Combinatorial Activation of STAT3 by EGF and Thrombin in Endothelial Cells

Matthew S. Waitkus
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COMBINATORIAL ACTIVATION OF STAT3

BY EGF AND THROMBIN IN ENDOTHELIAL CELLS

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Bachelor of Science in Biology
The Ohio State University
June 2007

Master of Science in Pharmacology
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Submitted in partial fulfillment of requirements for the degree

DOCTOR OF PHILOSOPHY IN REGULATORY BIOLOGY
WITH SPECIALIZATION IN CELLULAR AND MOLECULAR MEDICINE

at the

CLEVELAND STATE UNIVERSITY

JANUARY 2014
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External Reader
Dedicated to my family for their steadfast support,

and to the memory of my grandfather, Thomas Stewart.

Thank you for teaching me the value of working carefully and thinking deeply.
ACKNOWLEDGMENTS

I am deeply grateful to my advisor, Dr. Paul DiCorleto, for his support over the past four years. During my time in Paul’s laboratory, I have been extremely fortunate to be granted unrestricted intellectual freedom, outstanding scientific resources, and consistently sound guidance. I will always appreciate Paul’s dedicated intellectual and professional support, and I truly feel there was no better place for me to begin my scientific career.

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COMBINATORIAL ACTIVATION OF STAT3
BY EGF AND THROMBIN IN ENDOTHELIAL CELLS

MATTHEW S. WAITKUS

ABSTRACT

Endothelial cells line the luminal surface of blood vessels and form a regulatory interface between the bloodstream and underlying tissues. The endothelium responds to diverse, and potentially conflicting, environmental signals to regulate vessel growth, leukocyte adhesion, thrombogenicity, and vascular tone. Signaling pathways may interact, or “crosstalk,” in combinatorial signaling environments to enable cells to process disparate extracellular information at downstream signaling nodes and formulate appropriate biological responses based on combinations of extracellular stimuli.

We have reported that simultaneous stimulation of endothelial cells with EGF and thrombin synergistically induces expression of immediate early genes (IEGs) associated with growth and angiogenesis. We sought to characterize the molecular mechanism of EGF receptor (EGFR) and thrombin receptor (protease-activated receptor-1, PAR-1) crosstalk that regulates synergistic IEG induction. Using a bioinformatic comparison of IEG promoter regions, we identified the transcription factor STAT3 as a critical integrator of EGF and thrombin-activated signaling pathways. Depletion of STAT3 by RNAi completely abrogates synergistic IEG induction following EGFR/PAR-1 activation. Analysis of upstream signaling pathways by phosphoproteomic, RNAi, and
pharmacological approaches demonstrated that GSK-3α/β-dependent phosphorylation of STAT3 Ser\textsuperscript{727} is required for synergistic activation of the \textit{EGR1} promotor. Functionally, combinatorial EGFR/PAR-1 signaling suppresses EGF-induced proliferation and thrombin-induced leukocyte adhesion, and triggers a STAT3-dependent increase in endothelial cell migration.

Next, we used quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) to examine the inducible flux and abundance of endogenous STAT3 phosphoforms. We identified and characterized a STAT3 phosphoform that is simultaneously modified at Thr\textsuperscript{714} and Ser\textsuperscript{727} by GSK-3α/β. Both Thr\textsuperscript{714} and Ser\textsuperscript{727} are required for STAT3-dependent gene induction in response to coincident EGFR/PAR-1 activation. In this combinatorial signaling context, preventing formation of doubly phosphorylated STAT3, by depleting GSK-3α/β, is sufficient to disrupt signal integration and inhibit STAT3-dependent gene expression. LC-MS/MS analysis of human renal tumor extracts revealed that levels of doubly phosphorylated STAT3 are remarkably elevated in patients with clear cell renal cell carcinoma, suggesting that the GSK-3α/β-STAT3 signaling axis may be active in this disease. Collectively, our results describe a functionally distinct, noncanonical STAT3 phosphoform that is a critical integrator of disparate signaling pathways in endothelial cells and positively regulates STAT3 target gene expression in this context.
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<tr>
<td>3'UTR</td>
<td>3' Untranslated region</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>API</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
</tr>
<tr>
<td>EGR3</td>
<td>Early growth response 3</td>
</tr>
<tr>
<td>ELAM</td>
<td>Endothelial-leukocyte adhesion molecules</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-related kinase 1/2</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GAS</td>
<td>γ Interferon-activated site</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<td>------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>HB-EGF</td>
<td>Heparin-binding EGF</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HER3</td>
<td>Human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>HER4</td>
<td>Human epidermal growth factor receptor 4</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFNY</td>
<td>Interferon γ</td>
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<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>ISGF3</td>
<td>Interferon stimulated gene factor 3</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon stimulated response element</td>
</tr>
<tr>
<td>IkBs</td>
<td>Inhibitor of NFκBs</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>Mcl1</td>
<td>Myeloid cell leukemia 1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
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<tr>
<td>MKP-1</td>
<td>Mitogen activated protein kinase phosphatase-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MVEC</td>
<td>Microvascular endothelial cells</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor κ B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>p21</td>
<td>Cyclin dependent kinase inhibitor 1</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>PAR-1</td>
<td>Protease-activated receptor-1</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PGH2</td>
<td>Prostaglandin H2</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil containing kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SC</td>
<td>Stalk cell</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SSRE</td>
<td>Shear stress responsive element</td>
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<tr>
<td>STAT1</td>
<td>Signal transducer and activator of transcription 1</td>
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<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TC</td>
<td>Tip cell</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor α</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombin receptor agonist peptide</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor-2</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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1.1 Mechanisms of Endothelial Activation

