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Review

Signal persistence and amplification in cancer development and possible, related opportunities for novel therapies

Shea A. Ford [a], George Blanck [a,b,*]

[a] Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, United States
[b] Immunology Program, Moffitt Cancer Center and Research Institute, Tampa, FL 33612, United States

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ABSTRACT

Research in cancer biology has been largely driven by experimental approaches whereby discreet inputs are used to assess discreet outputs, for example, gene-knockouts to assess cancer occurrence. However, cancer hallmarks are only rarely, if ever, exclusively dependent on discreet regulatory processes. Rather, cancer-related regulatory factors affect multiple cancer hallmarks. Thus, novel approaches and paradigms are needed for further advances. Signal pathway persistence and amplification, rather than signal pathway activation resulting from an on/off switch, represent emerging paradigms for cancer research, closely related to developmental regulatory paradigms. In this review, we address both mechanisms and effects of signal pathway persistence and amplification in cancer settings; and address the possibility that hyper-activation of pro-proliferative signal pathways in certain cancer settings could be exploited for therapy.

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* Corresponding author at: 12901 Bruce B. Downs Bd. MDC7, Tampa, FL 33612, United States. Tel.: +1 813 974 9585; fax: +1 813 974 7357.
E-mail address: gblanck@health.usf.edu (G. Blanck).

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1. Introduction

Cancer represents a series of stages beginning with small levels of uncontrolled growth in situ, which rarely has a clinical impact, and ending with extensive metastasis, cancer cell invasion of multiple organs, and organ failure. Through this series of steps, cancer is defined by hallmarks [1], such as uncontrolled growth, resistance to apoptosis, tissue remodeling, evasion of the immune system, angiogenesis, and the capacity to colonize distant tissues.

These cancer development steps and their phenotypic hallmarks have been thought to arise because of a series of mutations, whereby each succeeding mutation facilitates the next hallmark [2]. However, many aspects of data accumulated over the last 30 years do not fit this model of cancer hallmark development. For example, recent, extensive sequencing of multiple isolates of cancer in the same patient demonstrates extensive functional variation in somatic mutations that are likely related to cancer development [3,4]. More importantly, individual regulatory proteins thought to be intimately associated with one cancer hallmark have turned out to have a bewildering array of effects on many cancer hallmarks [5]. The extensive, multidimensional nature of the cancer regulatory protein literature is too vast to summarize here, but this issue has been at least partially addressed recently [5]. Thus, many proteins thought to regulate the cell cycle regulate apoptosis [6–8]; and many proteins originally thought to regulate the cell cycle regulate tumor cell immune functions and extra-cellular matrix destruction [9–12].

Thus, novel paradigms of regulatory protein function in cancer development are needed. We have been addressing the question of whether differences in the levels of regulatory proteins, rather than a strict consideration of their absence or presence, provide a clearer understanding of how cancer develops, as well as providing novel considerations of screening for early stage cancers and for designing therapies [6] (Fig. 1). In this review, we first address how signal transduction mechanisms can have discreet effects with different levels of activity. We then address the possible application of these mechanisms and their effects in understanding several cancer settings.

Two important areas that will not be covered in this review are the roles of metabolic enzymes or their regulators (e.g. PKM2 kinase) in cancer-cell progression and computer modeling of signal amplification and signal crosstalk [13,14]. In particular, Gaudet et al. recently described the effects of fold changes of NF-κB, rather than simple presence or absence, and followed up with computational modeling of the NF-κB amplification process and effects [13].

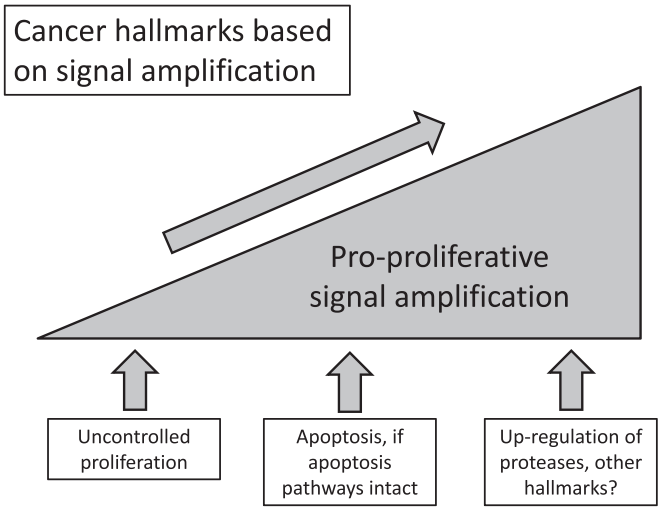

Fig. 1. Summary of a signal amplification paradigm of cancer hallmark development.

