocks MDM2 activity, permitting p53 to accumulate and induce apoptosis or senescence. Alternatively, balance can be restored as p53 activates MDM2 and blocks ARF; in turn, MDM4 blocks p53 activity. MDM2 also regulates itself and MDM4 by ubiquitination. Note that, although the human ortholog of MDM2 has been designated HDM2, for simplicity both are referred to here as MDM2, and no distinction is made between gene and protein names.

stability are regulated by C-terminal acetylation, methylation, ubiquitination, sumoylation, and neddylation (8-12).

Most relevant to this discussion is regulation of p53 stability. In brief, stability of p53 is tightly regulated in a feedback loop by the ubiquitin ligase MDM2, whose expression is in turn dependent on p53 {Figure 1; (13, 14)}. MDM2 activity is blocked by ARF, whose expression is dependent on E2F-1 transcription factor (15). To complete the loop, p53 inhibits ARF, and ARF blocks E2F-1 (16). These feedback loops are often disrupted in tumor cells by point-mutation, deletion, or gene-amplification of these regulators. (17-20). Recently, the importance of the MDM2 homolog MDM4 in this scheme has been established. MDM4 inhibits p53 transcriptional activity by direct binding (21, 22). In addition to destabilizing p53, MDM2 also destabilizes itself and MDM4, eventually freeing p53 to resume activity (23).

The activity and stability of p53 are also regulated by protein kinases such as CHK1, CHK2, ATM, and ATR, that respond to DNA damage sentinels such as BRCA1 (Figure 1). These kinases phosphorylate p53

directly and influence its stability and activity (24). Only a few examples are cited here that are relevant to the present topic.

3. p53 MUTATIONS AND BREAST CANCER

3.1. Familial

Familial cancer susceptibility syndromes reveal the critical role of p53-related signaling in tumorigenesis. An estimated 5-10% of breast cancers are due to germline mutations (25, 26). One of the best known syndromes is Li-Fraumeni, in which mutation of p53 itself predisposes to a broad range of cancers, including breast cancer, early in life (27). Analysis of numerous Li-Fraumeni kindreds has revealed many different mutations, mostly in the DNA binding domain (28). Loss of p53 in a mouse model produces a similar phenotype, normal development but early death due to multiple cancers (1).

Other familial syndromes stem from mutations in regulators of p53, especially those involved in sensing and signaling DNA damage. For instance, mutations in BRCA1 and 2 account for about one third of hereditary breast cancers (25). The protein kinases ATM and CHK2 activate p53 by phosphorylation of the N-terminus (29, 30). Mutations in ATM account for Ataxia-Telangiectasia, while CHK2 deficiency causes Li-Fraumeni-like syndrome. Interestingly, about 1% of the general population and 5% of breast cancer patients carry predisposing mutations in CHK2 (31). Another protein kinase, LKB1, interacts with and stabilizes p53, co-activating expression of the cell cycle inhibitor p21/WAF1 (32). Mutation of LKB1 causes Peutz-Jeghers syndrome (33, 34).

3.2. Spontaneous

At least one third of nonfamilial breast cancers bear mutations in p53 (35). These have been catalogued by the International Agency for Research on Cancer, which has assembled an online database of all known p53 mutations in various cancers and cancer cell lines, including 1400 mutations found in breast cancer (36). In contrast with other tumor suppressors, most of these mutations do not truncate the protein but rather are missense mutations in the DNA binding domain that inhibit the ability of the protein to bind to promoters (37). This property allows the mutant protein to behave as a dominant-negative inhibitor of co-resident wildtype p53 (36). A recently emerged nuance to this story is that there appear to be two classes of p53 target promoters, a high-affinity class comprising cell cycle arrest genes, and a lower affinity class comprising pro-apoptotic genes (38). Because it is in the tumor cell's interest to arrest cell division in response to DNA damage, some may re-program p53 to recognize the former class but not the latter (39-41). The detection of mutations in the DNA binding domain of p53 is associated with aggressive tumors and poor prognosis (42). Such mutations are about three times more frequent in tumors with a basal or high-ErbB phenotype than in tumors with a luminal profile (43).

As in familial breast cancer, mutations in signaling proteins that compromise p53 function are

common. The p53 inhibitors MDM2 and MDM4 are each amplified in 5-6 % of breast cancers (44, 45). These cells usually have wildtype p53, suggesting MDM2 and MDM4 as therapeutic targets (46). The stability and activity of p53 are also regulated by phosphorylation, and several phosphatases have been found to act on p53, including PP1, PP2A, PPMID and CDC14. PPMID is often amplified in breast cancer, especially in very aggressive tumors. Again, amplification is associated with the presence of wildtype p53 (47).