The vascular endothelium is a monolayer of cells that lines the entire luminal surface of the vasculature and forms a regulatory interface between circulating blood components and underlying tissue compartments. The endothelium covers a network of blood vessels that exceeds 100,000 km in aggregate length, with a surface area of approximately 5000 m$^2$ (1). Its massive size and distribution into all organs and tissues gives the endothelium the capacity to monitor physiological perturbations throughout the entire body (1, 2). By virtue of its juxtaposition with circulating blood components, the endothelium is critically situated to sense and respond to blood-borne stimuli on a systemic scale, making it the body’s largest homeostatic organ (2).
Although it was originally considered a passive barrier, modern research has uncovered numerous mechanisms by which the endothelium actively maintains vascular homeostasis. Under physiological conditions, the endothelium prevents vasospasm, resists leukocyte adhesion, inhibits smooth muscle cell proliferation, and acts as a non-thrombogenic surface for the entire circulatory system (2–4). The endothelium also acts as a selective physical barrier by actively regulating the passage of macromolecules and leukocytes into and out of the bloodstream (3). These physiological roles are influenced by environmental stimuli that cause the endothelial phenotype to fluctuate between antagonistic functions of vascular homeostasis. At different times or locations, the endothelium can be non-adhesive or hyperadhesive to leukocytes, procoagulatory or anticoagulatory, and act as a vasoconstrictor or vasodilator (2). In this way, the endothelium displays a remarkable phenotypic plasticity to adapt to environmental changes. These phenotypic changes are usually normal, adaptive responses to the changing vascular environment and are an essential contribution to vascular homeostasis.

Localized, non-adaptive changes to endothelial physiology can also alter its phenotype to induce changes in vessel tone, leukocyte adhesiveness, coagulation, and the production of autocrine and paracrine factors, including vasorelaxants and vasospastic substances (2, 3). These aberrant alterations are collectively referred to as *endothelial dysfunction*, and they are involved in the initiation and progression of numerous cardiovascular diseases. *Endothelial activation* is a distinct term that describes the process by which bloodborne and environmental stimuli cause endothelial cells to undergo dramatic functional changes and acquire new physiological properties (1).
Proper endothelial function is critical for maintaining vascular health, and endothelial dysfunction may cause or contribute to numerous vascular diseases (2, 5). This chapter will provide a brief overview of normal endothelial physiology that will serve as a foundation for an in-depth explanation of the molecular mechanisms underlying endothelial cell activation, as well as the contribution of endothelial cell activation to the genesis and progression of vascular diseases.

1.1.1 Morphology, Barrier Function, and Biomechanical Signal Transduction

As a continuous monolayer of cells, the vascular endothelium forms an extensive regulatory interface between the bloodstream and underlying tissues (6). Abundant intercellular junctions including tight junctions, gap junctions, and adherens junctions allow the endothelium to function as an active physical barrier (7–10). The relative abundance of these junctions varies between anatomic locations and confers site-specific specialization of endothelial permeability (3). Arteries and blood vessels of the brain contain more tight junctions, which restrict macromolecular flux between intravascular and extravascular compartments. Gap junctions, which consist of connexin proteins (11), link the cytoplasm of neighboring endothelial cells, and likely play roles in intercellular communication (10). Adherens junctions, which consist of cadherin proteins, are important for endothelial cell organization, growth, and migration (1, 10).

The modulation of intercellular junctions via post-translational modifications of junction proteins plays a role in the regulation of vascular permeability. The mechanisms vary according to stimuli, but permeability is usually increased by phosphorylation of junction proteins, actin-myosin-dependent morphological changes, and an increased
number of intercellular gaps in the endothelial monolayer (3, 12–16). For example, phosphorylation of the protein occludin, a component of tight junctions, can positively or negatively regulate its expression depending on the type of mechanical force acting upon the endothelium (13, 16). Furthermore, various inflammatory stimuli, including TNFα and thrombin, activate the Rho-associated coiled-coil containing protein kinases 1 and 2 (ROCK1, ROCK2) (17–19). Activated ROCKs positively regulate myosin light chain phosphorylation to increase actin-myosin contractility and endothelial permeability (20). Under physiological conditions, these changes are controlled and reversible, and represent adaptive control of macromolecular flux between the bloodstream and tissues.

Situated in direct contact with circulating blood, endothelial cells are constantly exposed to a variety of hemodynamic forces including shear stress, hydrostatic pressure, and cyclic strain. Shear stress is particularly significant because it is transduced by endothelial cell-surface proteins to activate signaling pathways that regulate changes in cell morphology, growth, and gene expression (21). The endothelial response to shear stress depends on the type and rate of flow. For example, exposure of endothelial cells to steady laminar flow may induce cell-cycle arrest in G₀ or G₁ by various mechanisms including p53 activation and induction of p21 (22, 23). In contrast, exposure to disturbed blood flow increases endothelial cell proliferation (6, 24, 25). Additionally, laminar flow causes endothelial cells to become aligned and elongated in the direction of flow (26), while disturbed flow causes endothelial cells to adopt a more polygonal shape without a clear orientation (6, 27).

