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Tumor suppression by the EGR1, DMP1, ARF, p53, and PTEN network

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Abstract

Recent studies have indicated that EGR1 is a direct regulator of tumor suppressors including TGF β 1, PTEN, and p53. The Myb-like transcription factor Dmp1 is a physiological regulator of the Arf-p53 pathway through transactivation of the *Arf* promoter and physical interaction of p53. The *Dmp1* promoter has binding sites for Egr proteins, and *Egr1* is a target for Dmp1. Crosstalks between p53 and Pten have been reported. The Egr1-Dmp1-Arf-p53-Pten pathway displays multiple modes of interaction with each other, suggesting the existence of a functional network of tumor suppressors that maintain normal cell growth and prevent the emergence of incipient cancer cells.

Keywords

EGR1; DMP1 (DMTF1); ARF; p53; PTEN; tumor suppression; signaling; transcription; nucleus; nucleolus

Introduction

The *Early Growth Response 1 (EGR1)* gene encodes a zinc-finger transcription factor of 59 kilo Daltons (kDa), a nuclear protein that contains three zinc fingers of the Cys₂His₂ subtype. The EGR1 GC-rich consensus sequence, 5'-GCG(T/G)GGGCG-3' and 5'-TCC(T/A)CCTCCTCC-3', have been identified in the promoter regions of genes for transcription factors that mediate cell growth, differentiation, or apoptosis (1–3). EGR1 can compete with the transcription factor SP1, which binds to extremely GC-rich sequences (so called 'SP' box), and is involved in the constitutive expression of housekeeping genes (4, 5). Because the consensus sequence for SP1 and EGR1 binding overlaps, EGR1 often displaces SP1 from gene promoters (6, 7). The gene targets mediated by Egr1 in response to ionizing radiation (IR) include TNF α , p53, Rb and Bax, effectors of apoptosis. Based on these, Egr1 is a pivotal gene that initiates signal transduction events in response to IR leading to either growth arrest or cell death in tumor cells. EGR1 also controls tissue repair, wound healing,

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Conflicts of Interest

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fibrosis, atherosclerosis, and other stress-related responses (2). Finally, EGR1 regulates cell response to hypoxia, promotes the formation of new blood vessels, and triggers tumor angiogenesis (2).

The transcriptional activity of EGR1 is inhibited through direct interaction with the NF- κ B-binding proteins named NAB1 and NAB2 (8). Mice lacking both *Nab1* and *Nab2* show severe congenital hypomyelination of peripheral nerves, with Schwann cell development arresting at the promyelinating stage (8). Their expression is also inducible, albeit delayed in comparison to Egr1 induction (8), creating an early negative feedback to ensure that the Egr1 activity is transient. Hence deregulated expression of NAB proteins in disease may contribute to alteration of EGR1 function. For instance, elevated expression of NAB2 in endothelial cells reduces angiogenesis whereas loss of NAB2 in prostate cancer contributes to increased EGR1 activity (9).

EGR1 protects normal cells from transformation by inducing apoptosis or growth arrest upon DNA damage (1–3). A strong evidence for EGR1's pro-apoptotic activity is that *Egr1*^{-/-} mouse embryo fibroblasts (MEFs) are resistant to apoptosis induced by IR (10, 11). Although *Egr1*-deficient mice do not spontaneously develop tumors, they show accelerated tumor growth in a two-step carcinogenesis model of skin cancer (12). UV-B radiation of keratinocytes induces EGR1 expression through activation of NF- κ B (p65/RelA), which mediates apoptosis and acts as a protection mechanism against the tumorigenic effect of UV-B. These observations support the hypothesis that EGR1 inhibits the growth of DNA damage-induced tumors. Thus *EGR1* has been considered to be a tumor suppressor gene rather than an oncogene (10–13; reviewed in 14; Fig. 1).

Two independent mechanisms, i.e. the induction of the epithelial cell suppressor TGF β 1 and induction of p53 family proteins, have been pointed out to explain the tumor-suppressive properties of EGR1 (14, 15; Fig. 1). Numerous other genes that affect cell growth such as *Insulin-like Growth Factors (IGFs)*, *TGF β* , *Fibronectin*, *PDGF*, *PTEN*, *WT1*, *Dmp1*, *Arf*, and *p53* genes have consensus sequences for EGR1 on the promoter suggesting direct binding of EGR1 and transactivation (14–21). Both the *Dmp1* and *Arf* promoters have binding sites for Egr1/2/3 (19; Fig. 2a) and our microarray analysis has shown that Egr1 is a target for Dmp1 (20). It has thus been hypothesized that Egr1 participates in the regulation of tumor suppressor gene networks involving these proteins, especially Dmp1, Arf, and p53 (Fig. 1). Any defects in this network may lead to the unopposed action of EGR1 on TGF β 1 that favors tumor progression. In this review, we discuss the importance of these molecules that have potential tumor suppressive activities.

