STKE Signaling Collection
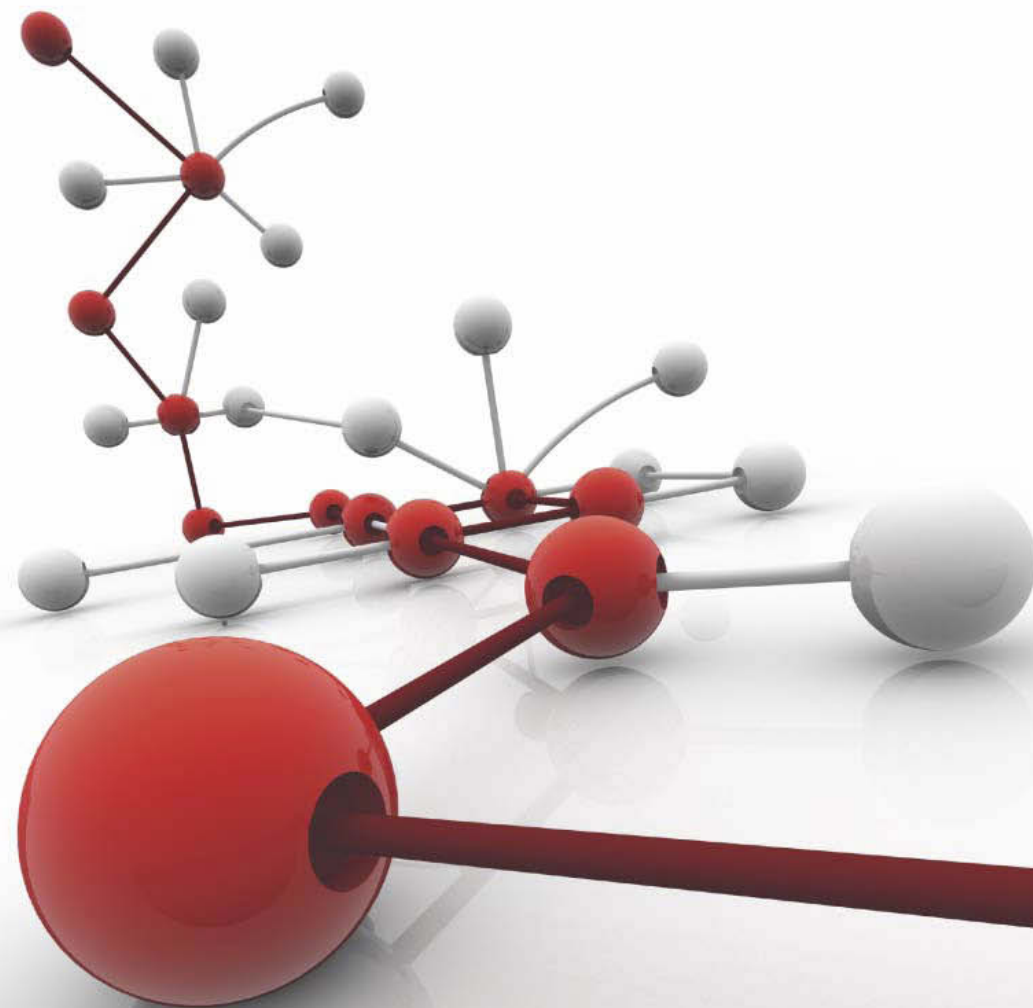
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Science's *STKE*: The Authoritative Source for Cell Signaling Information

Nancy R. Gough

Science's *Signal Transduction Knowledge Environment* (*STKE)* is an on-line resource and information management tool that enables experts and novices in cell signaling to find, organize, and utilize information relevant to processes of cellular regulation. "Signal transduction" refers to the bio-chemical processes by which cells respond to cues in their internal or exter-nal environment. Because signal transduction mechanisms are the natural control circuits that regulate biological systems, they provide potent targets for development of therapeutic agents to combat disease or otherwise alter the behavior of biological systems.

A cornerstone of STKE is the database of cell signaling, which is freely accessible through the Connections Maps. STKE provides access to the data through machine-generated interactive pathway diagrams. The information in the Connections Maps database is organized into canonical (generalized information from multiple experimental systems) and specific (detailed species-, tissue-, and cell-specific information) pathways, composed of ca-nonical and specific components, respectively. The structure of the database into canonical and specific data, as well as the population of the database by scientific experts, make the Connections Maps a unique and extremely valuable tool for research and education. This collection, which includes the pathway diagrams of the Epidermal Growth Factor Pathway, the T Cell Signal Transduction pathway, and the Estrogen Receptor Pathway, is a small sample of the many pathways available in the Connections Maps database. Explore the supporting data for these featured pathways and oth-ers by visiting the STKE Connections Maps.

STKE is a recognized journal, providing unique literature articles by signaling researchers. These include comprehensive Reviews, short focused Perspectives, and Protocols that describe detailed experimental methods. The STKE Virtual Journal is a digital library of primary research articles from participating publishers, which allows STKE users access to the full text and abstracts of topically relevant literature (signal transduction –related articles identified through a text-mining algorithm).

This collection highlights three STKE articles that provide new in-sights into the pathways described in the featured Connections Maps. A Perspective by Mullin discusses how leakage of epidermal growth factor across epithelial cells may contribute to cancer. A Perspective by Sprent discusses whether T cell activation may involve transfer of molecules from

the antigen-presenting cells. A Perspective by Biswas *et al*. describes how estrogen may reduce inflammation by inhibiting the transcription factor NF-κB.

STKE also represents a vibrant online community, with discussion forums, comments associated with STKE articles, and a directory of signaling researchers. At STKE, you will find a list of events (meetings, conferences, and workshops) of interest to the signaling community and links to editor-reviewed web sites. In addition to these resources, STKE provides educational resources for instructors and students, including an updated glossary of cell signaling terms and abbreviations and digital teaching resources, which include animations, slides, and lecture notes, as well as course organization information, such as syllabi and descriptions for creating student discussion forums and journal clubs.

Through personalization tools, such as online folders, customizable display settings, and customizable saved searches, STKE encourages users to be interactive and to help refine the site to meet their needs. STKE also provides a current-awareness function through various types of e-mail alerts, as well as the summaries of current research articles written by the STKE editorial staff.

Access to many of the STKE features, including the Community and Resources sections, and the Connections Maps requires only registration (which is completely free). Access to the full text and PDF of articles in the Virtual Journal, the STKE Reviews, Perspectives, and Protocols requires an individual subscription or access through an institutional site license. Please see the last page for subscription information or visit STKE (www.stke.org) today.

Epidermal Growth Factor Receptor Pathway

Joseph Schlessinger

(21 October 2005)

Description

This record contains general information about the epidermal growth factor receptor pathway collected across species.

The epidermal growth factor (EGF) receptor (EGFR) family is composed of four receptor tyrosine kinases (RTKs) designated EGFR, ErbB2 (also known as HER2 or neu), ErbB3 (HER3), and ErbB4 (HER4). Binding of EGF, or other members of the EGF family of growth factors, to the extracellular domain of these RTKs leads to receptor dimerization, activation of the intrinsic protein tyrosine kinase (PTK) activity, tyrosine autophosphorylation, and recruitment of various signaling proteins to these autophosphorylation sites located primarily in the C-terminal tail of the receptor. Tyrosine phosphorylation of the EGFR leads to the recruitment of diverse signaling proteins, including the adaptor proteins Grb2 and Nck, phospholipase Cγ (PLC-γ), Shc, STAT1, and other molecules that are described in more detail in this pathway. The evolutionary conservation of all the components of the EGFR signaling pathway in nematode, fruit fly, mouse, and man underscores the biological significance of this signaling pathway. Furthermore, aberrant regulation of the activity or action of EGFR and other members of the RTK family have been implicated in multiple cancers, including those of brain, lung, mammary gland, and ovary.

This Connection Maps of signaling by the EGFR and the Fibroblast Growth Factor Receptor Pathway (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_15049) describe the intracellular signaling pathways that are activated by binding of EGF to EGFR or by binding of FGF to FGFRs. Although EGFR is activated by binding of a single ligand molecule to the extracellular domain of the receptor molecule, the activation of FGFR requires the coordinated binding of FGF and heparan sulfate proteoglycan (HSPG) to the extracellular domains of FGFR. The comparison of the signaling pathways that are activated by EGF or FGF stimulation reveals the common and distinct components that mediate the pleiotropic responses induced by the two growth factors. This comparison also shows how a similar set of signaling components is subject to different stimulatory and inhibitory signals. The different connections between key components will alter the intracellular circuitry resulting in specific biological responses induced by EGF or FGF stimulation.

Pathway Details

URL: http://stke.sciencemag.org/cgi/cm/stkecm;CMP_14987

Scope: Canonical

Further Reading

J. Schlessinger, Common and distinct elements in cellular signaling via EGF and FGF receptors. *Science* **306**, 1506–1507 (2004).