Fluid shear stress also modulates endothelial gene expression. Shear stress response elements (SSRE) have been discovered in the promoters of many genes, and act
as critical *cis*-acting elements for shear stress-regulated gene expression (28, 29). Mechanotransducers, particularly integrins, at the endothelial cell-surface respond to shear forces to activate signaling pathways and transcription factors that regulate gene induction (30, 31). The exact mechanisms by which endothelial cell-surface proteins transduce mechanical forces is not known, but cytoskeletal proteins, receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and ion channels are all known to play a role (13, 14, 16, 21). The ability of endothelial cells to react to specific types of biomechanical forces emphasizes its role as a type of organism-wide sensory organ, capable of detecting and responding to a variety of hemodynamic forces depending on both the type and magnitude of the force.

Atherosclerotic lesions form at sites of disturbed blood flow in arterial vessels, suggesting that changes in shear stress may alter patterns of atheroprotective or atherogenic gene expression (6). Indeed, numerous reports suggest that mechanotransduction of laminar flow and high shear stress is adaptive and atheroprotective, while low-level shear stress or disturbed flow is atherogenic (6). The mechanisms by which disturbed blood flow contributes to lesion formation are not completely understood, but differences in the activation of signaling pathways between laminar and disturbed flow have been observed. Both types of flow activate intracellular signaling kinases and transcription factors including NF-κB, early growth response 1 (EGR1), activator protein 1 (AP1), and JUN N-terminal kinase (JNK) (21). Under laminar flow, the activation of these molecules is transient, increasing over several hours and then returning to basal levels. In contrast, EC exposed to disturbed flow show sustained activation of kinases and transcription factors, including NF-κB and JNK,
which coincides with increased expression of inflammatory genes, such as ICAM-1, VCAM-1, E-selectin, MCP-1, ET-1, and PDGF (6, 21, 32, 33). The differences in the kinetics of pathway activation and gene expression between laminar and disturbed flow may represent a potential basis for the non-random formation of atherosclerotic lesions at vessel bifurcations where blood flow is disturbed or slowed.

1.1.2 Regulation of Hemostasis and Vascular Tone

The endothelium acts as a regulator of vascular tone by synthesizing and secreting vasodilatory or vasoconstrictive substances that act on underlying smooth muscle cells. The most extensively studied endothelial-derived vasorelaxing factor is nitric oxide (NO) (4), which is synthesized by the endothelial enzymes endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). NO exerts a local vasorelaxing effect by diffusing into the smooth muscle cell and activating guanylate cyclase, thereby increasing levels of cyclic guanosine monophosphate (cGMP) (34). Higher levels of cGMP inhibit calcium entry into the smooth muscle cell, resulting in decreased vasoconstriction (35, 36). Endothelial cells are also capable of synthesizing vasoactive metabolites of arachidonic acid called eicosanoids. The most important eicosanoid for maintaining physiological vessel tone is the vasorelaxant prostacyclin. Endothelial cells express the enzymes cyclooxygenase-1,2 (COX-1, COX-2) which synthesize prostaglandin H2 (PGH2) from arachidonic acid (35). PGH2 can then be converted to a number of vasoactive metabolites, but endothelial cells express significant levels of the enzyme prostacyclin synthase (37), thus favoring the formation of prostacyclin.
Prostacyclin is secreted by endothelial cells and is a potent vasodilatory substance that acts on smooth muscle cells to decrease vascular tone (2).

Availability of NO is dysregulated in a number of vascular pathologies, including atherosclerosis and coronary artery disease (1). Reductions in NO availability occur though multiple mechanisms, including decreased NO production or stabilization, and inactivation by ROS (38, 39). Additionally, a number of endothelial-derived vasoconstrictive substances, including ET-1, angiotensin II, and PDGF antagonize the actions of NO and prostacyclin to induce smooth muscle cell contraction (Figure 1, top) (2). ET-1 is the most potent, and perhaps the most pathophysiologically important, vasoconstrictor reported to date. It is expressed as a propeptide by endothelial cells and is activated by proteolytic cleavage mediated by ET-converting enzyme (40). Multiple atherogenic factors induce ET-1, including lipoproteins, IFNγ, TNFα, thrombin and shear stress (42–45). Aberrant expression of endothelial-derived ET-1 may function in a number of vascular diseases, including atherosclerosis, where it is expressed in atherosclerotic vessels and contributes to pathophysiological vasoconstriction (46–48).

Under normal conditions, the endothelium actively participates in the prevention of blood clot formation. This non-thrombogenic property is maintained by the production of several endothelial-derived factors that act on platelets and enzymes of the coagulation cascade. Endothelial cells synthesize thrombomodulin which binds to thrombin and inactivates thrombin’s procoagulant activity (2, 49, 50). Additionally, endothelial cells synthesize the arachadonic acid metabolite prostacyclin (51), which was previously discussed in terms of its role as a vasodilator. Prostacyclin is a particularly potent inhibitor of platelet aggregation and platelet adherence to the endothelium (52, 53).
Under physiological conditions, endothelial-derived prostacyclin activates the prostacyclin receptor IP₁ on platelets to inhibit aggregation and adhesion (54, 55).