2. Mechanisms of signal transduction amplification

2.1. Development

In fact, there are several fields of biology where signaling levels and their impacts are the main paradigm, not the least of which is developmental biology. This review will emphasize cancer, but *developmental hallmarks* have long been understood to be fundamentally dependent on signaling and concentration *gradients*. For example, many regulatory regions require the binding of multiple molecules of the same transcription factor (TF), thereby rendering transcriptional regulation an exponential function of the TF concentration, in turn leading to what is effectively a discreet result from a specific concentration of a TF, for example the hunchback promoter response to a particular concentration the bicoid TF [15,16]. However, there has been little application of this developmental paradigm of concentration-dependent, molecular mechanisms to the function of somatic cells in the adult organism, with a few exceptions.

2.2. Class II transactivator induction

The interferon-gamma (IFN-γ) response has been extensively studied as basically the result of the presence or absence of IFN-γ, with of course, considerable success in illuminating the basic mechanisms of the pathway. However, responses to IFN-γ can be dependent on the amount of IFN-γ added to cells or on the duration of the presence of IFN-γ [17]. Major histocompatibility (MHC) class II induction in non-professional antigen presenting cells, such as skin fibroblasts or certain solid tumor cells, does not occur with short or low-level exposures to IFN-γ, even though these levels can lead to a maximal level of STAT1 phosphorylation, one of the very early steps in the MHC class II, IFN-γ response. This is because the duration of STAT1 phosphorylation is short lived [17]. And while this duration is enough for transcriptional activation of certain early response genes, it is not enough for activation of the MHC class II transactivator, CIITA. This is due to the fact that CIITA induction requires the *simultaneous* binding of both STAT1 and interferon regulatory factor-1 (IRF-1) to the CIITA promoter. However, due to the short lived, phosphorylated STAT1, these two regulatory proteins do not overlap in time. Thus, CIITA is not induced and MHC class II is not induced. In fact, this regulatory process complicates the interpretation of the IFN-γ pathway in tumor cells. While STAT1 knock-out mouse experiments have suggested that the presence of STAT1 in tumor cells is anti-tumorigenic, a more accurate interpretation is likely more complicated. For example, STAT1 induces the highly apoptotic IRF-1, as well as the immunogenic MHC class I and class II proteins. However, almost no human tumor line can be found to completely STAT1 negative [17,18]. Rather, almost all known examples demonstrate a partial or low level induction of STAT1, which fits with the anti-tumorigenic properties of the downstream aspects of the IFN-γ signaling and which raises the question of whether STAT1 activation alone has pro-proliferative and pro-metastatic functions?

3. Mechanisms and effects of signal transduction amplification in cancer settings

3.1. C-Jun N-terminal kinases (JNKs)

Alterations and mutations in the mitogen activated protein kinase (MAPK) signaling pathways are commonly associated with cancer. Different members of the MAPK family, for example the JNKs, influence cell fate through variable concentration and activity levels of MAPK, rather than simply because of the presence or absence of MAPK activity. JNKs specifically can be induced to switch between transient and sustained states [19]. Once in a sustained state, positive feedback keeps JNKs in a highly activated state that results in cellular apoptosis [19,20].

However, growth factor induced, transient activation of JNKs leads to cell survival and proliferation [19].

3.2. Extracellular signal-regulated kinases (ERKs)

ERKs are a subset of the mammalian MAPK family including MAPK1, MAPK3, MAPK6, and MAPK7. Anomalies in the ERK pathways are common in cancers and are often associated with RAS, B-RAF and C-RAF mutations. Differences in the level of ERK activation result in different effects on cellular fate, a pioneering discovery made by Blenis et al. with the understanding of how the SRF (c-fos) accumulation is dependent on sustained ERK activation [21].