Citation: J. Schlessinger, Epidermal growth factor receptor pathways. *Sci. STKE* (Connections Map, as seen October 2005), http://stke.sciencemag.org/cgi/cm/stkecm;CMP_14987.

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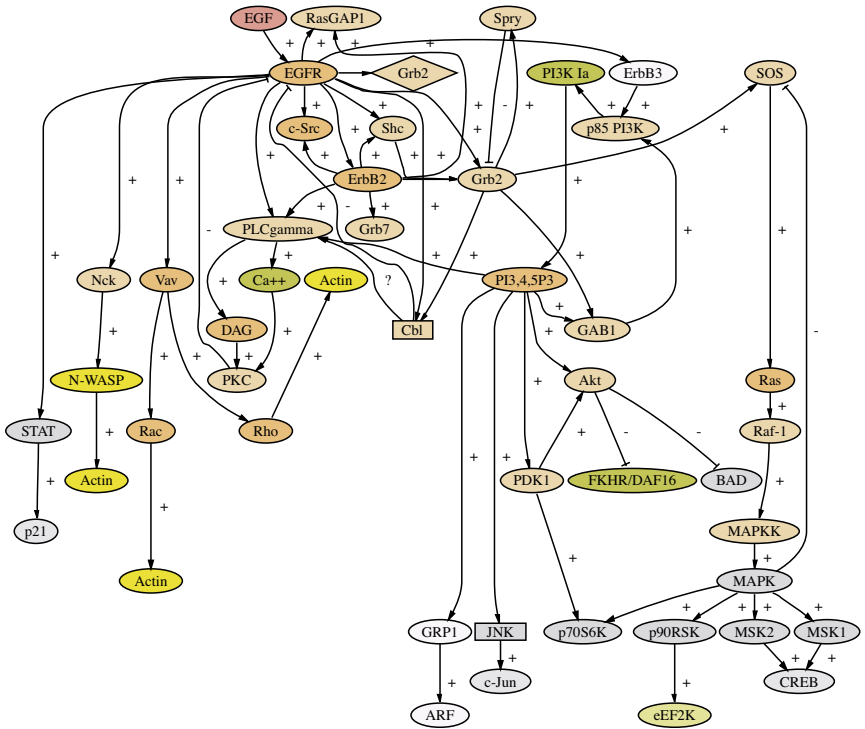
Epidermal Growth Factor Receptor Pathway. Pathway image captured from the dynamic graphical display of the information in the Connections Maps available 21 October 2005. For a key to the colors and symbols and to access the underlying data, please visit the pathway (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_14987).

Epithelial Barriers, Compartmentation, and Cancer

J. M. Mullin

(Published 20 January 2004)

Ninety-five percent of all cancer deaths—including those resulting from cancers of the colon, esophagus, breast, prostate, bladder, lung, pancreas, ovary, and liver—derive from epithelial tumors (*1*). This raises the question of what these diverse tissues have in common. They are, of course, all composed principally of epithelial cells. Although their differentiated properties vary, all of these different epithelial cell types share three features: (i) The cells have a relatively high rate of proliferation and turnover; (ii) all are polar, possessing structurally and functionally distinct front (apical or luminal) and back (basal-lateral or antiluminal) membranes; and (iii) the cells are knit together into an epithelial pavement that separates organ-specific luminal fluids from interstitial fluid (and blood) in the stromal fluid compartment. The high proliferative rate of epithelia is assumed to figure prominently in their high cancer proclivity, but this ignores, for example, the very low incidence of cancer in the small intestine, a tissue with very high cell turnover. Clearly, more is involved.

The compartmentation-oriented architecture of epithelia is a basic property of higher life forms and is in evidence very early in the game of life, with respect to both ontogeny and phylogeny, as seen in the blastocyst or the coelenterate (*2*, *3*). The polarity of epithelial cells allows, for example, for Na^+- and K^+-dependent adenosine triphosphatase proteins to normally occupy the basal-lateral cell membrane, whereas sodium channels or sodium-dependent sugar transport proteins occupy the apical membrane. This arrangement not only creates in part the electrical gradients across an epithelium, but also makes possible unidirectional fluid and solute transport, the bases for epithelial tissue reabsorptive and secretory functions throughout the body. Polarity also allows for the directional secretion of solutes synthesized by the epithelia. The hormone gastrin, for example, is secreted out across the basal-lateral membrane of gastric epithelia into the bloodstream, whereas epidermal growth factor (EGF) is secreted across the apical membrane of salivary and intestinal epithelia (Brunner's glands) into luminal fluid.

In any epithelial cell sheet, the barrier function has at least two components: the cells themselves and the tight junction (TJ) strands that circumferentially band each cell at the most apical point of the lateral intercellular space. These strands act as a gasket, preventing free interchange of most solutes between luminal and interstitial fluids along the lateral intercellular (paracellular) route. Composed of occludin and various members of the claudin family of proteins (*4*), the TJ strands can reject or retard certain solutes on the basis of their size and others on the basis of charge (*5*). The strands seem to create paracellular pores with evidence of specificity based on charge and size (*6*).

These considerations of polarity, compartmentation, and barrier function are the underpinnings of a fascinating development in biomedicine. There are certain times in any area of scientific research where one can witness a new concept taking shape and gaining acceptance. The involvement of epithelial barrier breakdown in the development of epithelial neoplasia is just such a concept at this time, although the "roots" of the concept go back many years (*7–10*). The concept involves the above three interrelated elements: (i) As a result of cell polarity, functional growth factor receptors are normally situated

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on the basal-lateral cell surface facing interstitial fluid and the bloodstream; (ii) growth factor proteins (the ligands for these receptors) are frequently compartmentalized at very high concentrations in luminal fluids within epithelial tissues; and (iii) early in the process of neoplasia, "distortions" occur in TJs such that relatively large solutes may pass across epithelial barriers that normally restrict their movement, a phenomenon one might call "lesional leak." Thus, the concept has developed that TJ disruption in premalignant neoplastic tissue can increase the likelihood that it will develop into a frank carcinoma because of the continuous stimulation of cell division of initiated (premalignant) cells that follows breakdown of the natural barrier between growth factors and their receptors.

The increasing awareness and acceptance of the concept at this time has likely been influenced by a "new" emphasis on the critical importance of cell and tissue organization that has emerged in the postgenomic era. If "ships are not exactly coming in" as yet, at least masts can be seen on the horizon. One such indicator consists of reports in the literature of tumor suppressor or modifier genes coding for TJ or TJ-associated proteins involved in maintaining epithelial and endothelial barrier function (*11–13*).

A consideration of the role of EGF in the gastrointestinal tract can perhaps best illustrate the rationale behind this concept. EGF is secreted in tremendous amounts across the apical membranes of epithelia in the salivary glands. It is present in saliva at concentrations much higher than those found in blood (*14*). Produced in the oral cavity, EGF flows down the esophageal epithelium and over the gastric epithelium, or more specifically, across the apical surfaces of esophageal and gastric epithelia. However, EGF receptors are normally situated on the basal-lateral cell surfaces of these and other epithelia (*15*). Furthermore, the epithelial barrier separates ligand from receptor because the TJ will normally reject solutes as large as EGF [molecular weight (MW) 6100; see, for example, (*16*)]. Thus, salivary-derived EGF should be without a function. This counterintuitive physical separation of ligand and receptor has, however, a reasonable explanation that suggests true adaptive value in the course of evolution. Any mechanical or infection-based tearing of the upper gastrointestinal epithelial barrier will result in localized and temporary reuniting of ligand and receptor. The ensuing motility and proliferative effects of EGF (*17*) on the epithelial cells should facilitate repair of the injury to the barrier. Barrier repair will then again separate ligand and receptor, making the process self-limiting.

Imagine ages back, when we swallowed bones with every meal. One can easily visualize adaptive advantage to such an elegant wound repair mechanism in an epithelium like the upper gastrointestinal tract. Wounding results in transepithelial leak of both a motility factor and a growth factor (EGF). The ensuing access to receptors, followed by cell spreading and cell division, should result in rapid wound healing. Signaling subsequently stops as ligands and receptors are segregated once more. This process would be valuable and conserved in various epithelial tissues. Indeed, the role of luminally derived EGF in gastrointestinal wound healing has support in research findings going back at least 10 years (*18–20*).

A similar scenario has just been described in airway epithelia for heregulin and the erbB receptor, members of the EGF and EGF receptor (EGFR) families, respectively (*21*). This recent research has shown not only segregation of secreted ligand (heregulin) and receptor (erbB) by airway epithelia, but also the ability of TJ disruption (by calcium chelation) to unite ligand and receptor, leading to receptor phosphorylation (activation). In similar fashion, physical wounding of an airway epithelial cell sheet allowed (luminal) heregulin to accelerate wound recovery by activation of (basal-lateral) erbB receptor. The authors speculate about the role of pathophysiological TJ disruption in this overall phenomenon of contact between an apical ligand and a basal-lateral receptor. Cystic fibrosis and asthma are mentioned as two such disease states.