Dmp1, a cyclin D binding myb-like protein 1 (Dmp1; also called Dmtf1), was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait (23, 24; 25–29 for reviews). Dmp1 physically interacts with any of the three D-type cyclins, each of which can inhibit its ability to bind to DNA (23, 24). Biological studies indicated that overexpression of Dmp1 causes cell cycle arrest in NIH 3T3 cells, the activity of which was attenuated by D-type cyclins independent of Cdks (24). Dmp1 (Dmp1 α) shows its activity as a tumor suppressor by directly binding to the *Arf* promoter to activate its gene expression, and thereby induces Arf- and p53-dependent cell cycle arrest (19, 30,

31; 32–36 for Ink4a/Arf reviews). Other transcriptional targets for Dmp1 include *CD13/APN* (37), *Areg*, *Thbs1*, *JunB*, and *Egr1* (20), suggesting that it is involved in signal transduction, cell proliferation, apoptosis, and angiogenesis. The activity of the Arf-p53 pathway is significantly subverted in *Dmp1*-deficient cells since they can easily give rise to immortalized cell lines that retain wild type p19^{Arf} and wild type p53 and are transformed by oncogenic Ras alone (38, 39). The murine *Dmp1* promoter is activated by oncogenic Ras as well as by constitutively active MEK1/2 and ERK1/2 in primary culture cells (19). Thus, Dmp1 is a key mediator between Ras-Raf-MEK-ERK mitogenic signaling and the Arf-p53 tumor suppressor pathway. Both *Dmp1* and *Arf* mRNA levels decrease at S-G2/M phase of the cell cycle since promoters are repressed by mitogenic signals mediated by E2Fs (40). Both promoters are repressed by and genotoxic signals mediated mainly the p65 subunit of by NF- κ B (41). Dmp1 (Dmp1 α) physically interacts with p53 to neutralize all the activities of Mdm2 on p53, indicating that this is the secondary mechanism of tumor surveillance in *Arf*-deficient cells (42, 43). Recent studies show that Dmp1 also transactivates *p16^{Ink4a}* although the effect is less than stimulation of *Arf* (44, 45).

Both *Dmp1*^{-/-} and *Dmp1*^{+/-} mice are prone to tumor development when neonatally treated with dimethylbenzanthracene (DMBA) or by IR (38, 39). Tumors induced by the *E μ -Myc* (39), *K-Ras^{LA}* (46), *neuNT* (activated mutant) (47), or *cyclin D1* (44) transgene were greatly accelerated in both *Dmp1*^{+/-} and *Dmp1*^{-/-} background with no differences between groups lacking one or two *Dmp1* alleles, suggesting a haploid-insufficiency of Dmp1 in tumor suppression (29, 39). Moreover, tumors from *E μ -Myc*, *K-Ras^{LA}*, or *MMTV-neuNT* mice rarely show *p53* mutation or *Arf* deletion, indicating that Dmp1 is a physiological regulator of the Arf-p53 pathway *in vivo* (39, 46, 47). The details of tumor-prone phenotypes in *Dmp1*-deficient mice, and the mechanisms for tumor suppression, their roles in human cancer are discussed in this review.

The tumor suppressive function of EGR1 is mediated by two independent pathways – i.e. TGF β - Fibronectin (FN) - Plasminogen Activator Inhibitor-1 (PAI-1), and DMP1-ARF-p53-PTEN signaling cascades (Fig. 1). We will focus on the latter since the former has been reviewed by Baron et al. (14).

Egr1 is a tumor suppressor *in vivo*

There have been two conflicting reports about the phenotypes of *Egr1*-deficient cells in culture (10, 48). Lee et al. reported that ES cells lacking *Egr1* exhibited similar growth rates and serum-induced gene expression profiles compared to *Egr1*^{+/+} cells (48). They were capable of differentiating into neurons, cardiac myocytes, chondrocytes, and squamous epithelium, suggesting minimal phenotypes caused by *Egr1* deficiency (48).

However, another report from a different lab reported that *Egr1*-null MEFs bypassed replicative senescence, exhibited a loss of DNA damage response and an apparent immortal growth, suggesting loss of p53 functions (10) in the absence of Egr1, just like *Arf*-null or *p53*-null cells (49–52). The tumor-suppressive function of *Egr1* has been demonstrated *in vivo* through the analyses of gene knock-out mice. Both wild type and *Egr1*-null mice were challenged with the two-step skin carcinogenesis model by treatment of skin with the mutagen DMBA, followed by treatment with the tumor promoter tetraphorbol acetic acid

(TPA) (12). Wild type mice developed tumors on an average 11 weeks after TPA induction. In contrast, *Egr1*-null mice exposed to the complete protocol (DMBA + TPA) developed tumors with an average time of appearance of 6 weeks, indicating that *Egr1* is a novel tumor suppressor *in vivo* (12). Spontaneous tumor development that is frequently observed in *Arf*-deficient mice (49, 50) has not been reported *Egr1*-deficient mice despite the striking phenotypes of *Egr1*^{-/-} MEFs (12). In summary, *Egr1*-deficient cells are prone to tumor development although the phenotypes are weaker than those observed in *Arf*-deficient cells.

Decreased expression of EGR1 in human cancer

EGR1 protein levels are decreased or absent in a variety of tumors including breast cancer (BC; 53), lung cancer (54–56), glioma (57), hepatocellular, and esophageal cancers (58). It has been reported that low level of EGR1 expression is associated with poor clinical outcome and resistance to therapy secondary to loss of pathways such as PTEN (54). Consistent with the finding in lung cancers, EGR1 decreases the malignant potential of human non-small cell lung carcinoma by regulating KRT18 expression (59).