In PC12 cells (neuroendocrine tumor cells of the medulla of adrenal glands), sustained ERK activation results in growth arrest, while transient ERK activation results in proliferation [22]. Induction of tumor suppressor p53 has been shown to sustain ERK1/2 activation and trigger cell death [23]. Photodynamic Therapy (PDT) is a type of cancer therapy that targets and kills malignant tumor cells. PDT-sensitive cells were discovered to have transient ERK activity, while PDT-resistant cells maintained sustained ERK1/2 activity [24]. Progesterone Receptor-B (PR) ligand normally causes rapid and transient activation of cytosolic ERK1/2, but PR-B induces robust and sustained ERK1/2 activation in breast cancer cells. The sustained ERK1/2 activity in breast cancer cells upregulates WNT-1 signaling, resulting in increased matrix metalloproteinase (MMP) activity. PR-B induced, sustained MAPK signaling appears to cause pro-proliferative effects in early breast cancer lesions. This sustained MAPK pathway also increases cyclin D1 protein expression [25].

3.3. Protein kinase C (PKCs)

PKCs are a family of proteins associated with regulation of signal transduction cascades activated by the phospholipase C pathway. PKCs require the binding of Ca^{2+} ions, phospholipids, and diacylglycerol (DAG) for activation. Transient activation of PKCs is associated with secretion and ion-influx [20]. Sustained activation of PKC is suggested to regulate proliferation, differentiation, apoptosis, migration, and tumorigenesis [20].

3.4. STAT3

STAT3 is normally not activated, i.e., phosphorylated, until induction by an extra-cellular ligand binding to a receptor. However, sustained STAT3 activation via phosphorylation is commonly linked to cancer progression. STAT3 phosphorylation is linked to cancer stem cell progression, increased tumorigenicity, and poorer disease-free survival. Sustained STAT3 activation is triggered by acylglycerol kinase (AGK) overexpression in esophageal squamous cell carcinoma, as well as in many forms of lung cancer, and in breast cancer [26]. AGK has the potential to directly facilitate sustained activation of STAT3. In particular, STAT3 activation is traceable to interleukin-6 stimulation, a process that is enhanced with overexpression of AGK. Overexpression of AGK has also been shown to facilitate high levels of NF-κB activation [26]. In prostate cancer, the activation of STAT3 by IL-6 leads to an increase in proliferation rate and decrease in patient survival. The high level of phosphorylated STAT3 observed in 82% of human prostate tumors protects these tumors from apoptosis. Patients with high levels of cytoplasmic phosphorylated STAT3 had significantly shorter time to death than patients with low cytoplasmic phosphorylated STAT3 [27]. MMP-7 is overexpressed in prostate cancer cells and linked to an increase in prostate cancer cell invasion. STAT3 is thought to bind to the human MMP-7 promoter sequence, thus inducing the action of MMP-7 [28]. Kaposi's sarcoma-associated herpesvirus (KSHV) infection has been shown to activate STAT3 during infection. After initial activation, STAT3 becomes inactive for 4 h. However, 12 h after infection, STAT3 activation is sustained for the duration of the infection. Inhibition of STAT3 activation in Kaposi's sarcoma causes cancer cell apoptosis [29].

Colitis-associated cancer (CAC) is associated with IL-6 induced, sustained STAT3 activation. Sphingosine-1-phosphate (S1P) induces sustained NF-κB activity to begin the process of STAT3 activation. STAT3 and S1P receptor-1 work together in a positive feedback loop to facilitate tumor growth and metastases [30]. The sustained STAT3 activity leads to cancer progression specifically through increased expression of target genes BCL, Cyclin D1, and Survivin that together increase cancer cell proliferation, angiogenesis, and tumor growth [31].