Although the process of luminal sequestration of growth factors certainly has adaptive value in this wound repair scenario, it does not "consider" situations of chronic TJ leak, which would allow luminal growth factors to have persistent, uninterrupted access to their receptors. Such chronic TJ leaks occur in epithelial cancers, which are largely a disease of our postreproductive years, a period in which evolution takes diminished interest. However, that period is now almost 50% of our life-spans.

The persistent and uninterrupted nature of such a transepithelial leak in cancer brings the discussion back a step to a more generalized picture of what transpires in the onset of cancer. It has been recognized for many years that the process of cancer development is not one, but a series of events, affecting a cell. In elegant experiments on mouse skin carcinogenesis, Boutwell defined at least two independent stages in the process (*22*). The first, termed "initiation," is a heritable irreversible change to a cell, as occurs for example in DNA alkylation by certain carcinogens. The second was described as a "promotional" event, which was thought to be extranuclear and reversible, and to require uninterrupted duration to achieve its tumorigenic goal. It is possible that this promotional stage is itself a cascade of events, with growth factor paracellular leak as one of its final stages. Interestingly, the "tumor promoter" class of chemicals that figured prominently in Boutwell's work—typified by 12-*O*-tetradecanoylphorbol 13-acetate (TPA or PMA)—is capable of inducing TJ leakiness at very low concentrations through its activation of one or more protein kinase C isoforms (*23*, *24*). This signaling pathway proceeds through the kinases MEK and ERK as downstream intermediates (*25*).

It has been known for almost 40 years that the TJs of epithelia are likely to be structurally altered in neoplasia (*26*). This has since been shown for ovarian cancer (*27*), breast cancer (*28*), glioblastoma (*29*), liver cancer (*30*), and colon cancer (*31*). However, leaky TJs could be just one of a myriad of characteristics of cancerous epithelia. On the other hand, precancerous conditions of the gastrointestinal tract such as aberrant crypt foci (*31*), Crohn's disease (*32*), gastric dysplasia (*33*), or Barrett's esophagus (*34*) also evidence TJ leakiness. This is of pivotal importance, because such leakiness can then play a role in the process. Moreover, tumor-promoting chemicals such as TPA have been shown to engender TJ leakiness to a wide range of solutes, including the growth factors EGF and insulin, which can thereby cross an epithelial barrier without degradation and enter the opposite fluid compartment while remaining biologically active (*35*, *36*). Interestingly, a related growth factor and potent epithelial oncoprotein, transforming growth factor–β (TGF- β), has been shown to itself have a TJ-disruptive effect on epithelia (*37*). Activation of the erbB2 receptor, a receptor for the EGF family of ligands whose overexpression is associated with breast cancer, causes TJ disruption in mammary epithelia (*38*). In each case, the TJ disruption will compromise barrier function and thereby potentially alter growth factor compartmentation.

Increasing the number of EGF receptors in epithelial cells has been observed to cause "misdirected" localization of receptors to the apical cell surface (*39*, *40*). This is pertinent because receptor number is characteristically elevated in transformed epithelia (*41*) and receptor expression in the cell surface facing luminal fluids is tantamount to (luminal) ligand diffusion to the intercellular space. Both would result in abnormal ligand-receptor interaction. In summary, the process of transformation/neoplasia is not protective of the two key properties of epithelia: their polarity and their barrier function.

Given a hypothetical scenario in which luminal growth factors cross epithelial cell layers at precisely the loci of transformed cells with altered TJs, these transformed epithelia would be placed under a chronic growth and motility stimulus. Entry of a luminal growth factor into an interstitial fluid compartment at foci of preneoplastic epithelia could create at least two interesting outcomes concerning the transformed state. First, the ligand could interact with the epithelial cells of the barrier itself, altering their own cell kinetics, acting in effect as a tumor-promoting event after their initial transformation
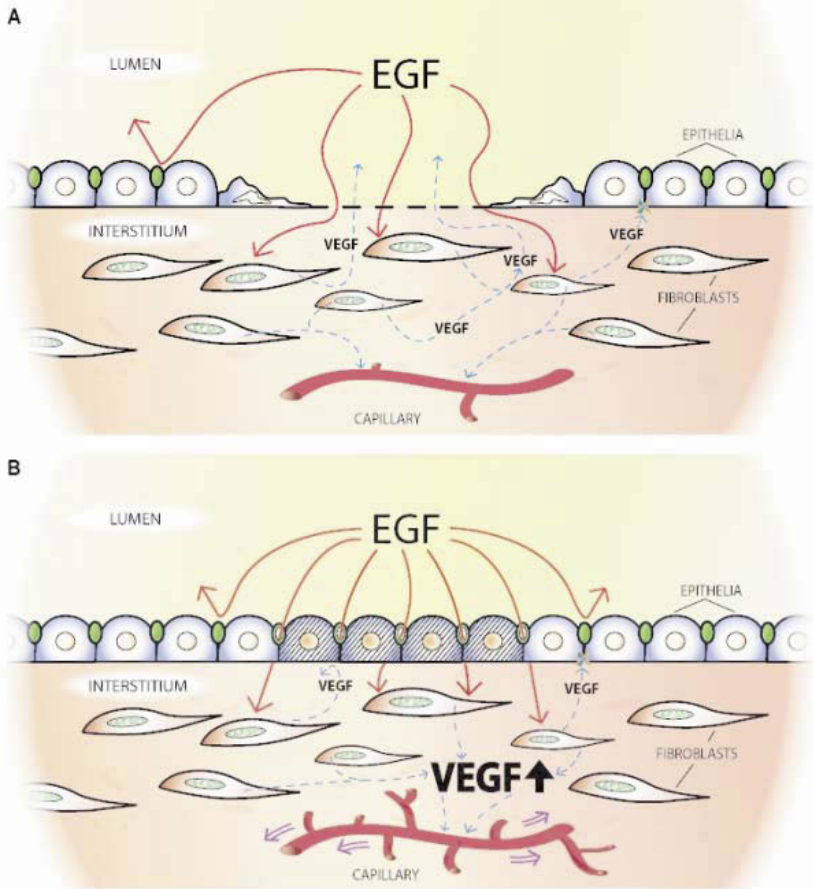
Fig. 1. (**A**) Illustration of epithelial wounding, where EGF from luminal fluid flows down its concentration gradient into the interstitial (stromal) fluid compartment. Entrance into this compartment gives luminally derived EGF access to EGF receptors on basal-lateral surfaces of epithelial cells, in turn engendering epithelial cell motility and proliferative changes that can accelerate reformation of the epithelial barrier. In addition to epithelial receptors, EGF can also access receptors on stromal fibroblasts. EGF is known to elicit increased VEGF synthesis and secretion (*53*, *54*). VEGF in turn can bind to endothelial receptors, thereby promoting angiogenesis (*55*). However, this second effect would be tempered by back-leakage of interstitial fluid constituents such as VEGF into the luminal compartment, thereby reducing their concentration in the stromal fluid. (**B**) In the case of preneoplastic foci, EGF leaks across distorted cell junctions and can produce similar effects on epithelial cells; however, this effect does not self-limit as in wound closure. It continues indefinitely and centers its promoting action on initiated, transformed epithelia. It would have similar action on stromal fibroblasts, namely, eliciting VEGF secretion into what will become the future tumor microenvironment. Unlike the situation with wounding, a "roof" (the epithelium), however leaky, exists across the stroma, which may reflect back VEGF (MW 46,000, substantially larger than EGF) and keep its concentration in the stroma higher than in the stroma below an epithelial abrasion. VEGF, vascular endothelial growth factor.

(Fig. 1). Second, the ligand could interact with receptors on fibroblasts in this stromal fluid compartment. In the case of EGF, this could stimulate these cells to produce vascular endothelial growth factor (VEGF), which could lead to a proangiogenic environment in this compartment, the putative future tumor microenvironment. Note that in this scenario, a key difference from wounding an epithelium (aside from the chronic, sustained nature of the leak) is that growth factors produced in response to the influx of the luminal growth factor may be trapped and thereby concentrated under the epithelium (if they are substantially larger than the luminal growth factor), whereas in a wound they can dissipate across the breach of the barrier.

Although the gastrointestinal tract, with salivary and Brunner's gland secretion of (luminal) EGF, provides a useful model to visualize this overall phenomenon, other epithelial tissues likely make use of similar restorative (and tumor-promoting) phenomena. One can find very high levels of EGF in luminal fluid (urine) of the urinary tract (*42*), as well as basal-lateral localization of EGF receptors (*43*, *44*). The situation regarding heregulin and the erb-2 receptor in airway epithelia was described earlier (*21*). Keratinocyte growth factor is secreted into luminal fluid of the uterus (*45*). TGF-â and insulin-like growth factors are secreted into breast milk, a luminal fluid that can potentially affect the mammary gland as well as the gastrointestinal tract of an infant (*46*, *47*).