Loss of chromosome 5, or a del(5q), is a recurring abnormality observed in 10 % of patients with a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) arising *de novo*, and in 40 % of patients with therapy-related MDS or AML (t-MDS/t-AML) (60–63). This led to the idea that 5q harbors one or more tumor-suppressor genes that have fundamental roles in the growth control of hematopoietic stem/progenitor cells. Cytogenetic mapping of commonly deleted regions (CDRs) centered on 5q31 and 5q32 identified candidate tumor-suppressor genes, including the ribosomal subunit *RPS14*, the transcription factor *EGR1*/*KROX20* and the cytoskeletal remodeling protein, *α-Catenin*, and *microRNAs 145, 146a* (62–66). Of note, the *EGR1* gene has been mapped to human chromosome 5q31, and thus has been implicated in the pathogenesis of human hematopoietic malignancies (60, 65, 66).

Paradoxical overexpression of EGR1 in prostate cancer

In good contrast to decreased expression of EGR1 in the majority of human malignancies, many observations support the notion that EGR1 contributes to prostate cancer progression (67–70). In prostate adenocarcinoma, the mRNA encoding *EGR1* is expressed at higher levels compared with their normal counterparts. The observation that levels of EGR1 expression correlate with Gleason scores and inversely correlate with the degree of differentiation of carcinoma cells also suggests that EGR1 is involved in cancer progression in the prostate (22). Mutation of p53 is associated with advanced prostate cancer as well as with metastasis and hormone independence. Sauer et al. showed that Egr1 overexpression correlated with an alteration of p53 activity caused by SV40 large T antigen or mutant p53 (71). In cells with altered p53 activity, Egr1 expression was abolished by pharmacological inhibition or RNAi silencing of p53. Although forced expression of wild type p53 was not sufficient to stimulate *Egr1* transcription, mutant p53 induced Egr1 (71). *Egr1* transcription was driven by the ERK1/2 pathway since it was abrogated by specific inhibitors of MEK. Induced Egr1 protein, in turn, increased the transcription of epidermal growth factor receptor (EGFR) ligands and downstream MEK/ERK cascades. Thus, mutant p53 initiates a feedback loop that involves ERK1/2-mediated transactivation of Egr1, which increases the secretion

of EGFR ligands (71). In short, EGR1 overexpression and mutant p53 are closely linked in prostate cancer progression.

EGR1 expression in the primary tumor correlates with radiation response in terms of control of the local tumor (72, 73). Therefore it should be an indicator for favorable therapeutic response. However, in the post-irradiated biopsies, EGR1 expression correlates with residual tumor and treatment failure (22, 72, 73). In this case, NAB2, the regulatory protein that represses the transcriptional activity of EGR1, is downregulated in human primary prostate carcinomas (8, 74, 75). Interestingly, EGR1 is overexpressed in the tumors of CR2-TAg mice where T antigen is expressed under the control of the *cryptidine 2 (CR2)* promoter and NAB2 expression is decreased (22, 67). Thus, both upregulation of EGR1 and loss of its repressor, NAB2, contribute to increased EGR1 activity in prostate cancer. It is hypothesized that when these two things happen together in cells with p53 dysfunction, the EGR1 expression behaves an invasive factor rather than tumor suppressor explaining why it is associated with radiation treatment failure. Overexpression of EGR1 in prostate cancer should be re-evaluated from the viewpoint of p53 because EGR1 (and also other p53-binding proteins) may stimulate mutant p53 associated with increased stem cell-ness, invasiveness/metastasis, and therapy resistance (76).

Tumor suppression by EGR1 – possible mechanisms

It has been reported that EGR1 overexpression increases the secretion of growth factors, cytokines, extracellular matrix proteins, and proteases (1–5). Conversely, EGR1 mediates growth factor-induced downregulation of E-cadherin by inducing an E-cadherin transcription repressor, Snail or Slug, which contributes to tumor invasion (77). A mutation of EGFR commonly found in lung cancer cells causes EGR1 overexpression and activation through activation of the ERK pathway (70, 78–80). Similarly, a mutation of B-RAF present in a high percentage of melanoma results in constitutive activation of ERK and upregulation of EGR1 (81). Thus constitutive activation of the ERK pathway in tumor cells appears to be a cause of high EGR1 expression caused by genetic defects affecting upstream regulators of the ERK signaling.

Sixty-six % of human melanomas have a mutation in the *B-RAF* gene where EGR1 is overexpressed (81, 82). Since RAF acts upstream of the ERK pathway, activating mutations of *B-RAF* result in ERK1/2 activity and EGR1 overexpression as described above. Importantly, constitutive RAF-MEK-ERK activation results in the induction and activation of the Dmp1-Arf-p53 pathway which should be beneficial for preventing incipient cancer cells undergo neoplastic transformation (19, 25; Fig. 1). Since there are extensive crosstalks between EGR1 and Dmp1-Arf-p53 pathway (explained below in detail), overexpression of EGR1 in cancer cells should have more favorable than ominous effects to predict the outcome of patients.