Another mechanism for constraining p53 is the loss of ARF. ARF inhibits Mdm2 activity by sequestering it in the nucleolus, thus preventing it from degrading p53 (48-51). The loss of ARF also prevents oncogene-induced senescence (52). Mice lacking ARF are highly susceptible to breast cancer (53).

4. RE-ESTABLISHING p53 FUNCTION IN TUMOR CELLS

Tumor suppressors are therapeutically challenging, both teleologically and practically. While oncoproteins can be targeted for inhibition by libraries of small molecules, it is the absence of the tumor suppressor that is pathogenic. The only therapeutic options are to either re-establish expression of the wildtype tumor suppressor or somehow compensate for its loss in the tumor cell. However, p53 is an exception to this rule for two reasons. First, p53 protein is nearly always present in some form in tumor cells, either mutant or wildtype. Second, p53 is negatively regulated by oncoproteins that *can* be targeted.

Thus current therapeutic approaches may target MDM2 to restore p53 to effective levels; re-activate mutant p53; or re-introduce wildype p53 by various strategies. These approaches are supported by recent studies showing that overexpression of p53 is sufficient to kill most breast cancer cell lines *in vitro* (54), and re-expression in p53-null tumors in mice drives tumor cells into senescence or apoptosis (55-57).

4.1. Relieving wild-type p53 from inhibition by MDM2
Under normal physiological conditions, the stability of p53 is tightly regulated by MDM2, resulting in a half-life of only twenty minutes. MDM2 binds to the N-terminal domain and ubiquitinates several positions at the C-terminus (58), thus causing p53 proteosomal degradation (59). In addition, by binding to the transactivation domain of p53, MDM2 inhibits its transcriptional activity (60). As discussed earlier, p53 and MDM2 form an autoregulatory feedback loop, each controlling the concentration of the other, and tumor cells can neutralize p53 simply by overexpressing MDM2 (61). Inhibitors of MDM2 are at various stages of development.

4.1.1. Peptides
The p53-MDM2 interaction region was first identified in yeast two-hybrid screens (62) and in immunoprecipitation experiments (63). Site directed mutagenesis clarified the importance of p53 residues

Leu14, Phe19, Leu22, Trp23 (64). Early studies focused on p53-derived peptides to inhibit p53-MDM2 interaction (59, 65). Stabilized peptides were observed to induce cell cycle arrest in HCT116 cells and apoptosis in SJSA-1 and JAR carcinoma cell lines (66). Fusion with a transducible Tat peptide was effective in stabilizing p53 and inducing cell death in a rabbit model of retinoblastoma (67).

4.1.2. Small molecule inhibitors
Small molecule inhibitors in principle can circumvent the delivery and stability problems of macromolecules. The best known of these are the nutlins. Nutlins are cis-imidazoline derivatives that bind to the p53 pocket on MDM2, displacing p53 from the p53-Mdm2 complex (68). The effect of nutlins is only observed in cells with wild-type p53, suggesting that nutlin's action is exclusively through the p53 pathway. Crystallographic analysis of the nutlin-MDM2 complex shows that nutlins project functional groups into the binding pocket and thus mimic the three critical p53 amino acids (Phe19, Trp 23 and Leu26) required for interaction between p53 and MDM2. The most active isomer, nutlin 3a, can induce p53-mediated apoptosis through both transcription-dependent and transcription-independent pathways in spite of low levels of ATM or high levels of Mdm2 (69). In retinoblastoma cells, nutlin 3a was found to increase p53 and p21 protein levels, and p53-mediated apoptosis occurred in a dose dependent manner (70). Nutlins are most effective against tumor cells that retain wildtype p53 and have normal to high levels of Mdm2 (71). However, Mdm2 interacts with proteins other than p53, e.g., HIF-1alpha; nutlin 3a was recently found to block this interaction, resulting in inhibition of the HIF1alpha target gene VEGF (72).

An older class of such compounds is the norbornanes. Like nutlins, these agents compete for the hydrophobic pocket of MDM2 normally occupied by p53. These compounds showed moderate activity in the MCF-7 breast cancer cell line and in other cell lines. The derivative Syc-7 showed about 5-fold selectivity between MCF7 cells and NEC normal cells; it stimulated p53 and p21 accumulation and hence apoptosis (73).