Endothelial production of prostacyclin, thrombomodulin, and other anticoagulant factors appears to be constitutive under physiological conditions, necessitating the rapid production of the procoagulant factors when clotting is required. Endothelial cells synthesize numerous thrombotic factors including tissue factor (TF) (56, 57), platelet activating factor (PAF) (58, 59), factor VIII (60), and von Willebrand factor (vWF) (61). TF is particularly important for the transformation of the vessel wall into a thrombogenic surface because it promotes the activation of factors IX and X of the coagulation cascade (1). Healthy vessels maintain a strict balance between thrombotic and anti-thrombotic factors, favoring the expression of anti-thrombotic factors under physiological conditions, and rapidly producing TF and other pro-coagulant factors following exposure to thrombotic stimuli (Figure 1, right) (2). Endothelial expression of the pro-thrombotic TF is induced by a variety of stimuli including thrombin, TNF, shear stress, and oxidized lipoproteins (2, 6). Additionally, changes in the relative expression of tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) contribute to increased thrombogenicity of the activated endothelium. Activated endothelial cells tend to express higher levels of PAI-1, which can be induced by oxidized lipoproteins and shear stress (62–64) and lower levels of tPA (2, 65). Together, these changes reduce the rate of fibrinolysis at localized sites of endothelial cell activation, creating a more thrombogenic environment.
1.1.3 Inflammatory Activation of the Endothelium

Under physiological conditions, the endothelium exists as a remarkably non-adhesive surface for circulating leukocytes. Various inflammatory agonists, including TNFα, IL-1, thrombin, and oxidized lipoproteins stimulate endothelial-leukocyte interactions by inducing endothelial expression of leukocyte adhesion molecules (ELAM) (3, 66–70). Leukocyte adhesion to the endothelium occurs in several steps. First, circulating leukocytes are captured by endothelial-expressed selectin molecules (E-selectin, P-selectin), and “roll” along the luminal surface of the blood vessel. The next step, firm adhesion, is mediated by adhesion molecules such as ICAM-1 and VCAM-1 that bind to leukocyte integrins and tether leukocytes to the endothelial surface. Diapedesis and transendothelial migration of leukocytes occur due to a combination of increased endothelial permeability and the expression of monocyte and T-cell chemoattractants by endothelial and smooth muscle cells (2, 3, 71). These changes are rapid and reversible in vivo. For example leukocyte rolling and adhesion is induced within one hour of TNFα administration in mice, and this effect specifically requires TNF receptors of the vascular endothelium, highlighting the important role of endothelial activation in vascular inflammation (66).

Inflammatory stimuli that activate endothelial cells converge on several common signaling pathways including NF-κB and MAPK signaling. The NF-κB family is composed of five transcription factors, RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). In unstimulated cells, these proteins exist as latent cytoplasmic factors in complex with inhibitors of NF-κB (IκBs) (72, 73). Inflammatory activation by TNF or IL-1 causes rapid degradation of IκBs via the ubiquitin-proteosome system (74, 75),
followed by translocation of NF-κB dimers to the nucleus where they bind to the promoters of inflammatory genes, including ELAM and leukocyte chemoattractants, to activate their transcription (3). MAPK signaling is also critical for inflammatory activation of endothelial cells in response to cytokines. For example, p38 activity is critical for induction of E-selectin and VCAM-1 in response to TNFα (68).

NF-κB signaling also regulates the formation and release of endothelial-derived microparticles. Microparticles are membrane vesicles less than 1 µm in diameter that are released from the cell in response to various stimuli. These microparticles can be generated by the same agents that induce adhesion molecule and cytokine expression (e.g., TNF and thrombin), and are functionally heterogenous (76). Depending on their lipid and protein compositions, these NF-κB-regulated microparticles can be pro-coagulant, chemotactic, or promote leukocyte adhesion to the endothelium (77). Circulating levels of endothelial-derived microparticles increase at early stages in atherosclerosis and may contribute to disease progression, in part, by reducing eNOS phosphorylation and the bioavailability of NO (77). The overall contribution of microparticles to vascular disease progression remains uncertain. However, as a functionally heterogenous group of bioactive substances, microparticles may represent an additional target of pathophysiologically relevant factors regulated by NF-κB transcription factors.

1.1.4 Growth-Factor Activation of the Endothelium and Angiogenesis

Normally quiescent under physiological conditions, endothelial cells can be activated by growth factors to acquire proliferative, migratory, and invasive properties.
Important stimulators of endothelial cell proliferation include fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), PDGF, and epidermal growth factor (EGF) (78). These substances also induce angiogenesis, the formation of new blood vessels from the preexisting vasculature.