Tumor suppression through DMP1 and ARF

Published studies indicate extensive crosstalks between the Dmp1 and Egr1 since the *Dmp1* promoter has Egr1/2/3 sites in the proximal region (19; Fig. 2a) and *Egr1* gene expression was downregulated in the lungs from *Dmp1*^{-/-} mice (20). Both human and mouse *Arf* genes

are direct transcriptional target for Dmp1 (Dmp1 α) (24). The *Arf* promoter has numerous binding sites for Egr1 (Inoue & Fry, unpublished data; ref. 30 for the *Arf* promoter sequence), indicating an essential role of Egr1 in Arf-mediated tumor suppression. We also found direct physical association between Dmp1 α and p53 in response to oncogenic and DNA damaging stress signaling (42), which must be important because physical interaction between Egr1 and p53 has been reported (71, 83). The tumor suppressive mechanisms of Dmp1 α are explained in detail later in a separate chapter.

The c-Myc protein is frequently (~80 %) overexpressed in human cancers regardless of the tissue of origin. Since it controls expression of hundreds of coding and non-coding genes that affect cell cycle entry, proliferation, differentiation, metabolism, and survival/death decisions of cancer cells, it is an excellent target for cancer therapy (84–86). Although deregulated c-Myc leads to tumor growth, it also triggers apoptosis by stimulating the Arf-p53 pathway to protect against unrestrained proliferation (87). Both c-Myc and Arf have apoptotic activities independent of p53 (88–90; reviewed in 91). In cells without p53, Arf directly binds to c-Myc protein and inhibits c-Myc-induced hyperproliferation and transformation with a concomitant inhibition of canonical c-Myc target gene induction (89). Consistently, Bonne et al. (92) showed that Arf was necessary for c-Myc to drive transcription of a unique, non-canonical target gene, *Egr1* (92). In contrast, c-Myc induced *Egr2* through a canonical mechanism that was inhibited by Arf (92). They further demonstrate that Egr1 is essential for p53-independent c-Myc-induced apoptosis. Therefore, Arf-binding switches the activity of c-Myc from a proliferative to an apoptotic protein independent of p53 through a unique non-canonical transcriptional mechanism (92, 93). Of note, Egr proteins play critical roles in the Arf-C/EBP β pathway, which is essential in oncogene-induced senescence (94), suggesting the universal role of Egr1 in eliminating incipient cancer cells to act as a tumor suppressor.

The nucleolus is a multi-domain enriched with proteins involved in ribosome biogenesis, cell cycle and apoptosis control, differentiation of stem cells and malignant transformation (34, 88, 90, 91). Arf is mainly localized to the periphery of nucleolus where is able to negatively regulate ribosome biogenesis (34). It has been shown that Arf promotes sumoylation of several interacting proteins such as topoisomerase I, Mdm2, p53, and Egr1 (95–97). Upon interaction with ARF, EGR1 acquires the ability to bind to specific targets such as PTEN through sumoylation, which, in turn regulates cell growth (98; the role of ARF in EGR1-PTEN pathway is discussed below in the PTEN section). Since EGR1 colocalizes with ARF under IGF-1 stimulation, they tested whether EGR1 also relocates to the nucleolus to interact with ARF (98). They showed that EGR1, mainly in the sumoylated form, colocalized with nucleolar markers such as fibrillarin and B23 in the presence of ARF (98). Then they identified a canonical EGR1-binding site on the *B23* promoter and that the levels of B23 expression were directly correlated with those of EGR1 (99). B23 regulated EGR1 stability and accumulation within the nucleolus through proteasome involvement, demonstrating the presence of the nucleolar B23-ARF-EGR1 network (99).

p53

p53 is a direct transcriptional target for Egr1 (12, 21, 100; Figs. 1 & 2b); Egr1 also binds with both wild type and mutant p53 (71, 83) although the significance of this interaction is still not clear since such physical interaction happens in the cytoplasm (83). *Egr1*-null MEFs bypassed the replicative senescence and exhibited an immortal growth with no sign of replication-dependent growth arrest, and also loss of DNA damage response, suggesting loss of p53 functions (10, 12). The properties of replicative senescence and DNA damage - induced growth arrest can be rescued by infection of the *Egr1*-null MEFs with a retrovirus expressing either p53 or Egr1. However, the Egr1-expressing retrovirus was ineffective in restoring replicative senescence or the DNA damage response in *p53*-null MEFs, indicating that Egr1 acts upstream of p53 (12). They also conducted GeneChip analyses of gene expression between wild type and *Egr1*-null MEFs and found that nineteen p53 target genes were identified. Electrophoretic mobility shift assay and chromatin immunoprecipitation confirmed that p53 was a direct target of Egr1 (Figs. 1 and 2(b)).

p63 and p73

Several Egr1-dependent properties of p53 are reflected in the functions of p73, a member of the p53 family which undergoes multiple splicing effects producing numerous isoforms (101–103). The regulation of p73 at the transcriptional level is controlled by two promoters that produce two main forms; one is named TAp73 because it contains the transactivating domain (TA) that gives p53-like activities (104–106). Ding et al. isolated a genomic DNA fragment in the 5' upstream region of the human *p73* gene and characterized the promoter region (104). The human *p73* promoter contained a putative TATA-box, and did not exhibit any homology to the *p53* gene (104). Transient transfection assays using progressive truncations of the *p73* promoter showed that the basal transcription was mediated by nucleotides –119 to +19 relative to exon 1, where putative Sp1, AP-2 and Egr-1/2/3 sites are located and CpG dinucleotides were especially concentrated (104). Consistently, Egr1 can upregulate the *p73* gene to induce the apoptosis of neuroblastoma cells (14).