Although this overview has focused on breakdown of ligand compartmentation in epithelial cancers, there are likely a number of other diseases of epithelial and endothelial tissues where one or another facet of this overall concept will come into play. Polycystic kidney disease is one possible example (*48*). Certain central nervous system disorders are another, as can perhaps be hinted at in the concomitant decrease of both blood-brain barrier TJ permeability and angiogenesis by src-suppressed C-kinase substrate, which is a protein kinase C substrate and potential tumor suppressor (*49*).

To underscore the fact that we are only beginning to understand the role of transepithelial growth factor leakage through TJs in wound healing and for neoplastic progression, there already are known facts that are either in opposition to this model or require its adjustment. There is, for example, evidence for apical growth factor receptors in many different cell types (*50, 51*). In addition, there is definitive evidence for normal transepithelial passage of growth factors by transcellular, transcytotic routes (*52*). The disease relevance of paracellular transit of ligands may reside in its relatively unregulated and abnormal nature, but the existence of apical growth factor receptors and of cellular-based ligand transport will require modifications to the overly simplified model presented here.

Clearly, we are just beginning to understand this phenomenon and the mechanisms responsible for it, both as they relate to wound repair and to their potential in neoplasia. Like any model, it needs to be continually tested and questioned. If it does hold true, it contains the promise of new points of interdiction in limiting the growth of a wide variety of epithelial cancers.

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T Cell Signal Transduction

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(21 October 2005)

Description

This record contains general information about T cell signal transduction collected across species.

Upon T cell receptor (TCR) engagement, Lck (a member of the Src family of protein tyrosine kinases) phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the cytoplasmic domains of the chains of the CD3 complex (Fig. 1). Subsequently, ZAP-70 (a member of the Syk family of kinases) is recruited by way of its Src homology-2 (SH2) domains, binding to the phosphorylated ITAM sites. Activated ZAP-70 propagates signal transduction through the phosphorylation of down-stream targets including the adapter molecules LAT and SLP-76. These adapters, in turn, facilitate phospholipase C-gamma 1 (PLC-γ1) activation, resulting in the cleavage of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The known signaling pathways that lead to T cell activation upon TCR engagement rely on IP3 and DAG second messengers. IP3 triggers calcium mobilization, which leads to activation of nuclear factor of activated T cells (NF-AT). DAG activates RasGRP and protein kinase C theta (PKC-θ), which in turn leads to ac-tivation of the Ras-mitogen-activated protein kinase (Ras-MAPK) and nuclear factor kappa-B (NF-κB) pathways respectively. CD45 and Csk have been identified as key proximal regulators of T cell signal transduction by modulating phosphorylation and, hence, enzymatic activity of the Src family kinases.

Pathway Details

URL: http://stke.sciencemag.org/cgi/cm/stkecm;CMP_7019

Scope: Canonical

Further Reading

A. L. Singer, G. A. Koretzky, Control of T cell function by positive and negative regula-tors. *Science* **296**, 1639-1640 (2002).

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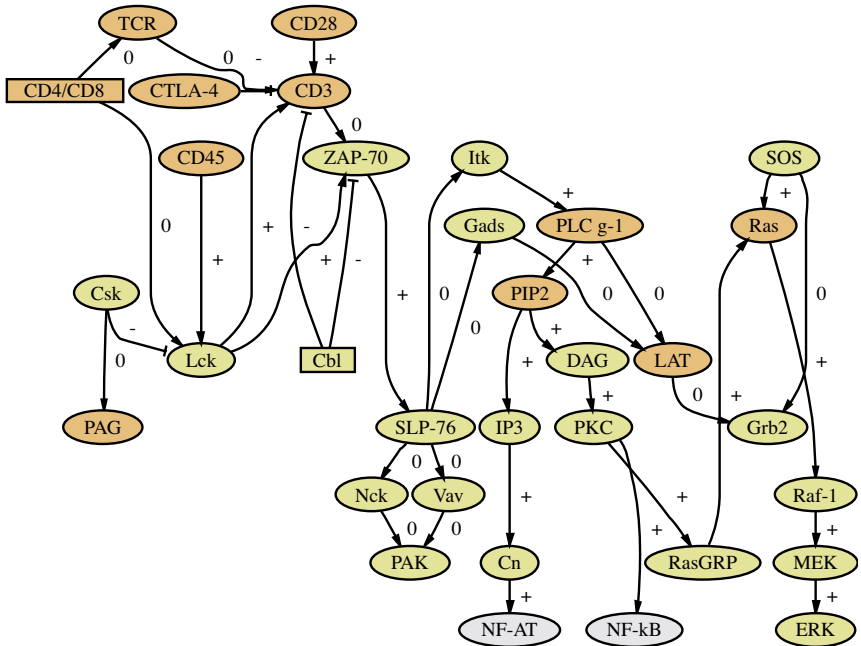
T Cell Signal Transduction. Pathway image captured from the dynamic graphical display of the information in the Connections Maps available 21 October 2005. For a key to the colors and symbols and to access the underlying data, please visit the pathway (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_7019).

Swapping Molecules During Cell-Cell Interactions

Jonathan Sprent

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Multiple studies have shown that interactions between cells of the immune system can cause surface molecules to move from one cell to another (*1*). An elegant example of cell-surface molecular exchange is provided by the recent finding that interactions between natural killer (NK) cells and their targets cause bidirectional receptor-specific transfer of molecules between these cells (*2*). Thus, interaction between inhibitory killer immunoglobulin (Ig)–like receptors (KIRs) on NK cells with major histocompatibility complex class I (MHC I) ligands on target cells can cause NK cells to absorb MHC I (*2–5*) and target cells to acquire KIR (*2*). The antigen-specific receptors on T (*1*, *6*, *7*) and B (*8*) cells can also absorb cell-associated ligands from antigen-presenting cells (APCs). However, in this case receptor-mediated absorption is largely unidirectional. Thus, for T cell receptor (TCR) interaction with MHC-peptide complexes on APCs, T cells absorb MHC-peptide complexes, but there is no reciprocal transfer of TCRs to APCs.

The notion that T cells can absorb molecules from APCs has a long history and dates back to the finding that murine T cells transferred to MHC-different hosts expressed Ig molecules with binding specificity for host MHC molecules (*9*). Subsequent studies showed that the Ig molecules on the T cells were derived from contaminating donor B cells and represented host-specific alloantibody, presumably bound to fragments of host MHC antigens held by the antigen-specific receptors on the donor T cells (*10*, *11*). Strong support for this antigen bridging model came from later studies showing that activated MHC-reactive T cells were able to bind membrane vesicles expressing specific MHC alloantigens and that the bound MHC ligands on the T cells then provided targets for binding of specific MHC alloantibody (*12*). The bound vesicles led to T cell proliferation, indicating that the absorbed material was immunogenic (*13*). Subsequent studies with T cell lines and clones demonstrated that T cell absorption of MHC ligands was antigen specific and that MHC II–restricted clones absorbed both MHC I and MHC II from APCs (*14*), implying that both specific and adjacent (bystander) ligands on APCs were absorbed (*13–15*). Studies with bone marrow chimeras showed that T cells could acquire MHC II in vivo, apparently from thymic epithelial cells (*16*, *17*). More recently, intercellular transfer has been shown to involve multiple cell-surface molecules and to apply to various cell types (see below).

Receptor/Ligand Interactions

When TCR transgenic T cells recognize specific MHC-peptide complexes on APCs, transfer of MHC to the responding T cells is largely mediated by TCRs (*6*, *7*). However, this is clearly not the only mechanism for absorption, because culturing purified polyclonal T cells with syngeneic APCs in the absence of foreign antigen leads to rapid absorption of MHC and other molecules from the APC, even in the presence of monoclonal antibody to the TCR (*7*). This TCR-independent absorption by T cells is controlled in part through recognition by the CD28 receptor protein of its ligand B7 (B7-1 or B7-2).