Another class of inhibitors that bind to the same pocket is the chalcones, derivatives of phenoxy acetic acid and phenoxymethyl tertazole (74). These inhibitors were of low to moderate affinity in blocking p53 and MDM2 interaction, but a boronic acid derivative had a much higher binding affinity (75).

The fungal metabolite chlorofusin binds to the N-terminus of MDM2 but the exact mechanism has not been determined. Its *in vivo* efficacy was low (76, 77).

RITA, a novel furan derivative, binds to p53 and changes its conformation in the N-terminal region. This makes p53 incapable of binding to MDM2 and hence bypasses MDM2-driven degradation (78).

An alternative approach is to inhibit the E3 ligase activity of MDM2. Lai *et al.* reported 3 molecules, an

anilidosulfonamide, a bis- (amidinophenyl)-urea and a benzoylimidazolone which antagonize p53 ubiquitination by inhibiting E3 ligase activity of MDM2 (34).

4.1.3. Antisense and siRNA

MDM2 has also been attacked at the level of gene expression, first by antisense technology and now by RNA interference. Antisense oligos interfere with RNA splicing, translation and/or transport by binding to 5' cap region, 3' poly A tail or the splicing site of pre-mRNA (79).Various antisense oligonucleotides, modified to improve cellular uptake and reduce degradation by nucleases have been tested (80). Oligo AS5 (phosphodiester oligonucleotide or PO-OLIGO) binds to the translation start codon of MDM2 and decreases the MDM2 protein levels by 3-5 fold at concentration of 100-400nm (81). The p53 protein level and activity were increased following antisense treatment. Like the small molecule inhibitors, antisense oligonucleotides increased apoptosis in a p53-dependent manner (78, 82). Mixed backbone oligonucleotides reduced tumor growth in MCF-7 mouse xenografts and increased the efficacy of chemotherapeutic agents (83-85).

RNA interference by siRNAs has been much more successful at suppressing gene expression in mammals than antisense strategies, although stabilization and delivery methods require further optimization. Nevertheless, the use of siRNA to suppress MDM2 has proven effective *in vitro* and was recently used successfully to treat breast cancer *in vivo* (39, 77).

4.2. Restoring function to mutant p53

Mutant p53 is often present at high concentrations in tumor cells because p53 mutation disrupts a negative feedback loop (Figure 1). Mutant p53 cannot transactivate MDM2; lack of MDM2 ubiquitin ligase activity leads to accumulation of p53. In addition, certain mutations render p53 resistant to MDM2-mediated destabilization (86). Thus reactivation of mutant p53 may trigger massive apoptosis in p53-mutant cells. Most such mutants bear missense mutations in their DNA binding domains, and several compounds that redress such lesions have emerged from pharmaceutical libraries.

4.2.1. PRIMA-1

PRIMA-1 (p53 reactivation and induction of massive apoptosis -1) is a small molecule (2,2-Bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one) that restores normal function to mutant p53 by unknown mechanisms. PRIMA-1 induces massive apoptosis in a variety of tumor cells including the breast cancer cell line MDA-MB231 (87-89). PRIMA-1 restores the wild-type conformation to mutant p53 both in the test-tube and in living cells. It also prevents unfolding of p53 upon heating at 37°C (90). The pro-apoptotic action of PRIMA-1 seems to require mutant p53, as it induces the expression of the p53 target genes p21 and MDM2 only in mutant p53 cell lines (91).

4.2.2. CP-31398

CP-31398 is a styrylquinazoline derivative that stabilizes a subset of mutant p53 molecules and restores

native conformation (92-95). Treatment with CP-31398 causes cells to undergo either cell cycle arrest or apoptosis. It blocks the ubiquitination and degradation of p53 thus increasing the p53 level, but this action is not observed in human papillomavirus cells expressing the p53-binding protein E6 (96). Unlike PRIMA-1, CP-31398 is also active against cells expressing wildtype p53, sensitizing them to chemotherapeutic drugs (97-99). For example, it was active against melanomas and carcinomas carrying mutant p53 in a xenograft model (100).

4.2.3. Maleimide

The maleimide derivatives MIRA-1 and MIRA-2 have been shown to restore the active conformation to mutant p53 and preserve the activity of wildtype p53 upon heating. Treatment of tumor cells with these reagents induces p21, MDM2 and PUMA in mutant p53-dependent manner. MIRA-3 was effective against p53 mutant tumors in a mouse xenograft (101).