The 2nd *p73* promoter regulates the expression of a set of isoforms are known as Np73; they lack the same transactivating domain, and, therefore generates effects distinct from those of p53 (101–103, 107). Consistently, many of the roles of Np73 are oncogenic, having distinct set of target genes that can be transcriptionally regulated. Of note, Np73 is not induced by Egr1 (100). Kartasheva et al. (107) examined whether Np73 α might modulate cellular transcription in the absence of p53. Np73 α increased the expression of *EGR1* and *CDC6*, whereas it decreased the mRNA levels of *c-MYC*, *Cyclin A2*, *NF κ B1*, *ODC1*, and *RET* finger protein. They concluded that the impact of Np73 α on gene expression was not limited to p53-responsive genes. Although the p63 family of gene products that are related to p53 may play a role in tumor suppression, they appear not to be regulated by Egr1. Both the *p63* and *p73* genes are infrequently mutated in cancers (102) although p53 is mutated in half of human cancers (108). In summary, TAp73 and TAp63 could act in place of p53 in *p53*-deficient cancer cells when an apoptosis response is required to remove damaged cells.

PTEN

The *phosphatase and tensin homolog (PTEN)* tumor suppressor gene encodes a pro-apoptotic factor that is frequently suppressed or mutated in a variety of human cancers (reviewed in 109–111). Notably, Stambolic et al. reported that *PTEN* is a direct transcriptional target for p53 (112; Fig. 1). Freeman et al. subsequently showed that PTEN regulates p53 protein levels and transcriptional activity through both phosphatase-dependent and -independent mechanisms (113). Consistently, the p53 protein levels are dramatically reduced in *Pten*^{-/-} cells. Reintroducing wild type or phosphatase-dead PTEN mutants lead to a significant increase in p53 stability. Importantly, PTEN physically associated with endogenous p53 (113). Finally, PTEN regulated the transcriptional activity of p53 by modulating its DNA-binding activity. Their study provides a novel mechanism by which loss of *Pten* can functionally control the tumor development by modulating p53 activity (113; reviewed in 114). Then the same group showed that PTEN regulates *Mdm2* expression through the P1 promoter (115; the p53-responsive element is in the P2 promoter). In *Pten*-null cells and prostate cancer tissues, the *Mdm2* P1 promoter activity was up-regulated, resulting in increased L-Mdm2 expression and enhanced p90^{Mdm2} isoform production (115). Furthermore, PTEN controls *Mdm2* P1 promoter activity through its lipid phosphatase activity, independent of p53. Their results provided a novel mechanism for *Pten* in controlling Mdm2 oncoprotein functions and p53 activity (115). Mayo et al. reported that retroviral transduction of PTEN into U87MG (PTEN-null) glioblastoma cells increased p53 activity and expression of p53 target genes and induced cell cycle arrest (116). U87MG/PTEN(+) glioblastoma cells were more sensitive than U87MG/PTEN(-) cells to death induced by etoposide. Their results established a direct connection between the activities of two major tumor suppressors and that they act together to respond to stresses and malignancies (116).

Virolle et al. showed that that *PTEN* transcription was upregulated by Egr1 after irradiation in wild type, but not *Egr1*-deficient mice *in vivo* (17). They found that Egr1 specifically bound to the Egr1-consensus sequence 5'-GCGGCGGCG-3' in the *PTEN* promoter. Consistently, inducing endogenous Egr1 by exposing cells to ultraviolet light upregulated the expression of PTEN leading to apoptosis where *Egr1*^{-/-} cells showed resistance. Therefore, loss of Egr1, which often occurs in human cancers, contributed to the radiation resistance of cancer cells through downregulation of PTEN (17). The functional link between Egr1 and PTEN has been reported in other studies (117–120) confirming the critical role of Egr1-PTEN signaling in tumor suppression. Finally, it was reported that miR-183 functions as an oncogene by targeting the Egr1 and PTEN tumor suppressor genes, again proving the functional link between these two tumor suppressors in cancer (121).

ARF plays an important role in the EGR1-PTEN signaling. Yu et al. reported a novel Akt-EGR1-ARF-PTEN axis, in which PTEN activation *in vivo* requires p14^{ARF}-mediated sumoylation of EGR1 (122). This modification was dependent on the phosphorylation of EGR1 at S350 and T309 by Akt, which promoted interaction of EGR1 with ARF at K272 in its repressor domain by the ARF/Ubc9/SUMO system. EGR1 sumoylation is decreased by ARF reduction, which also exhibited reduced amounts of PTEN (122). Thus functional

inactivation of any of the tumor suppressors in this axis - PTEN, EGR1, and ARF - causes some degree of dysfunction of the others (122).

Characterization of the EGR1-PTEN pathway has translational values in lung cancer clinics. In non-small-cell lung cancer, it was shown that that EGR1 expression strongly correlated with PTEN expression, which was associated with longer overall and disease-free survival after surgical resection of tumors compared with patients with low levels (54, 123). Thus measuring the level for EGR1 will make it possible to identify patients at high risk for recurrence; this may also identify patients who have tumors resistant to therapy secondary to loss of pathways regulated by PTEN. Very recently, Ryu et al. reported that paradoxical induction of growth arrest and apoptosis by EGF is caused by the upregulation of PTEN by activating EGR1 in human lung cancer cells (119), demonstrating that the EGR1-PTEN signaling cascade activation plays an essential role in cancer therapy (Fig. 1).