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Thus, especially for CD4+ T cells, absorption of both B7 and MHC molecules from APCs by polyclonal T cells is much higher with normal T cells than with T cells from CD28−/− mice (7, 18). CD28-mediated absorption by T cells is apparent with resting T cells but is much higher with activated T cells, presumably reflecting the higher expression of CD28 on these cells. In contrast to CD28-B7 interaction, T cell absorption through interaction of the T cell adhesion molecule LFA-1 (lymphocyte function–associated antigen–1) with ICAM-1 (intercellular adhesion molecule–1) interaction is quite weak, which is surprising because LFA-1 is thought to be a much more effective adhesion molecule than CD28 (7). The role of additional receptor/ligand interactions in T cell absorption, such as the uptake of OX40L from APCs (19) and of various adhesion molecules during transendothelial migration of activated T cells (20), is less clear.
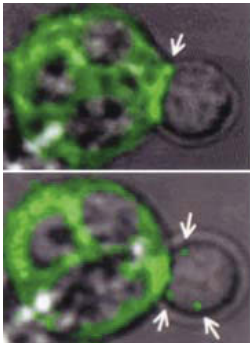

Fig. 1. Rapid internalization of APC-derived MHC I by CD8+ T cells. Naïve TCR transgenic 2C CD8+ T cells, which are specific for MHC I L^d plus QL9 peptide, were incubated with transfected Drosophila cell APCs expressing L^d linked to green fluorescent protein (GFP). In the presence of QL9 peptide, contact of a 2C CD8+ cell (**top**) with the L^d-GFP-expressing APC (**bottom**) caused L^d capping on the APC at the site of T-APC contact by 5 min (top). By 20 min, small fragments of L^d were internalized by the T cells (bottom).The data are adapted from (6).

Mechanism of Absorption

Because the molecules transferred can involve both specific and bystander ligands, it is likely that the molecules are absorbed in the form of membrane fragments during cell-cell contact. The simplest explanation is that, in some receptor/ligand interactions, the avidity of interaction is sufficient to cause small pieces of plasma membrane to be ripped off the surface of the donor cell. This scenario is especially applicable to cell-cell interactions that involve immune synapse formation; that is, where local association with the cytoskeleton can augment and stabilize receptor/ligand interactions and even lead to local membrane fusion (6, 21–25). Dissociation of the cells may then cause small pieces of membrane to be pinched off and transferred from one cell to another. For NK cells, capture of molecules from target cells is a metabolically active process that requires the actin cytoskeleton, intracellular adenosine triphosphate, Ca^{2+}, and functional protein kinase C (23). For T cell uptake of molecules from APCs, the cytoskeleton is crucial for ligand internalization but is less important for initial uptake onto the cell surface, especially when high-avidity receptor/ligand interactions are involved (26).

In considering the role of the cytoskeleton, it is curious that cell to-cell movement of the bound molecules can be unidirectional or bidirectional, depending on the receptor/ligand interaction concerned. How the direction of transfer is controlled is unclear. KIR-mediated uptake of MHC I ligands from target cells is enhanced when the latter are pretreated with cytochalasin D or latrunculin B, agents that disrupt the cytoskeleton (2). Conversely, such treatment reduces the reciprocal transfer of KIR to the target cells. These findings suggest that the direction of transfer is somehow influenced by differential association of the ligands with the cytoskeleton, but the mechanisms involved are still obscure (1).

As an alternative to direct transfer at the immune synapse, the molecules might be acquired through binding of membrane vesicles that are shed or secreted by the donor

cells. For T cell uptake of molecules from APCs, this question has been addressed by culturing these cells together or separated by a porous but cell-impermeable membrane in Transwells (*7*, *26*). In some situations, absorption through a Transwell membrane is very limited, especially when naïve T cells are used and the density of ligand on the APC is low. Under these conditions, T cell uptake of molecules from APCs generally requires direct cell-cell contact. In other situations, however, efficient absorption of molecules can occur through a Transwell membrane. In this case, high-avidity receptor/ligand inter-actions are essential. Thus, for TCR-independent CD28-mediated absorption, uptake via a Transwell membrane is prominent only with activated T cells; that is, cells expressing a high density of CD28. Likewise, TCR-dependent absorption in Transwells requires a high density of peptide/MHC ligand on the APC.

Many cell types, including dendritic cells (DCs) (*27–31*), B cells (*32*, *33*), and T cells (*15*, *34–36*), shed membrane fragments in the form of exosomes (*1*, *37*, *38*). These are Transwell-permeable small (50 to 100 nm) membrane vesicles derived from fusion of multivesicular endosomes with the plasma membrane. In APCs, the release of exosomes is especially prominent for immature DC (*27*, *29*, *30*, *37*). After peptide loading, exo-somes from immature DCs are strongly immunogenic, although only after absorption and cross-presentation of peptides by mature DCs (*29*, *30*). Uptake of exosomes by DCs is receptor/ligand-specific and requires DC expression of αVβ3 integrin, LFA-1, and ICAM-1 (*31*, *38*); these molecules attach to various ligands on the exosomes, including milk fat globule E8 (also called lactadherin), phosphatidylserine, and the tetraspanins CD9 and CD81. APC-derived exosomes also bind to T cells, including both naïve and activated T cells (*39*). For naïve CD8+ T cells, binding is strongly dependent on TCR–MHC I–peptide interaction. There is also a strict requirement for LFA-1–ICAM-1 interaction; that is, exosomes have to express ICAM-1 as well as specific MHC-peptide complexes (*39*). To be immunogenic after binding, the exosomes also have to express B7, presumably to provide costimulatory function. With combined expression of MHC I–peptide complexes, ICAM-1, and B7, the exosomes are directly stimulatory for naïve CD8+ cells in the absence of APCs or cytokines.

Fate of Absorbed Molecules

The transfer of molecules during cell-cell contact is quite rapid. Thus, T cell uptake of MHC-peptide complexes from APCs is prominent within 5 min of initial cell culture (*6*, *7*). The absorbed material is detectable on the cell surface for several hours but then gradually disappears, largely through internalization. MHC-peptide complex internaliza-tion is apparent within 10 to 20 min (Fig. 1) and parallels that of TCR down-regulation, suggesting that intact TCR-MHC-peptide complexes are internalized. Similar rapid in-ternalization applies to molecules absorbed by NK cells, DCs, and B cells (*1*, *3*, *8*, *31*). For DCs and B cells, the absorbed ligands are directed to endosomes, which allows the processing of antigen and loading of peptides onto MHC II molecules. In CD8+ T cells, internalized MHC I molecules are destroyed in lysosomes (*6*, *7*). In certain situations, such as the binding of exosomes (or iccosomes, for immune complex–coated bodies) by follicular DCs in germinal centers, the absorbed material may remain on the cell surface for prolonged periods (*40*).

Biological Significance

The transfer of cell-surface molecules during cell-cell interaction could be an epiphe-nomenon, an inevitable by-product of high-avidity receptor/ligand interaction. Even so, the immunological consequences of this phenomenon are considerable. Thus, for APCs, stripping these cells of specific MHC-peptide complexes by T cells during early stages of the immune response may lead to preferential stimulation of high-affinity T cells (*41*). For T cells, absorption of MHC I–peptide complexes from APCs makes CD8+

cells transiently sensitive to lysis by adjacent cytotoxic T lymphocytes (CTLs) (*6*); such fratricide could serve to limit immunopathology caused by overexuberant CTL activity. T cell absorption of MHC and other ligands could also allow these cells to act as APCs for other T cells (*15*, *18*). Another possibility is that absorbing membrane fragments from APCs and other cells may facilitate cell-cell dissociation, thus allowing T cells to move from cell to cell and enter the circulation to mediate effector function (*7*). As for T cells, the capacity of NK cells to remove membrane fragments during target cell killing may facilitate cell detachment and thereby improve the efficiency of killing. Conversely, KIR uptake by target cells could prevent NK cells from repeated scanning of the same targets (*2*). For B cells, internalization and then processing of cell-associated antigens may be a vital prelude for enabling B cells to present surface MHC II plus peptide to T helper cells (*8*).

This article centers on cells of the immune system, but it seems highly likely that intercellular transfer of cell-associated molecules is of broad biological significance. For example, the secretion of exosome-like membrane vesicles may have an important role in tissue development (*42*) and also in fertility (*43*). Cell-to-cell spread of intracellular pathogens such as HIV could be augmented by transfer of the HIV-binding chemokine receptor CCR5 to CCR5$^-$ cells (*44*) and of hitchhiking of HIV in exosomes (*38*). Exosomes can also transmit prions (*45*). These findings highlight the physiological importance of intercellular membrane exchange, but there is clearly much more to discover about this intriguing topic. Information on the various receptor/ligand interactions controlling ligand transfer is still limited, and it will be especially important to establish whether the transferred ligands can contribute to cell signaling, either on the cell surface or after internalization.

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Estrogen Receptor Pathway

John D. Norris and Donald P. McDonnell*

(21 October 2005)

Description

T*his record contains general information about the estrogen receptor pathway collected across species.*

The estrogen receptor (ER) is a ligand-dependent transcription factor. Numerous proteins and processes impinge on ER function, which leads to an unexpected level of complexity in the actions of this hormone. Both positive and negative regulators are shown in the pathway.

Pathway Details

*URL*: http://stke.sciencemag.org/cgi/cm/stkecm;CMP_7006

Scope: Canonical

Further Reading

D. P. McDonnell, J. D. Norris, Connections and regulation of the human estrogen receptor. *Science* **296**, 1642-1644 (2002).