4.2.4. HDAC inhibitors and p53

One means by which the cell stabilizes p53 is acetylation of its C-terminus. Accordingly, several histone deacetylase (HDAC) inhibitors have been tested for the ability to stabilize p53. One such inhibitor, depsipeptide FR901228 has been shown to increase p53 acetylation at K373/K382 which is accompanied by recruitment of p300 to the p53 C-terminus and induction of p21 expression. The acetylated p53 has a longer half-life due to a significant decrease in p53 ubiquitination (102).

Another study has shown that histone deacetylase inhibitors such as FR901228 and trichostatin A were preferentially cytotoxic to cells with mutant p53. It has been suggested that HDAC inhibitors initiate degradation of mutant p53 either by restoring or mimicking p53 transactivation functions (103). Upon DNA damage, sudden restoration of p53-like function by HDAC inhibitors is highly cytotoxic to cells with mutant p53.

4.3. Re-introduction of p53 by gene therapy

A Holy Grail of gene therapy is to re-establish p53 or indeed any tumor suppressor gene in all of the cells of a tumor. As recent studies have shown, performing this molecular genetic trick using the Cre-Lox system in p53-deficient tumors in mice prompted tumor regression in all cases (104-106). However, the technology to accomplish this in human tumors requires further maturation.

4.3.1. Retrovirus-mediated gene therapy

As a delivery vehicle, retroviruses have the advantage of producing long term expression, at least theoretically. Their integration into the genome requires the infected cell to be in S phase, but cancer cells are usually cooperative in this regard. A bigger drawback is that viral integration is random and therefore potentially mutagenic; the use of retroviral therapy to treat an immunodeficiency was halted after therapy-induced leukemias emerged (107). In addition, transduction of p53 cDNA with retrovirus vectors produces mutations due to retroviral replication errors, which limits the efficacy of retroviral delivery (108). Nevertheless, the method has been useful in establishing

the efficacy of viral vectors against tumor cells *in vitro*. For example, retroviruses encoding p53 produced growth arrest or apoptosis in p53-deficient H358 lung cancer cells and in a human glioma cell line while having no effect on p53-wildtype cells (109).

4.3.2. Adenovirus-mediated gene therapy

In contrast to retroviruses, adenoviruses do not integrate but persist as an episome and can generate high expression levels. They have a very broad host range, but infectability does vary considerably among breast cancer cell lines (110). Hematopoetic lineages lack receptors for the adenovirus serotype and hence cannot be infected without re-engineering of the adenovirus-vector (111). In addition, adenoviruses commonly infect human airways, so adenoviral administration *in vivo* rapidly generates a strong immune response, severely compromising its utility as a gene-therapy agent (112). Various strategies for evading this obstacle have been developed, and adenoviral therapies are in clinical trials (102, 113, 114).

Adenoviruses expressing p53 from a CMV promoter have been employed analytically to identify p53-induced genes and to determine the range of tumor genotypes that are capable of responding to restored expression of p53 (115, 116). A commercial version of this virus has been developed by Introgen for cancer therapy. Advexin (INGN 201) is a replicatively impaired recombinant E1-deleted serotype 5 adenoviral vector that was very effective *in vitro*. Treatment of p53 null PC-3 cells induced high levels of p53 and p21 gene expression *in vitro* and inhibited tumor growth in nude mice following orthotopic injection. (117, 118). Early clinical trials demonstrated safety and limited efficacy against a range of cancers, and Advexin is now in Phase III trials in the US (119-121). Meanwhile, a similar construct called Gendicine produced by SiBiono (Shenzhen) has been approved for use against a broad range of cancers in China. Its efficacy is difficult to evaluate (122).

Another gene therapy approach targets p53-deficient cells by exploiting adenoviral biology. Adenovirus EIA protein has been shown to bind pRb, bypassing the pRb check point and thereby releasing free E2F (123). EIA also induces the expression of p14ARF, which inhibits MDM2 and promotes subsequent accumulation of active p53 protein in the nucleus (124). This leads to cell cycle arrest and apoptosis via the p53 target gene p21 and BAX protein, respectively (125, 126). To circumvent this problem, the adenovirus EIB region encodes a 55KDa protein which binds and inactivates p53 in infected cells (127-129). It also exports p53 to the cytoplasm (130) where p53 is degraded by another viral protein, E4 orf6 (131).