The role of EGR1-PTEN axis was also evaluated from the viewpoint of epithelial-mesenchymal transition (EMT) that plays a critical role in cancer metastasis. Kim et al. (120) studied how genes affect cancer cell invasion and EMT through investigating the molecular mechanisms of 2'-benzoyloxycinnamaldehyde (BCA; anti-tumor drug candidate). Cell migration was inhibited significantly by BCA both *in vitro* and *in vivo*. Moreover, BCA inhibited Snail and Vimentin expression as well as β -catenin nuclear accumulation (120). Importantly, suppression of EGR1 by siRNA subverted the inhibition of matrigel invasion by BCA, indicating that EGR1 was responsible for the BCA effect, resulting in decreased EMT and invasion through PTEN activation (120). Therefore the EGR1-PTEN signaling axis is critical in the EMT of colon cancer metastasis, and points to the role of BCA as a therapeutic agent for colon cancer.

Tumor suppression by Dmp1

Activation signals for Dmp1

We have been searching for positive and negative regulators for the *Dmp1* promoter as well as those for *Arf*. We found that the *Dmp1* promoter was efficiently activated by oncogenic Ras^{V12} as well as by constitutively active c-Raf, Mek1/2, and Erk1/2 in primary cells (19; Fig. 1). p19^{Arf} did not significantly accumulate in response to oncogenic Raf signaling in *Dmp1*-null cells that were refractory to Raf-mediated cell cycle arrest, suggesting the critical role of Dmp1 as a mediator of oncogenic Ras-Raf-Mek-Erk signaling and the Arf-p53 tumor suppressor pathway (19). Consistently, *K-Ras*^{LA} lung tumorigenesis was significantly accelerated in both *Dmp1*^{+/-} and *Dmp1*^{-/-} mice with larger tumors with invasion/metastasis in *Dmp1*-deficient tissues (46). The *Dmp1* gene was hemizygously deleted in ~40 % of lung carcinomas from wild type *K-Ras*^{LA} mice, demonstrating the primary involvement of *Dmp1* in lung carcinogenesis (46). We also found that loss of heterozygosity (LOH) of h*DMP1* was found in ~35 % of human non-small cell lung carcinomas, especially those that retain wild type *INK4a/ARF* and/or *p53* (46), suggesting that lung cancers with h*DMP1*-loss may constitute a novel disease entity. We also conducted GeneChip microarrays using *Dmp1*^{+/-} and *Dmp1*^{-/-} lungs and found that other transcriptional targets for Dmp1 α include *Areg*, *Thsp1*, *JunB*, and *Egr1* (20), suggesting that it is involved in signal transduction, cell proliferation, angiogenesis, and/or metastasis.

HER2/neu is a representative molecular marker for BC; the gene is amplified and the protein is overexpressed 30 % of human BC (47, 124). We characterized the signaling pathway between HER2/neu and Dmp1 using *MMTV-neu* mice as a model (47; 125 for a review of *MMTV* models). Both Dmp1 and p53 were induced in pre-malignant hyperplastic lesions from *MMTV-neu* mice where mammary carcinogenesis was significantly accelerated in both *Dmp1*^{+/-} and *Dmp1*^{-/-} mice with more aggressive phenotypes with invasion/metastasis (47). We also observed selective deletion of *Dmp1* in >50 % of wild type HER2/neu mammary carcinomas, while the involvement of *Arf*, *Mdm2*, or *p53* was rare. Tumors from *Dmp1*-deficient mice showed significant downregulation of *Arf* and *p21*^{Cip1}, showing p53 inactivity and more aggressive phenotypes than those from *Dmp1* wild type *neu*-transgenic mice (47). Consistently, *MMTV*-driven expression for *Flag-Dmp1α* indicated that it has tumor suppressive activity *in vivo* (45; 28, 126, 127 for reviews). Thus, our study shows the pivotal roles of *Dmp1* in HER2/neu-p53 signaling and BC prevention (28, 128).

Inhibitory signals for Dmp1

Both genotoxic and oncogenic stress activates the nuclear factor-kappa B (NF-κB) and p53 proteins; however, the p53 activity is antagonized by NF-κB signaling suggesting the presence of crosstalks (129). The *Dmp1* promoter was repressed by treatment of cells with anthracyclins and UV-C, non-classical NF-κB activators (41; Fig. 2a). Following anthracyclin/UV-C treatment, p65 (RelA) and other subsets of NF-κB proteins were bound to the *Dmp1* promoter (41). Repression of *Dmp1* transcription by anthracyclins depended on the unique NF-κB site on the promoter. Among NF-κB proteins, p65 played the major role in *Dmp1* repression since downregulation of p65 by shRNA significantly attenuated the response of the promoter by anthracyclins/UV-C (41). Likewise, the amount of Dmp1 bound to the *Arf* promoter decreased significantly upon anthracyclin treatment; this treatment, in turn, downregulated the p19^{Arf}. We also showed that repression of the *Arf* promoter by p65 or anthracyclins depended on Dmp1 (41). Compared to wild type cells, both *Dmp1*^{-/-} and *Arf*^{-/-} cells showed resistance to anthracyclin-induced cell death. Non-immortalized *p65*-knockdown cells were much more sensitive to anthracyclins than wild type cells, indicating the role of p65 in protecting cells from apoptosis (41). Thus, the Dmp1-Arf pathway is repressed by NF-κB in response to genotoxic stress, which implicates a novel mechanism of p53 inactivation by NF-κB (129).