The primary event governing the initiation of angiogenesis is the formation of an endothelial cell sprout from a preexisting endothelial cell monolayer (79). Environmental cues, primarily in the form of growth-factors such as VEGF, stimulate quiescent endothelial cells to become migratory and invasive (79, 80). This phenotypic change coincides with a reduction in cell-to-cell contacts and a corresponding increase in vascular permeability (80). Based on relative amounts of VEGF receptor-2 (VEGFR-2), a “tip cell” (TC, high VEGFR-2 expression) is selected to lead an endothelial cell sprout to form a new vessel (81). The TC leads migration by following chemoattractant guidance cues and degrading extracellular matrix (ECM) proteins. Other activated endothelial cells, termed “stalk cells,” (SC) follow behind TCs, maintain a proliferative phenotype, and form a rudimentary new vessel lumen (Figure 1, top-right). Together, TCs and SCs constitute the endothelial cell sprouts that continue to migrate until an adjacent sprout is reached. Sprouts then fuse together in a process called anastomosis. Following anastomosis, the motile and proliferative phenotypes of TCs and SCs are gradually lost, and cell-to-cell junctions are reestablished to form a proper lumen and allow for blood flow. The endothelium plays an additional role in forming a mature neovasculature by synthesizing and depositing ECM proteins, as well as synthesizing growth-factors like PDGF which recruit pericytes to support the new vessel (79).
Figure 1. Mechanisms of endothelial cell activation. Clockwise from the left: Blood vessels are lined by a single cell-thick layer of endothelial cells surrounded by extracellular matrix proteins and concentric layers of smooth muscle cells. Endothelial cells synthesize and secrete a variety of growth-factors and vasoactive substances (top) to regulate smooth muscle cell growth, motility, and vessel tone. Endothelial cells also respond to growth-factors to create angiogenic sprouts to form new blood vessels (top right). Endothelial cells actively regulate the hemostatic/thrombotic balance of circulating blood by modulating production of pro-coagulant and anti-coagulant factors (right). Inflammatory activation of endothelial cells (bottom) is caused by a wide-variety of factors and is implicated in numerous disease states, including atherosclerosis. Inflammatory factors like TNF induce expression of leukocyte adhesion molecules and chemokines that recruit leukocytes to sites of inflammation, permit leukocyte rolling and firm adhesion to endothelial cells, and facilitate the extravasation of leukocytes into the subendothelial space. The accumulation of monocytes in the subendothelial space, and the activation of monocytes by oxidized lipoproteins and endothelial-derived factors is a critical event in the initiation of atherosclerosis. (From Waitkus, Harris, and DiCorleto, “Mechanisms of Endothelial Activation” in The Encyclopedia of Medical Immunology, 2014. ISBN 978-0-387-84827-3.)
1.1.5 Significance of Endothelial Activation

As the body’s largest homeostatic organ, the vascular endothelium forms a marvelous non-thrombogenic surface lining every vessel in the body. Its function is critical for maintaining vascular health, and its dysfunction is associated with the genesis and progression of various vascular diseases. As a dynamic biological interface, the endothelium has the capacity to sense environmental changes, synthesize vasoactive substances, interact with other cell-types, regulate vascular permeability, and form collateral vessels from the preexisting vasculature. Dysregulation of these processes is present in many vascular diseases, and endothelial dysfunction often precedes atherosclerosis and associated conditions, suggesting that endothelial activation may play a causative role in serious vascular illnesses.

Although the endothelium is known to participate in the genesis and progression of vascular diseases, the specific triggering events of endothelial activation that distinguish pathophysiological dysfunction from physiological adaptation are still incompletely characterized. Therefore, a more thorough understanding of endothelial dysfunction may lead to descriptions of causative events underlying endothelial dysfunction for distinct disease states. Such knowledge may allow for better risk-stratification of patients before major adverse events, as well as the identification of novel opportunities for therapeutic intervention to prevent or treat vascular diseases.

1.2 Mechanisms of Thrombin Signaling through Protease-activated Receptors

Previous studies in our laboratory have focused on the actions of thrombin in regulating inflammatory activation of vascular endothelial cells (67, 68, 82–84).
Thrombin is well known for its role in regulating blood coagulation, but it also activates a family of receptors termed protease-activated receptors (PARs) to initiate intracellular signaling pathways in numerous cell types (85). In this way, PARs critically link the process of thrombin generation following tissue injury to injury-induced cellular responses via activation of cell surface receptors. Further, thrombin-dependent activation of PAR-1 on endothelial cells is a potent inducer of endothelial-leukocyte adhesion molecules (ELAM), and may therefore provide a link between tissue injury and inflammation.

1.2.1 Structure and Function of Protease-activated Receptors

The PARs are members of the family of G-protein coupled receptors (GPCRs) that comprises the largest class of transmembrane signaling proteins in the human genome (86). GPCRs have an extracellular NH$_2$-terminus, seven distinct transmembrane helices with alternating intracellular and extracellular “loop” regions, and an intracellular COOH-terminus (Figure 2). For most GPCRs, ligand binding to an extracellular pocket formed by the transmembrane helices causes conformational changes in the receptor that activate heterotrimeric G-proteins via nucleotide exchange. By this mechanism, GPCRs act as transmembrane guanine nucleotide exchange factors (GEF) that trigger GDP-GTP exchange and activation of G-proteins when the appropriate extracellular cue is present.

PARs are a unique among GPCRs because they are irreversibly activated via protease-mediated cleavage of their NH$_2$-terminus (87). Thrombin cleaves a 41 amino acid domain (Figure 2, red) from the NH$_2$-terminus of PAR-1, thus exposing a new NH$_2$-terminus (Figure 2, blue). The newly exposed NH$_2$-terminal domain acts as a tethered
ligand by binding to the second extracellular loop of PAR-1 to activate G-protein-dependent signaling (Figure 2, right) (88). A peptide agonist composed of the sequence Ser-Phe-Leu-Leu-Arg-Asn, termed thrombin-receptor agonist peptide (TRAP), that corresponds to the newly exposed NH₂-terminus of PAR-1 can activate PAR-1-dependent signaling pathways independent of receptor cleavage (89, 90).