3.5. MMP2 and MMP9

MMPs are associated with tissue remodeling, angiogenesis, and metastasis. In QG90 cells derived from human small-cell lung cancer carcinoma, hyaluronan A (HA) activates the secretion of MMP2, but requires focal adhesion kinase (FAK). FAK signaling induces MMP-2 secretion as well as sustained MAPK activation. Elevated MMP2 secretion contributes to the destruction of the extracellular matrix, contributing to tumor invasion and metastasis and showing a clear correlation with poor prognosis. In small-cell lung cancer, fibronectin activates MMP-9 secretion in the same way HA stimulates MMP-2 secretion, but the fibronectin–MMP9 pathway also requires FAK signaling [32].

Ovarian cancer cells demonstrate sustained elevation of p42/p44 MAPK activity that appears to be required for proteolytic mechanisms associated with malignancy. Sustained activation of p42/44 MAPK augments the expression of MMP-2 and is apparently required for the expression of MMP-9 [33]. IL-28A stimulates the expression of MMP-2 and MMP-9 through activation of MAPK in bladder cancer cells. However, a threshold level of IL-28A exposure is required for induction of *any* MMP9 in 5637 and HT1376 bladder carcinoma cells, although induction of MMP-2 appears to occur at the lowest level of IL-28A exposure. And, a *threshold* overexpression of the MAPK signaling pathway is required for migration of bladder cancer cells [34], as cells with constitutive *low levels* of MAPK signaling do not migrate.

3.6. MMP8

MMP8 is the first member of the MMP family that appears to serve a protective role in cancer. Overexpression of MMP-8 in M-4A4 cells leads to a decrease in metastasis. Knock-down of MMP-8 in NM-2C5 cells leads to an increase in metastasis [35]. Sustained expression of MMP-8 in breast cancer cells is deleterious to the long-term growth of tumor cells. Transient expression of MMP-8 induces expression of IL-6 and IL-8, which has been shown to be pro-malignant. It is has been hypothesized that early, transient MMP-8 activity triggers an acute inflammatory response, but only sustained, elevated MMP-8 expression impairs cell growth in breast cancer [36]. MMP8 acts as a tumor suppressor by increasing adherence to the extracellular matrix to decrease tumor invasion and metastasis [37,38]. MMP8 has been shown to inhibit melanoma growth and tumor formation in vivo [39]. However, MMP8 activity may have different effects depending on the tissue type and the stage of cancer progression. Although MMP8 is tumor suppressive in certain forms of breast cancer and melanoma, MMP8 is correlated with poor prognosis in both patients with head and neck squamous cell carcinoma, ovarian cancer and bladder cancer [39].

3.7. CD30 signaling and NF-κB

The CD30 receptor is a member of the tumor necrosis factor receptor superfamily and is highly expressed in activated T- and B-cells. Normally, CD30 mediates the signal transduction that leads to NF-κB activation. Anomalous, constitutive NF-κB activation is common to many tumor types, and a reduction in the level of tumor cell NF-κB can lead to tumor cell apoptosis. However, reports have also indicated that, what might be termed “hyper-activation” of NF-κB, in particular by LPS, is

associated with apoptosis [40]. Also, the blast cells of anaplastic large cell lymphoma (ALCL), very similar to the Reed–Sternberg (R–S) blast cells in Hodgkin's disease, are sensitive to NF-κB associated apoptosis, while the R–S cells depend on NF-κB for proliferation. This distinction has never been resolved, but one report [8] points to the possibility that NF-κB would function in a hyper-activated state in the ALCL cells in contrast to the Reed–Sternberg cells. Hirsch et al. [8] demonstrated that promoter reporter constructs representing three different genes that are responsive to NF-κB are activated at only a low level in R–S cells but are hyper-activated in ALCL cells, following CD30 signaling. Authors made the case for CD30 insensitivity in the case of the R–S cells, but regardless, constitutive NF-κB activity was comparatively modest in the R–S cells and induced NF-κB activity at strikingly high levels in the ALCL cells. Interestingly, primary lymphoma of the bone (PBL) is another rare example, like ALCL blasts, where there is detection of cytoplasmic but not nuclear NF-κB, raising the question of whether nuclear NF-κB in PBL would lead to apoptosis? And finally, NF-κB has been linked to apoptosis in non-cancer, neuronal settings [40].