Dmp1-interacting proteins

Overexpression of the *Dmp1* gene in mouse NIH 3T3 cells inhibits their entry into S phase (24). The cell cycle arrest depended on the ability of Dmp1 to bind to DNA and transactivates gene expression, and was specifically antagonized by co-expression of D-type cyclins, including cyclin D1 point mutants (D1K112E, K114E) that do not bind to Cdk4 (24). Studies from other laboratories showed that overexpression of D-type cyclins inhibited transcriptional activities of other Myb proteins, v-Myb and B-Myb (130, 131; reviewed in 132), suggesting a functional link between D-type cyclins and Myb-like proteins.

We recently showed that Dmp1α co-immunoprecipitates with p53, but not Mdm2, or Arf (42). These two proteins directly interacted through p53's carboxyl-terminus and Dmp1's DNA-binding domain. Dmp1α expression antagonized p53's ubiquitination by HDM2 both

in cell and *in vitro*, and restores p53's nuclear localization that was lost with HDM2 expression (42). Moreover, Dmp1 α stabilized p53-DNA complexes on genomic DNA that contained p53-consensus sequences, which were either supershifted or disrupted with antibodies to Dmp1 (43). Dmp1 α -p53 interaction significantly increased the levels of p53 independent of Dmp1's DNA-binding, and hence the *p21^{Cip1}* promoter activity was synergistically induced by co-expression of Dmp1 α and p53 in *p53^{-/-};Arf^{-/-}* double knockout cells (42). The induction of *p21^{Cip1}* and *Bbc3 (Puma)* by genotoxic doxorubicin treatment was more seriously affected in *Dmp1^{-/-}* and *p53^{-/-}* tissues than in *Arf^{-/-}* (42). Lysates from mice injected with doxorubicin showed that Dmp1 bound to *p21^{Cip1}*, *Bbc3*, and *Thbs1* genomic regulatory regions in a p53-dependent fashion (43). These data indicate that Dmp1, but not Arf, is actively involved in DNA damage response, justifying further investigation of the role.

How cyclin D1 sends oncogenic signals to the p53 pathway has remained a mystery since it is not a transcription factor. We demonstrated that cyclin D1 physically interacts with Dmp1 α to activate the *Arf* and *Ink4a* promoters to eliminate incipient cancer cells to undergo cell cycle arrest or apoptosis (44). Indeed, cyclin D1 or D1T286A-induced mammary carcinogenesis was accelerated in *Dmp1^{+/-}* mice with more aggressive phenotypes of tumor cells and distant metastases, proving that cyclin D1-Dmp1 α interaction plays essential roles in quenching oncogenic cyclin D1 signaling *in vivo* (44; reviewed in 132). In summary, Dmp1 α stimulates the p53 pathway by direct transactivation of the *Arf* promoter in response to oncogenic stress (19, 30, 38, 39, 44–47) and through direct physical interaction with p53 in response to genotoxic stimuli or when *Arf* is not available for tumor suppression (42, 43; Fig. 1).

We recently analyzed more than 100 human BC specimens: LOH of hDMP1 was found in 42 % of BCs (133). This finding is significant since the frequency of hDMP1-loss is equivalent to that of *INK4a/ARF* or *p53* in human BC. LOH of hDMP1 was found in a mutually exclusive fashion with that of *INK4a/ARF* and/or *p53* in the same samples in 80–90 % of the cases (46, 133), suggesting that hemizygous hDMP1 deletion might define a new disease entity with a different response to therapy. The hDMP1 locus encodes at least three splicing variants - hDMP1 α , β , and γ with antagonizing functions (134). Recent studies suggest essential role of aberrant RNA splicing in the pathogenesis of cancer (135–139). The hDMP1 α gene corresponds to murine *Dmp1 α* that positively regulates the p19^{Arf}-p53 pathway (134). Conversely, the hDMP1 β and γ isoforms lack the DNA-binding domain, and hDMP1 β is dominant-negative over hDMP1 α in *CD13* and *ARF* induction (134, 140). Forced expression of hDMP1 β stimulates cell proliferation in a p53-independent fashion (141; 27, 142, 143 for reviews). Of note, the hDMP1 β/α ratio was increased in 33 % of our BC specimens (141). The overexpression of DMP1 β stimulated the growth of breast epithelial cells and was associated with significantly shorter survival of BC indicating that it is a novel oncogene and a novel prognostic factor for human cancer (141, 142).