![Thrombin-mediated Activation of PAR-1](image)

**Figure 2. PAR-1 Activation by Thrombin.** Thrombin cleaves a 41 amino acid sequence of the PAR-1 NH₂-terminus (shown in red). Following cleavage, the newly generated NH₂-terminus acts as a tethered ligand by binding to the extracellular domain of PAR-1 and activating intracellular signaling pathways.

PAR-1 can be activated by several proteases including thrombin, TF-VIIa-Xa, activated-Protein C, plasmin, MMP-1 (90–95). Cleaved and activated PAR-1 couples to the G-protein subunits Go₁₂, Go₁₃, Go₄, Go₅, and Gβγ (85, 96, 97), and activates a
multitude of signaling pathways including MAPKs, Src family kinases, PI3K-AKT, phospholipase C, Rho-associated protein kinases, and transactivation of receptor tyrosine kinases (68, 82, 98–103). Through these signaling pathways, thrombin transduces the information of tissue injury to generate the appropriate cellular responses of inflammation, proliferation, and cellular migration (67, 102, 104, 105).

1.2.2 Thrombin-activated Signaling Pathways in Endothelial Cells

Endothelial cells line the luminal surface of the vasculature and are exposed to thrombin following tissue injury. Endothelial cells predominantly express PAR-1, and exposure to thrombin stimulates multiple PAR-1-dependent signaling pathways including activation of MAPKs and Src family kinases (68, 106). Activation of endothelial PAR-1 by thrombin induces expression of leukocyte adhesion molecules (68, 84) and leukocyte adhesion to an endothelial monolayer (67, 82). Further, thrombin stimulates angiogenesis and endothelial cell migration by directly activating endothelial PAR-1 (84). Kinney et al. showed that thrombin induces ex vivo endothelial cell sprouting from murine aortic rings (106), and others have shown that low levels of thrombin induce endothelial differentiation into tube-like structures on a basement membrane extract (105).

These results are largely consistent with earlier observations that exogenous thrombin or TRAP administration enhances wound healing and neovascularization of dorsal incisions in rats (107). In contrast, Connolly et al. reported that PAR-1 knockout mice did not have significantly altered wound healing responses compared to wild-type littermates (108). However, the same laboratory and others also reported that ~50% of PAR-1 deficient mice die at embryonic day 9.5, likely due to severe defects in the
development of yolk sac vasculature (109–111). Another report demonstrated that PAR-1 knockout mice displayed altered vascular remodeling following arterial injury, and that PAR-1-deficient mice did not show injury-induced increases in vessel and lumen diameters that were observed in wild-type mice (112).

Collectively, existing literature suggests that thrombin-dependent activation of PAR-1 increases localized inflammation, facilitates neovascularization following injury, and increases cellular proliferation and migration. The fact that ~50% of PAR-1−/− embryos die at ~E9.5 suggests that PAR-1-dependent processes, particularly vascularization, are critical for normal development. The elucidation of novel PAR-1 signaling mechanisms may therefore reveal unappreciated therapeutic opportunities to enhance wound healing and neovascularization following tissue injury, as these processes are limiting factors that regulate the regeneration of functional tissues. Furthermore, aberrant thrombin generation in advanced cancers creates a hypercoagulable state that contributes to tumor-associated angiogenesis and metastasis (113). Therefore, understanding mechanisms by which thrombin/PAR-1 signaling cooperates with oncogenic signaling pathways, including EGFR and STAT3, may reveal novel aspects of the tumor microenvironment that facilitate tumor vascularization, growth, and metastasis.

1.3 Mechanisms of EGF Receptor Signaling

The EGF receptor (EGFR) is a member of the ErbB family of RTKs that includes HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. The ErbB receptors are single-pass transmembrane receptors with extracellular ligand binding domains, a transmembrane domain, and a cytosolic tail with a tyrosine kinase domain (Figure 3). The four receptors
form a variety of homodimers and heterodimers in response to the EGF family of ligands. EGFR, HER3, and HER4 directly respond to a number of EGF family members (Figure 3). Some of these ligands bind multiple family members (HB-EGF), while others are specific to a single receptor (EGF). To date, there is no known ligand for HER2 (114). Structurally, HER2 is constitutively in an “open” state, poised for dimerization, but does not readily homodimerize (115, 116). Instead, HER2 forms functional heterodimers with other ErbB family members to initiate intracellular signaling cascades (114).

**Figure 3. The ErbB family of receptors and their ligands.** ErbB receptors can be activated by numerous endogenous ligands. Binding of a ligand to a receptor facilitates homo- and heterodimerization, autophosphorylation, and activates downstream signaling pathways including MAPK and AKT signaling.