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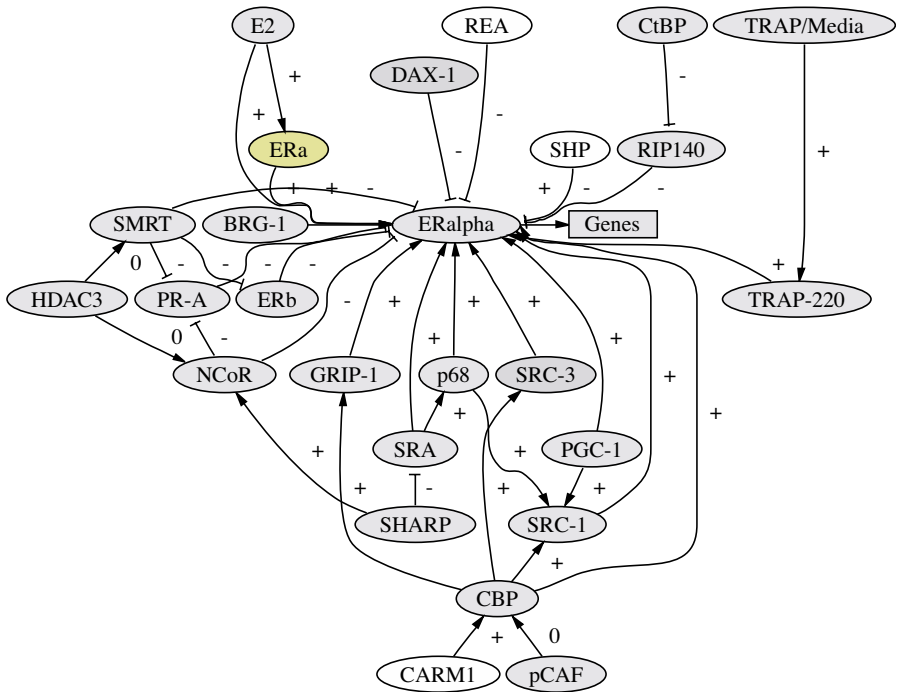
Estrogen Receptor Pathway. Pathway image captured from the dynamic graphical display of the information in the Connections Maps available 21 October 2005. For a key to the colors and symbols and to access the underlying data, please visit the pathway (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_7006).

Crossroads of Estrogen Receptor and NF-κB Signaling

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In a recent report, Chadwick *et al*. (*1*) presented an innovative concept regarding the mode of action of the ER when it interacts with selective ligands. These authors demonstrated that the nonsteroidal compound WAY-169916 blocked proinflammatory signals mediated by the transcription factor NF-κB. Inhibition of NF-κB signaling required binding of the selective ligand WAY-169916 to either of the two forms of ER (ERα or ERβ) and, apparently, this pathway-selective anti-inflammatory effect was achieved through a nonclassical mode of action by the receptor. In animal models, systemic administration of WAY-169916 reversed disease states caused by an abnormal inflammatory response. Furthermore, WAY-169916 failed to induce familiar effects of estrogen, mediated by its classical ligand-dependent function in promoting gene expression. Thus, WAY-169916 appears to be an anti-inflammatory agent that requires ER for its action and blocks proinflammatory signaling by NF-κB.

What brought these authors to examine the effects of an ER ligand on inflammation? In clinical medicine, states of estrogen excess and in particular pregnancy ameliorate symptoms of inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease (Crohn's disease and ulcerative colitis), and multiple sclerosis. These effects are attributed to the increased concentrations of estrogen produced during pregnancy. Furthermore, molecular approaches have documented antagonistic cross-talk between the NF-κB and ER pathways by demonstrating the ability of estrogen-activated ER to quell NF-κB signals. The mechanisms can only be inferred but may include direct protein-protein interaction, inhibition of binding of NF-κB to DNA, or unbalanced sharing of transcriptional coactivators. Chadwick *et al*. (*1*) exploited cross-talk between ER and NF-κB pathways by developing an ER ligand that selectively inhibited NF-κB and inflammation, without inducing classical estrogen effects.

The ovarian hormone estrogen controls cell proliferation and differentiation in reproductive organs such as the uterus, pituitary gland, mammary gland, and ovary. However, estrogen has other effects in humans, among which are effects on the skeletal, cardiovascular, and nervous systems that influence bone density, concentrations of blood lipids, and cognitive function. The NF-κB transcription factor is activated by a multitude of stimuli, including cytokines, growth factors, viral and bacterial infections, and various mediators of cell stress (*2*, *3*). In health, NF-κB signaling is required for the normal inflammatory response caused by immune activation. NF-κB is linked to disease states by way of overactivity, usually a consequence of aberrant stimulation by otherwise normal signals.

In the canonical view of ER signaling, estrogen and related sex steroids bind to the ER to promote formation of a receptor homodimer, which is released from cytoplasmic chaperones to enter the nucleus and to transactivate responsive target genes (Fig. 1). This

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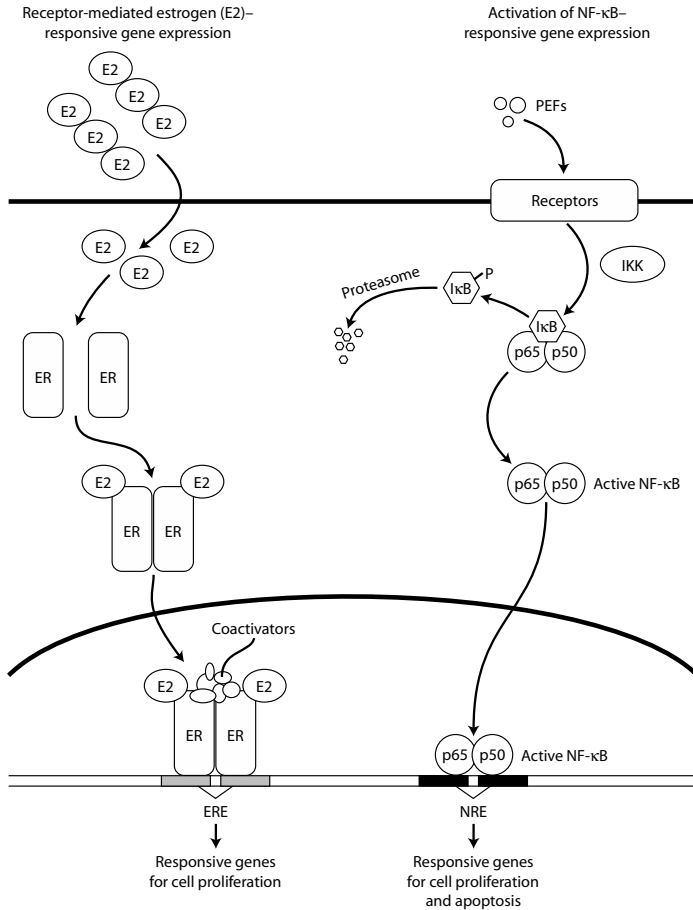
Fig. 1. ER and NF-κB signaling pathways. (**Left**) Fundamentals of ER signaling. This elementary scheme illustrates the genomic actions of the ER, which entails the expression of estrogen-responsive genes. The ovarian hormone estrogen (E2) interacts with its cytoplasmic receptor (ER). On estrogen binding, monomeric ER forms a dimer in a process chaperoned by heat shock protein 90. ER bound to E2 is released from the cytoplasm, and the ligand-activated factor traffics to the nucleus where it binds to ER response elements (ERE) in the promoter region. In the presence of specific coactivators, ER initiates transcription of responsive genes, which are necessary for cell proliferation and differentiation of cells in reproductive organs of the female. (**Right**) Activation of NF-κB. NF-κB is a transcription factor that exists as homo- or heterodimers of Rel (reticuloendotheliosis) family proteins. One group, the processed members of the family, includes RelA (p65), RelB, and c-Rel. The second group contains the unprocessed members p105 (precursor to p50, NF-κB1) and p100 (precursor to p52, NF-κB2), which are cleaved to generate p50 and p52 in the cytoplasm. All these proteins have the common Rel homology domain (RHD), which contains DNA binding, nuclear localization, transactivation, and the IκB-binding domains. The p65-p50 heterodimer is the most commonly detected and most abundant form of NF-κB in different cell types.

classical signaling by estrogen and ER may be designated "genomic" signaling. The system is activated by estrogen to perform normal functions in female reproduction, or it can mediate abnormal proliferation of mammary and endometrial cells in breast and uterine cancer. In the NF-κB signaling pathway, pleiotropic extracellular factors interact with cell surface receptors and cause the release of active NF-κB (a family of at least five distinct subunits, which form homodimers or heterodimers when activated) from phosphorylated cytoplasmic inhibitory protein κB (IκB). IκB-kinase (IKK) and, perhaps, other protein kinases catalyze phosphorylation of IκB (*4*). Like ER, activated NF-κB is translocated to the nucleus, where it binds to the promoter regions of a cohort of target genes and ultimately influences cell proliferation and evasion of cell death (apoptosis).