Onyx-015 adenovirus is a human group C adenovirus that contains an 827bp deletion in the E1B region and a point mutation that generates a stop codon preventing expression of truncated E1B-encoded 55KDa protein (132). Thus Onyx-015 is incapable of binding to p53 and neutralizing it. The end result is that the virus can replicate only in cells that lack p53 function. These cells propagate the virus and lyse while normal cells are unfazed (133). The system is teleologically appealing because it is effective on p53-deficient cells regardless of how they came to be that way.

Onyx-015 was effective *in vitro* against cervical and colon carcinoma, glioblastoma, and pancreatic adenocarcinoma cells lacking functional p53, with an efficiency comparable to wild-type adenovirus (134, 135). The growth of mouse xenografts was also inhibited (123, 136, 137).

The Onyx viruses were developed and shepherded through clinical trials by Onyx Pharmaceuticals. Against head and neck cancers, Onyx-015 reduced tumor size by about two-thirds (138), and stable disease was achieved in about half of a cohort of chemo-resistant colorectal cancer patients (139). Still, the drug failed to gain approval from the FDA due to insufficient evidence of increased patient survival. However, the virus has since been resurrected as "H101" by Sunway Biotech (Shanghai) and is presently undergoing clinical trials in China (111, 140).

5. CONCLUSIONS AND PERSPECTIVES

After being mistaken for an oncogene for the first ten years following its discovery, p53 has come to be recognized as the central player in stress response and tumor suppression. Consequently, the p53 literature is expanding so fast that every waking moment could be spent reading about new facets of p53 biology.

This focus on p53 is vindicated by recent developments using mouse models. Three groups have demonstrated independently that the restoration of p53 expression in various tumor models causes regression by apoptosis and senescence, leading to clearance by the immune system (141-143). These results validate the p53-restorative strategies described herein. In addition, these studies should finally lay to rest the persistent assertion that senescence is an *in vitro* artifact irrelevant to tumor response.

However, a related mouse model has thrown cold water on the efforts to restore p53 by inhibiting MDM2. Mice lacking both MDM2 and p53 develop normally, but if p53 expression is restored during adulthood, the now unbuffered p53 activity in normal cells is frequently lethal, leading to ablation of multiple tissues (144). Of course, it may be possible to circumvent this problem by modulating dosage and timing.

The ability to regulate p53 expression at will *in vivo* has led to another therapeutically relevant surprise. The pathology caused by chemotherapy and subsequent p53 damage-signaling is apparently unnecessary for tumor suppression. Christophorou *et al.* discovered that irradiation-induced pathology could be prevented by keeping p53 expression off until a few days following irradiation; this delay did not compromise the ability of p53 to suppress lymphoma (145).

From both biological and therapeutic perspectives, one of the most interesting frontiers of p53 research is the interplay between p53 and its sister proteins p63 and p73. All three have multiple splice variants, some with very different properties and different patterns of expression in normal and neoplastic tissues, and there is evidence for both functional antagonism and redundancy (reviewed recently by Murray-Zmijewski *et al.* (146)). These intra-family dynamics add yet another layer of complexity to be considered.

A fast-closing frontier is the characterization of the Pifithrome, the full retinue of genes that are activated or repressed by p53. Prodigious efforts have resulted in the identification of at least 300 genes that are regulated up or down by p53 (147, 148). These efforts were motivated by the rationale that, if downstream pro-apoptotic targets are identified that can somehow be reactivated in cancer cells by a small molecule approach, then p53-mutant cells could be re-sensitized to chemotherapy. Finding the right target and the right drug could obviate efforts to restore p53. Since many p53 targets are cell type-specific, the most effective treatment for breast cancer would likely continue to differ from that for other tumor types.

Finally, the simple but hard-won model of p53 as a tumor suppressor is under revision. This was prompted by the realizations that p53 can distinguish between cell cycle target genes and those devoted to apoptosis, and that the ability to arrest cell division in the face of massive genomic insult is in the interest of the tumor cell, in contrast to its ability to apoptose. Indeed, expression of the cell cycle target p21 is often robust in cancer cells and is almost never lost (149). Thus, inhibitors of p53 that prevent arrest may be useful in some situations to sensitize damaged p53-positive tumor cells to mitotic catastrophe (150). This complexity and enduring capacity for novelty ensure that the study of p53 signaling will continue to be one of the most productive and exciting interfaces between basic and translational cancer research for some time to come.

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