EGFR is activated by EGF, HB-EGF, TGFα, betacellulin, epiregulin, epigen, and amphiregulin (117). In the absence of these ligands, EGFR is predominantly in a “closed”
conformation in which contacts between extracellular domain II and IV sequester the
dimerization arm from other receptors (118). Ligand-binding to extracellular domains I
and III of EGFR causes EGFR to adopt an “open” conformation in which the
dimerization arm is exposed (Figure 4) (118). In this ligand-bound conformation, EGFR
readily forms homodimers or heterodimers with HER2 (114, 116). Intracellularly,
dimerization facilitates physical association of the cytosolic tails of EGFR monomers.
Multiple lines of evidence suggest that the active state involves one monomer acting as
an allosteric activator for the kinase domain of the other monomer, creating an
asymmetric dimer in which only one kinase domain is activated (119, 120). The active
kinase domain of one monomer transphosphorylates the other, and the resulting
phosphotyrosine residues of EGFR cytosolic tail act as binding sites for adaptor protein
with SH2 or phosphotyrosine-binding domains (114, 121). These adaptor proteins
activate numerous intracellular signaling cascades that ultimately elicit changes in gene
expression and cellular phenotypes.
Figure 4. Mechanism of ligand-mediated EGFR dimerization. Ligand binding causes EGFR monomers to adopt an “open” conformation in which the dimerization arm of the second extracellular domain is extended. Dimerization leads to kinase activation and phosphorylation of tyrosine residues in the EGFR cytosolic tail.

1.3.1 Overview of EGF Receptor Signaling Pathways

EGFR activation initiates a number of signaling pathways including MAPK, PI3K, Src-family kinases, NF-κB, and STATs (122–125). Several phosphotyrosine residues on the EGFR cytosolic tail act as binding sites for the adaptor protein Grb2 which, in turn, initiates the canonical pathway of MAPK activation via RAS and RAF. Further, EGF induces STAT3 Tyr705 phosphorylation (123), and this pathway may play a role in oncogenesis and drug resistance in head and neck squamous cell carcinoma (126).
Recently, Guo and Stark reported that EGFR induces NF-κB activation by a mechanism that requires the tyrosine kinase Fer in lung cancer cells (124).

In 1996, Daub et al. observed that activation of GPCRs rapidly leads to the phosphorylation of EGFR and Neu (HER2 ortholog) and termed this phenomenon “transactivation” (127). Prenzel et al. subsequently determined that EGFR transactivation by LPA or carbachol required metalloprotease-mediated cleavage of HB-EGF (100). By this mechanism, GPCR activation leads to autocrine activation of RTKs such as EGFR, thus facilitating a type of cellular crosstalk between cell surface receptors (Figure 5). These studies formed the basis for a large number of subsequent investigations of GPCR-mediated transactivation of EGFR. Transactivation of EGFR functions in diverse cellular processes including proliferation, migration, and invasion (128–130).
Figure 5. GPCR-mediated transactivation of EGFR. Activation of GPCRs such as PAR-1 activated MMPs that, in turn, cleave and release growth-factors from their pro-peptide precursors. Released growth factors (e.g. HB-EGF can activate EGFR in an autocrine fashion.

1.3.2 Endothelial EGF Receptor Signaling

EGF was first shown to increase the rate of endothelial cell proliferation by Gospodarowicz et al. in 1978 (131). In this study, EGF increased the proliferative response of human endothelial cells, but not bovine endothelial cells, in the presence of calf serum. Further, the EGF-induced proliferative response was potentiated by simultaneous stimulation with thrombin, indicating some interaction between EGF and thrombin-activated signaling pathways. A subsequent study by a different group reproduced the effects of EGF on HUVEC, but was unable to reproduce the potentiation of EGF-dependent proliferation by thrombin (132).
Given its capacity to stimulate cell proliferation, EGF was investigated for its ability to enhance corneal endothelial cell proliferation, migration, and wound closure. EGF was found to be a potent inducer of corneal endothelial cell migration in mitotically inhibited corneal endothelial cells (133). A subsequent study demonstrated that EGF administration increased the rate of endothelial wound closure in excised corneas (134). Furthermore, both EGF and TGFα, an EGF family member, were found to induce angiogenesis in vivo (135). These results suggested that EGF is an endogenous inducer of angiogenesis and wound closure, and could potentially act as a therapeutic agent to stimulate tissue regeneration.

Studies throughout the 1980s and 1990s characterized the binding of EGF to various endothelial cells from different species, as well as the proliferative and migratory response to EGF by these cells. However, more recent studies presented evidence that EGFR was not expressed on either MVEC (136) or HUVEC (136–139). It is important to note that these studies predominantly relied on immunoblot analyses to assess endothelial EGFR expression, and, in at least two cases, compared EGFR expression in endothelial cells to cancer cell lines with overexpression of EGFR (136). Earlier studies employed radioligand binding assays of EGF to endothelial cells to identify EGF-EGFR binding, and it is likely that the superior sensitivity of radioligand binding assays relative to immunoblots is one of the reasons for the conflicting reports of endothelial EGFR expression in the literature.

We have reported that EGF induces HUVEC migration in vitro, in contrast to the results of others (84, 137). Another group performed a direct comparison of EGF and VEGF-induced angiogenesis in vivo, and demonstrated that EGF induces
neovascularization to the same extent as VEGF in the mouse cornea (140). Mehta et al. reported that activation of EGFR by HB-EGF induces HUVEC migration and tube formation via the MAPK and PI3K pathways (141). More recently, Maretzky et al. found that VEGF-induced endothelial cell migration requires EGFR activity, suggesting that there exists some degree of crosstalk between these major vascular signaling pathways (142). These results suggest that endothelial EGFR function is an important and understudied regulator of endothelial cell physiology, and may significantly contribute to postnatal angiogenesis in vivo. It is therefore critical to improve our knowledge of endothelial EGFR functions so that we may begin to understand the extent to which endothelial EGFR signaling contributes to vascular physiology and disease.