NF-κB is located in the cytoplasm in most cells (with the exception of B cells) in an inactive state sequestered with IκB protein. Pleiotropic extracellular factors (PEFs), including ligand triggering of cell surface receptors, initiate phosphorylation cascades that lead to activation of IKK. Activated IKK phosphorylates IκB, marking it for degradation by proteasomes, thereby releasing and allowing translocation of the active NF- κB dimer into the nucleus. The activated NF-κB then binds to its NF-κB response element (NRE) in the promoter region of responsive genes and aids their expression. NF-κB is a multifunctional transcription factor and modulates the expression of genes that influence cell cycle progression, regulated cell death (apoptosis), inflammatory reactions, immune response, metastasis, stress-related genes, and integrated viral genes.

The cell proliferative actions of both estrogen and active NF-κB are mediated by increased expression of the cell cycle regulatory protein cyclin D1. Cyclin D1 forms complexes with cyclin-dependent kinases 4 and 6 (Cdk4 and Cdk6), and the holoenzyme phosphorylates the retinoblastoma protein (Rb), which causes the release of the transcription factor E2F-1. Free E2F-1 then augments expression of specific genes responsible for driving S phase and cell cycle progression (Fig. 2). Thus regulation of cyclin D1 is a point in the "crossroads" at which estrogen and NF-κB signaling merge, and in this case, both promote cell cycle progression (Fig. 2). In the immune system, NF-κB activates inflammatory cells and propels inflammation. In female reproductive organs, estrogen and its receptor act on target genes to cause mixed proliferative and differentiation effects. Estrogen signaling through ER also inhibits NF-κB activation, in a manner that appears to be independent of gene transcription, a so-called "nongenomic" effect of estrogen and ER signaling (Fig. 2). Therefore, the crossroads of these two pathways, exemplified by the inhibition of NF-κB activation, is potentially important in cells that simultaneously carry signaling traffic over both routes.

Myeloid and lymphoid progenitor cells, mature lymphocytes, and neutrophils express ERα, ERβ, or both receptors (*5*). Furthermore, knockout mice devoid of expression of one or both receptor isoforms show problems with immune system development and even develop a mixed myeloproliferative and lymphoproliferative disorder (*6*). NF-κB activation is detected in inflammatory diseases that are associated with increased amounts of inflammatory cytokines (*7*, *8*). Some of these cytokine's genes are direct targets that are both expressed in response to NF-κB activation and can feed-forward to activate NF-κB by causing release of NF-κB from IκB. This can amplify the immune response and prolong inflammation, either in a physiologic response or in a disease state. Estrogen or a selective compound like WAY-169916 can act through the nonclassical and anti-inflammatory pathway between ER and NF-κB and can inhibit the inflammatory chain reaction.

Human breast cancer is another disease state where the NF-κB and ER pathways intersect. Sixty percent of human breast cancers express ER (the ER-positive cancers), and the predominant effect of ER and estrogen is to stimulate cancer cell growth. A probable consequence of the cross-talk between ER and NF-κB in breast cancer cells was observed in human breast tumor specimens (*9*). Active NF-κB detected by DNA bind-
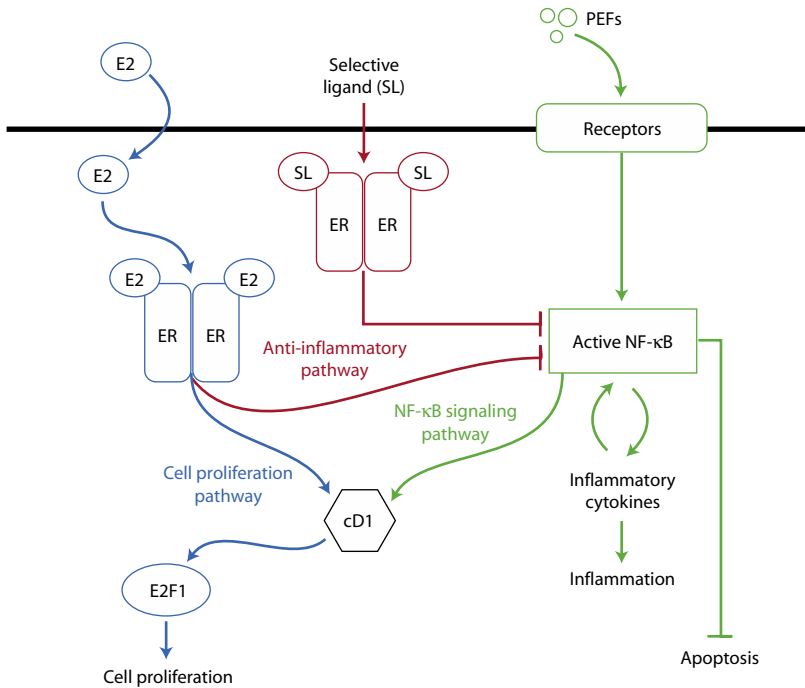
Fig. 2. Crossroads of ER and NF-κB signaling. Estrogen interacts with ER (Fig. 1), which initiates a sequence of events leading to modulation of the expression of responsive genes (blue arrows). Estrogen can also bind to ER and exert nongenomic effects, which inhibit NF-κB activation and the cellular response to inflammation (red arrows). The outcomes of these two diverging actions of estrogen are tissue specific. In reproductive tissues and cells, proliferation predominates and in inflammatory cells, NF-κB is inhibited and proliferation retarded. WAY-169916 is a nonsteroidal ER ligand that can selectively inhibit NF-κB in inflammatory cells, by using the first intersection of these two pathways. NF-κB is activated in steps (Fig. 1) and transits into the nucleus, where it binds to NRE in the promoter regions of responsive genes. In the G1 phase of the cell cycle, the NF-κB and ER pathways that control cell proliferation intersect a second time by controlling the expression of cyclin D1. Cyclin D1 activates the cyclin-dependent kinases 4 and 6 (Cdk4 and Cdk6), which phosphorylate the retinoblastoma protein (Rb) and release the transcription factor E2F-1 from the RB-E2F-1 complex. E2F-1 drives the expression of genes responsible for cell cycle progression and cell proliferation. In immune cells (lymphocytes, neutrophils, and macrophages), NF-κB also stimulates the production of cytokines and inhibits apoptosis of immune effector cells, thereby amplifying the process of inflammation. Estrogen activates both the proliferative and anti-inflammatory pathways of ER, whereas the selective action of WAY results in only the anti-inflammatory effect.

ing was found in extracts of the majority of tumor samples that lacked ER expression (ER-negative tumors), whereas DNA bound NF-κB was practically absent in ER-positive specimens. It is likely that ambient estrogens bound to ER and suppressed NF-κB activity in ER-positive human breast tumor samples. Because primary human breast tumors were taken from patients before treatment, the effect of hormone therapy on NF-κB status in specimens from treated patients has not been addressed.

Restoration of NF-κB signaling during antiestrogen treatment might send proliferative signals to mammary cancer cells. Pharmaceuticals are available that inhibit NF-κB, some of which are in clinical use (for example, bortezomib, a proteosome inhibitor that stabilizes IκB). Many other low-molecular-weight compounds under development target the very specific phosphorylation of IκB by IKK and the subsequent release of active NF-κB. These drugs may find their way into the treatment of both ER-negative breast cancer (with generally high constitutive activity of NF-κB) and perhaps hormone-resistant ER-positive breast cancer.

Currently available drugs that target estrogen signaling can be divided into the selective estrogen response modulators (SERMs), which act as partial agonists; completely antagonistic agents (fulvestrant), which cause rapid degradation of receptor; and the aromatase inhibitors, which inhibit estrogen synthesis. Neither the SERM raloxifene nor the antagonist fulvestrant fully inhibits NF-κB activation produced by interleukin 1. In patients with ER-positive breast cancer, the consequences of estrogen blockade are less certain. Resistance to SERMs is at least partly explained by their acquiring agonist activity with continued usage. Whether NF-κB is reactivated in parallel with the resistance to SERMs is potentially an important issue but remains an unanswered question. Furthermore, if NF-κB activity is modulated during antihormone treatment, will this be beneficial to or detrimental for patients with ER-positive breast cancer? Blocking the chronic repression of NF-κB activation by estrogen signaling, as well as its release by antiestrogen treatment, may play some role in resistance to endocrine therapy of breast cancer.

WAY-169916 requires the presence of one of the ER receptor isoforms for its inhibition of NF-κB. Both fulvestrant and raloxifene antagonized the effect of WAY-169916 in vitro, and fulvestrant blocked NF-κB inhibition in an animal model of inflammatory bowel disease (that is, it blocked the beneficial effect of WAY-169916). Aromatase inhibitors are often prescribed for postmenopausal women with ER-positive breast cancer. In this circumstance, ER is freed from its natural ligands and remains available for binding to a selective ligand like WAY-169916. This natural "back door" into NF-κB activation machinery provides a way to inhibit NF-κB by co-opting the ER in ER-positive breast cancer.