With regard to mechanisms of NF-κB signal amplification, sustained activity of NF-kappaB can be facilitated by flotillin-1, which has been shown to facilitate the recruitment of the TNF-alpha receptor to lipid rafts [41]. The TNF-alpha receptor is thereby presumably more efficient at maintaining NF-κB activation. In pancreatic ductal adenocarcinoma (PDAC), hyper-O-GlcNAcylation of the p65 (RelA) subunit of NF-kappaB contributes to sustained NF-κB activation as well as an anti-apoptotic state. Suppression of hyper-O-GlcNAcylation decreased oncogenic NF-κB activity, and was shown to impair primary tumor growth. Inhibiting sustained NF-κB activity caused the death of PDAC cells by apoptosis [42]. Finally, unphosphorylated STAT3 stabilizes NF-κB binding to DNA and enhances NF-κB expression in chronic lymphocytic leukemia cells [43].

3.8. Patient tissue

Inhibitor of DNA Binding (Id) protein is a negative regulator of cell transcription linked to metastasis. Patients exhibit varying levels of Id-1 expression, with a correlation of expression level to the level of metastasis [44]. Thus, Id-1 represents an example of how proteins related to cancer development can be expressed at different levels, with different effects for the different levels, rather than being responsible for a specific result with the simple presence or absence of the protein. Similar results were observed for patient tissue levels of flotillin-1, discussed above as a possible facilitator of sustained activation of NF-κB [41]. Rho GDP dissociation inhibitor 2 (RhoGDI2) was recently recognized as a gene responsible for suppressing metastasis in bladder cancer. Patients with large amounts of RhoGDI2 demonstrated a lower mortality risk compared to patients with lower amounts of RhoGDI2. Reduced RhoGDI2 expression is associated with venous and lymph node metastasis [45]. Once again, this RhoGDI2 scenario indicates that the presence or absence of a cancer-related protein is not a suitable paradigm for assessing the impact of the protein on cancer development. Undoubtedly, there are many other assays of patient samples that do not reflect a mere presence or absence of a cancer-relevant protein [46]; thus the question will persist, at what point does the level of a protein indicate a particular prognosis? In each case, it can be argued that the read-out of results, when considering a gradation of a patient expression levels, is only arbitrarily established as a discreet result. However, this issue will not be resolved until more sophisticated approaches make a clear distinction between completely positive and negative results traceable to a particular level of patient protein function. For example, it is possible to detect certain levels of certain regulatory proteins, and with a sufficiently sophisticated approach, establish that such a level does not lead to detection of *any metastasis*. Such a question may be addressed with highly sensitive approaches, such as PCR-detection of circulating tumor cells. It is conceivable that in all cases the level of circulating tumor cells falls along a gradient of expression for a particular regulatory protein for all expression levels measured. However, it is also possible that there is no detection of circulating tumor cells until a regulatory protein level exceeds a threshold. Indeed this type of result could be obtained with a significant level of confidence, with enough patient samples, whereby a slope of a line of circulating tumor cell detection, plotted against regulatory protein expression levels, would fall to zero on the Y axis long before the regulatory protein expression level fell to zero.

4. Copy number variation (CNVs) and gene dosage effects

CNV refers to the abnormal duplication or deletion of various regions of the genome, and can represent a wide variety of variations, e.g., *DHFR* gene amplification in cells selected for methotrexate resistance [47] to trisomy 21, resulting in Down's syndrome. Trisomy 21 leads to a higher incidence of acquired acute lymphoblastic leukemia, acute myeloid leukemia, and acute megakaryocytic leukemia (AMKL) and is associated with an increased mutation rate in the transcription factor gene *GATA1*, possibly traceable to increased transcription of the *GATA1* gene, in turn traceable to over-expression of chromosome 21 genes [48,49]. The increased mutation rate of *GATA1* in combination with the overexpression of *ETS2* and *DYRKIA* genes, which reside on chromosome 21, leads to reduced apoptosis, in ways not fully understood, particularly because both these genes appear to have tumor suppressing effects for other tissues [48]. A high resolution genomics analysis of partial trisomies indicated the importance of an 8.35 megabase region of chromosome 21 in AMKL development [50], including the *RUNXI*, *ERG*, and *ETS2* genes, all implicated in cancer development in non-Down syndrome settings [51–53]. In sum, Down syndrome related leukemia development is intimately associated with a change in the level of potential oncoproteins and not simply regulated by the presence or absence of these proteins.