Future directions for research

We have reviewed the mechanisms for tumor suppression by EGR1 and DMP1 proteins. Interestingly both of these have tumor suppressive activities dependent on p53 although the

mechanisms are not completely understood for the latter. For instance, it is currently unknown whether any of the DMP1 proteins are involved in Fibronectin, PAI-1, TGF β , and PTEN signalings. It is also unclear whether p63 or p73 are critical targets for DMP1 for tumor suppression. p53 is a critical transcriptional target for Egr1 while Dmp1 transactivates the *Arf* promoter and also physically interacts with p53 for tumor suppression. In case of DMP1, the full-length isoform (DMP1 α) has been shown to have tumor-suppressive activity while the short β isoform is oncogenic independent of p53 (141). Currently the activity of DMP1 γ and its interacting partners are unknown. Future research will focus on the physiological and oncogenic role of DMP1 γ and other splice variants from the *DMP1* locus.

One intriguing issue is the crosstalks among EGR1, DMP1, and WT1 transcription factors. Some of the DNA-binding consensus sequences are common between EGR1 and WT1 (144). Although DMP1 and WT1 do not share DNA-binding properties, DMP1 is a critical target gene for WT1 for transcriptional repression (145). Since overexpression of WT1 is very common in human leukemias (146–149), WT1-binding to the *DMP1* promoter may be a critical mechanism for inactivation of the ARF-p53 pathway in human malignancies. Interestingly all of these transcription factors (Egr1, Dmp1, and WT1) directly bind to p53 (42, 83, 150). In case of Dmp1 α , it inhibits all the activities of HDM2 in p53 regulation. Although *Egr1* is a pivotal gene that initiates signal transduction events in response to IR, the significance of Dmp1 induction by DNA damage response (42) is still not clear. These issues should be clarified in the future to get a better picture of signaling networks involving EGR1, DMP1, and p53 in tumor suppression.

Another feature that is common between DMP1 and EGR1 is the control of gene expression by microRNAs. It has been reported that EGR1 expression is dysregulated by numerous microRNAs (151–155) while microRNA-155 promotes bladder cancer growth by repressing the tumor suppressor DMP1 (156). Thus it is highly possible that both of these genes contribute to tumor suppression without genomic DNA deletion or promoter methylation. That is one promising direction of research in the future.

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Figure 1. Signaling cascades involving EGR1, PTEN, DMP1, ARF, and the p53 network. Published studies showed that Dmp1 receives oncogenic signals from mutant Ras (19) or overexpressed HER2 (47) and directly transactivates the *Arf* promoter (30). This p19^{Arf} induction, in turn, neutralizes all the activities of Mdm2 and activates p53. Our study shows that the Dmp1 protein directly interacts with p53 and blocks the activities of Mdm2, especially in epithelial cells and T lymphocytes (42). Dmp1 also stabilizes p53 binding to transcriptional target genes (43). These two mechanisms will act synergistically to boost the p53 activity to prevent tumorigenesis. The *Dmp1* promoter has binding sites for Egr1 (19) and the transcription factor Egr1 is one of the target genes for Dmp1 that has tumor-suppressive activity (20). Egr1 mediates p53-independent c-Myc-induced apoptosis via a non-canonical ARF-dependent transcriptional mechanism (92, 93). Both the *Dmp1* and *Arf* promoter has numerous binding sites for Egr1 where Egr proteins play critical roles in the Arf-C/EBPβ pathway (94) in oncogene-induced senescence. In addition Arf promotes sumoylation of several interacting proteins such as Egr1, topoisomerase I, Mdm2, and p53 (95–97) suggesting extensive crosstalks. Both PTEN and p53 are transcriptional targets for EGR1 (12, 21). In addition to Egr1-Dmp1-Arf-p53-Pten signaling, EGR1 transactivates the *Fibronectin* (FN) and *TGFβ1* promoters for tumor suppression (14). The signaling pathways

that connect p53 and Dmp1 α (negative feedback), DMP1 β and RB are currently under investigation (discontinuous lines).

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Figure 2. The structure of the mouse *Dmp1* (a) and human *p53* (b) promoters.

a. Mouse *Dmp1* promoter (19). The major transcription initiation site is shown by an arrow. The DNA sequences for potential transcription factor-binding site for the proximal promoter regions (−414 base pairs from the transcription initiation sites) are shown. The mouse *Dmp1* promoter has potential binding sites for AP1 (19), E2F:DP (40), NF-κB (41, 47), Ets/Elk (19), EGR1/2/3 (19), AML1a, GATA, C/EBP and others. It was reported that Akt, a downstream kinase of receptor-type tyrosine kinases, phosphorylates IKK. This, in turn, phosphorylates IκB and releases NF-κB, leading to transcriptional activation of *Dmp1* (47).

Akt also phosphorylates Myc-associated zinc finger protein MAZ at Thr385, which is released from the *p53* promoter, leading to transcriptional activation of *p53*. This is a novel mechanism for p53 activation during oncogenic activation mediated by Akt (157). **b.** Human *p53* promoter (21). The 5' end of the major transcription initiation sites 5'-CTTCTC-3' is shown by an arrow. The human *p53* promoter has binding sites for NF- κ B (158), p53 (159), c-Myb, E2F:DP (160), YY1 (161), and others. In addition, EGR1 directly binds to (TCC)_n sequences of the *p53* promoter (−139 to −186) (21) for transactivation. This sequence has the binding sites for c-Myb and YY1 as well. The regulatory sites on the human *p53* promoter by p73 or p53 have been mapped by Wang and El-Deiry (159).