In cultured cell systems and animal models (*9–11*), NF-κB activation has been implicated in breast cancers, particularly those driven by two members of the Erb family of receptors: ErbB1 (epidermal growth factor receptor EGFR) or ErbB2 (HER2 or Neu). Increased expression of ErbB2 due to increased gene copy number (amplification) is found in 20% of human breast cancers, and at least one-half of these ErbB2-positive tumors are ER-positive. Furthermore, most patients with ER-positive cancers receive endocrine therapy with estrogen-receptor inhibitors The consequences of releasing NF-κB from inhibition by estrogen during endocrine attack on breast cancers are unknown and only speculative. Compounds such as WAY-169916 might be beneficial even for therapy of hormone-resistant breast cancers by decreasing the effects of NF-κB activation. Thus, the existence of a crossroads of estrogen and NF-κB signaling traffic could provide rationale for targeting NF-κB activation in both ER-negative and ER-positive breast cancers during endocrine treatment.

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Quantitation of Activated Transcription Factors

To date, screening large numbers of compounds to determine their effect on transcription factor activation has been limited by a lack of suitable assays. Existing methods such as Western blot, gelshift and reporter gene assays lack sensitivity & reproducibility. They also require the running and developing of gels or the transfection of plasmids into cell lines, which makes them difficult to adapt for high-through-put screening.

In this study, an ELISA-based method[1] that assays transcription factor activation is used to study translocation and DNA-binding activity of NFκB p50. Competition assays are also performed to demonstrate the assay's specificity.

Materials & Methods
Cell culture and preparation of cell extracts
HeLa cells (American Type Culture Collection; Manassas, VA) were cultured in minimum essential medium and 10% FBS (Invitrogen; Carlsbad, CA). To activate NFκB, the cells were stimulated with 20 ng/ml TNF-α (R&D Systems; Minnesota, MN) for 30 minutes at 37°C. Nuclear and cytoplasmic extracts were prepared from the unstimulated and stimulated cells using the Nuclear Extract Kit (Active Motif; Carlsbad, CA).

Measurement of DNA-binding activity
The TransAM™ NFκB p50 Kit (Active Motif) was used to assay DNA-binding activity of NFκB p50 in nuclear and cytoplasmic extracts made from unstimulated and stimulated HeLa cells. For competitive binding experiments, 5 μg of nuclear extract from stimulated cells were assayed in the presence of wild-type or mutated competitor

oligonucleotides. TransAM NFκB Kits contain a 96-well plate comprised of twelve 8-well strips. Each well is supplied coated with oligonucleotide that contains a consensus-binding site for NFκB (5´-GGGACTTTCC-3´). This sequence is also present in the wild-type competitor oligonucleotide. The mutated competitor oligonucleotide contains three mutated bases, destroying the NFκB binding site. The p50 primary and HRP-conjugated secondary antibodies, Wash & Binding Buffers and Developing & Stop Solutions are supplied in the TransAM Kit. Figure 1 presents a schematic of the TransAM procedure. These steps are detailed below:

1. *Bind transcription factor to consensus site.*
   30 μl of Complete Binding Buffer is added to each well to be used. For competitive binding, Complete Binding Buffer contains 1, 5 or 20 pmol of the wild-type or mutated competitor oligonucleotides. Increasing amounts (0.625, 1.25, 2.5, 5.0 & 10.0 μg) of nuclear and cyto-plasmic extracts made from the unstimulated and TNF-α stimulated HeLa samples were diluted in 20 μl Complete Lysis Buffer and added to the wells. All samples were tested in duplicate. Blank wells containing only 50 μl Complete Binding Buffer were also assayed. After a 1-hour incubation at room temperature (RT) with mild agitation, the wells were washed 3X with 200 μl Wash Buffer.
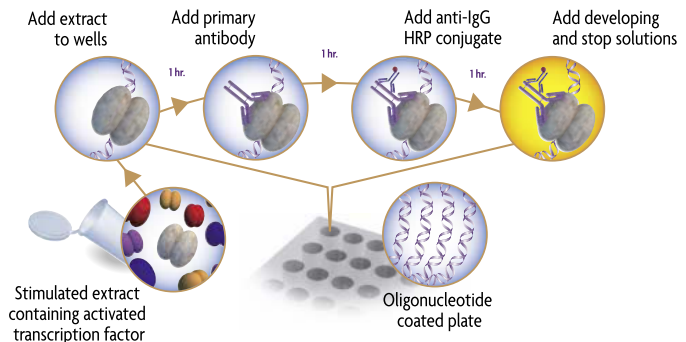

Figure 1: Schematic of the TransAM method.

2. Binding of primary antibody.

100 µl of a 1:1000 dilution of rabbit polyclonal NFκB p50 antibody was added to each well and incubated at RT without agitation for 1 hour. After the incubation, the wells were washed 3X with 200 µl Wash Buffer.

3. Binding of secondary antibody.

100 µl of a 1:1000 dilution of HRP-conjugated, anti-rabbit secondary antibody was added to each well and incubated at RT without agitation for 1 hour. After the incubation, the wells were washed 4X with 200 µl Wash Buffer.

4. Colorimetric reaction.

100 µl TMB substrate was added to each well for 10 minutes before adding 100 µl Stop Solution. Optical density was then read at 450 nm using a CERES UV 900C spectrophotometer (Bio-TEK Instruments Inc., Winooski, VT). All results shown are averages of the two duplicate samples after subtracting the average of the two Blank values.

Results

Activation and translocation of NFκB p50

Figure 2 demonstrates that the TransAM assay effectively measures the DNA-binding activity of NFκB p50. The linearity of the figure shows that the signal is directly proportional to the quantity of transcription factor present. The assay also confirms that stimulation results in both translocation and activation of the DNA-binding activity of NFκB p50 as only nuclear extracts from stimulated cells test positive.
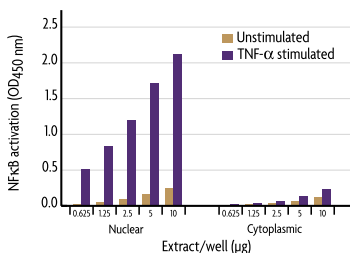

Figure 2: Nuclear and cytoplasmic extracts prepared from unstimulated and stimulated HeLa samples are assayed using the TransAM NFκB p50 Kit.

Reference: 1. Renard, P *et al.* (2001) *Nuc Acids Rsch* 29(4):E21.

Assay specificity

The specificity of the transcription factor/DNA interaction is confirmed through use of the wild-type and mutated competitor oligonucle-otides. Figure 3 demonstrates that increasing amounts of wild-type competitor oligonucle-otide reduce the signal of the nuclear extract. In contrast, addition of even a large excess of mutated competitor oligonucleotide, which does not contain a consensus-binding site for NFκB, does not affect the signal strength. As the p50 antibody in the assay has already been shown to be specific for NFκB p50 by Western blot (data not shown), these results confirms that the measured signal is NFκB p50 bound to its consensus-binding site.
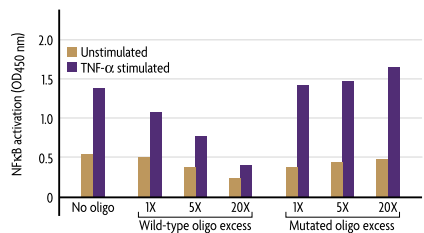

Figure 3: DNA-binding assays are performed in the presence of wild-type or mutated competitor oligos.

Conclusion

Because the TransAM assay specifically measures transcription factor that is bound to its target DNA, it quantifies transcription factor activation. While the extreme complexity of transcription regulation dictates that multiple study systems and numerous assay methods be employed in drug discovery efforts, this ELISA-based method has a unique combination of features (high-throughput format, compatibility with both cell and tissue samples, high-sensitivity, quantitative rather than qualitative data) that make it a powerful addition to existing methods for studying transcription factor activation. The TransAM technology supplied by Active Motif is now available for studying over 30 different transcription factors. For complete information, visit www.activemotif.com.

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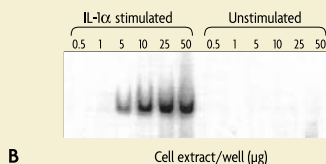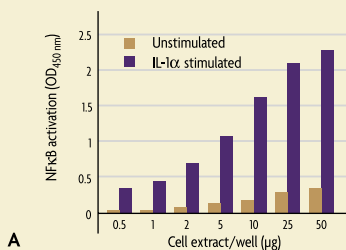
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* Patent pending

A

B   Cell extract/well (μg)

TransAM is more sensitive/easier to quantify than gelshift. Increasing amounts of whole-cell extract from WI-38 cells stimulated with IL-1α for 30 minutes are assayed using the TransAM NFκB p50 Kit (A) or by gelshift (B).

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