In yeast cells, heterozygous deletion of certain genes can cause increases in the rate of the cell cycle and decreases in apoptosis rates [54]. The majority of the yeast genes evaluated in these haploproficiency studies have human orthologs with analogous connections to cancer cell functions, for example, *UBX4*, *IDP1*, *IDP2*, *MSH2*, *RAD1*, *TOP2*, *NBP2*, *MUS81*, *RAD54*, *DBF2*, *STP22*, and *PBS2*. These 12 genes have also been shown to have copy number reduction in more than 25% of cancer samples for various cancer types [54].

In a recent study, we have noted that CNVs represented in the COSMIC database indicate that many cancer types have reduced copies of apoptosis-effector genes while maintaining or increasing copy numbers of proliferation-effector genes [55].

5. Exploiting signal amplification for therapy: apoptosis resulting from the hyper-activation of pro-proliferative pathways

While signal amplification, as a mechanism of discreet effects in cancer cells, is in its infancy as a paradigm, a recurring result above is that hyper-activation of pro-proliferative pathways leads to apoptosis. This phenomenon has been extensively studied in T-cell biology, where negative selection is due to hyper-activation of the T-cell receptor but less activation of the T-cell receptor leads to T-cell proliferation [56,57]. Furthermore, the retinoblastoma tumor suppressor protein (Rb) reduces IFN-γ induced apoptosis, and slow growing cancer cells have been reported as having greater resistance to anti-cancer therapies [44,58,59].

Many TFs that lead to the activation of genes that facilitate the transition through S-phase, such as the histone genes, also lead to the activation of genes that cause apoptosis [6]. Given the nature of cancer as uncontrolled cell growth, consideration of hyper-activation of signaling pathways that lead to progression through S-phase, as a strategy to induce apoptosis, has been highly limited. However, there would likely be more active application of this strategy, particularly in experimental or pre-clinical settings, with a plausible mechanism that would account for pro-proliferation genes and pro-apoptosis genes sharing the same

TFs and account for the completely different results of the two sets of genes, namely proliferation versus apoptosis. We have recently proposed such a mechanism, using genomics and bioinformatics approaches, whereby we have determined that pro-proliferation genes are larger than pro-apoptosis genes and have more active chromatin region per gene [6]. Furthermore, the TF binding sites that are shared among the pro-proliferation genes and pro-apoptosis genes occur, on average, more commonly in the pro-proliferation genes. Thus, a model that fits both these data, and the data that indicate that hyper-activation of pro-proliferative pathways leads to apoptosis, would be as follows. As TFs become active, as a result of stochastic processes, the TFs first encounter and activate pro-proliferation genes. If the cell progresses properly through S-phase, possibly as a result of cell volume expansion or cell division, the shared TFs never accumulate to such an extent as to significantly encounter the smaller pro-apoptosis gene targets. However, if the shared TF set accumulates at a high rate, the pro-apoptosis genes become significantly occupied and activated by the TFs, i.e., exemplifying a feed-forward mechanism associated with failed progression through S-phase. Initial data from our lab is consistent with this mechanism of apoptosis induction for IFN-γ treatment and for the role of Oct-1in IFN-γ induced apoptosis [60]. And fascinatingly, a 40-year old conundrum of high dose estrogen therapy for breast cancer, which led to conflicting results, including tumor regression in some settings, has never been explained [61].

If the above feed-forward mechanism of apoptosis is supported by additional data, it is possible that cancer therapy could include an assessment of exactly which TFs in a given cancer activate apoptosis pathways still intact in the cancer cell. In other words, is it possible that identification of intact apoptosis pathways could lead to identification of pro-growth signaling pathways that, if hyper-activated, would lead to cancer cell apoptosis.

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