Given its anatomic location, the endothelium is constantly exposed to a variety of disparate stimuli within the vascular microenvironment. Consequently, endothelial cells not only respond to specific agonists per se, but also integrate different, potentially conflicting, signals and formulate appropriate biological responses to maintain vascular health. Signal transduction cascades are well defined with regard single ligand/receptor models, but less is known about how endothelial cells respond when they are exposed to multiple stimuli simultaneously. Importantly, previously published results from our laboratory suggest that simultaneous exposure of endothelial cells to EGF and thrombin triggers synergistic induction of immediate early genes, but the mechanism of signal integration is unknown.
1.4 Mechanisms of STAT3 Activation

Named for its dual role as a signal transducer and transcription factor (143), STAT3 is a highly studied protein that regulates numerous physiological processes (e.g. angiogenesis, oncogenesis). This chapter will provide a brief summary of the discovery and characterization of STAT transcription factors, with a specific focus on STAT3. A detailed review of STAT3 literature will follow that summarizes the complex regulatory mechanisms that exist for modulating STAT3 activity in a diverse set of microenvironments. This review will therefore provide a proper context with which to understand the results of our STAT3 studies, and will ultimately highlight the novelty of our results and the extent to which they enhance the fundamental understanding of STAT3 biology.

1.4.1 The Discovery of STATs

The discovery of STAT transcription factors was primarily the result of work in the laboratory of Dr. James E. Darnell Jr. that focused on identifying DNA-binding proteins that bound specific IFN-stimulated response elements (ISREs) in the promoters of IFN α/β-responsive genes. Studies from the Darnell lab revealed that promoters of the IFN-inducible genes *IFIT2* (*ISG54*) and *ISG15* contained a conserved DNA element ~100 base pairs upstream from the transcription initiation start site (144, 145). By using this DNA element as a probe, Levy et al. were able to detect binding of a large protein complex, termed ISGF3, to the ISRE in the 5’ region of *IFIT2* (146). Subsequent work by Xin-Yuan Fu revealed that ISGF3 is composed of four distinct proteins with masses of 113, 91, 84, and 48 kDa (147). These proteins formed a multisubunit complex that
coimmunoprecipitated with anti-sera against the 113 or 91 kDa protein (148). Furthermore, it was observed that the 91 kDa subunit of ISGF3 also mediated activation of IFN-γ-dependent genes by binding to distinct DNA sequences termed GAS elements (gamma-IFN-activated site) in the promoters of IFN-γ-responsive genes (149, 150). Tyrosine kinase inhibitors, but not a broad-spectrum serine kinase inhibitor, prevented ISGF3 formation in response to IFN-α, and the 91 kDa protein that bound to GAS elements was found to be phosphorylated at Tyr\textsuperscript{701} (151, 152), suggesting that inducible phosphorylation was critical for activity of these transcription factors. The 91 kDa protein was termed STAT1 (signal transducer and activator of transcription 1), named for its dual role as a signal transducer and transcription factor in response to IFNs (143).

Shortly after the discoveries that uncovered STAT1 activation in response to IFNs, STAT3 and STAT4 were cloned from a cDNA library by Zhong et al. in the Darnell laboratory (153). Over a short period of time, multiple labs working in parallel discovered that STAT3 is activated by IL-6 or EGF via tyrosine phosphorylation, and that STAT3 binds to GAS elements in the promoters of IFNγ-inducible genes (123, 154, 155). These studies revealed that STAT1 and STAT3 were similar, but functionally distinct members of a larger family of transcription factors, and raised important questions regarding the specificity of STAT3 activation, as well as the significance of STAT3-mediated transcription.

### 1.4.2 Canonical STAT3 Signaling Mechanisms

Original studies of STAT3 signaling found that IL-6 or EGF treatment causes rapid phosphorylation of STAT3 at Tyr\textsuperscript{705} (123). This phosphorylation coincides with
formation of STAT3 homodimers and STAT1-STAT3 heterodimers that bind to GAS elements. However, the exact mechanisms regulating Tyr\textsuperscript{705} phosphorylation and the kinases involved remained unclear after these initial reports. Janus kinases (JAKs) were known to regulate interferon signaling (143), and it was found that JAK1 was rapidly tyrosine phosphorylated in response to IL-6 (156). Both STAT3 and JAK1 physically associated with the signal transduction protein gp130 upon IL-6 stimulation, suggesting that there was a direct link between JAK1 and STAT3 phosphorylation. Indeed, subsequent studies demonstrated that human fibrosarcoma cells lacking JAK1 exhibited remarkably reduced STAT3 phosphorylation in response to IL-6 (157). The same study found that JAK2 and TYK2 are also activated in response to IL-6, but are insufficient for STAT3 tyrosine phosphorylation, demonstrating a critical and non-redundant role for JAK1 in STAT3 Tyr\textsuperscript{705} phosphorylation.

Shortly after the JAK1-STAT3 connection was established, STAT3 was found to have a second site of phosphorylation that was sensitive to H7 kinase inhibitors (158). This site was determined to be a serine phosphorylation, and Wen et al. found that phosphorylation at this site was critical for maximal transcriptional activity of STAT3 (159). STAT3 serine phosphorylation was subsequently mapped to Ser\textsuperscript{727}, and was found to have no effect on STAT3 DNA-binding activity (160). Additional studies found that Ser\textsuperscript{727} phosphorylation was critical for STAT3-dependent gene induction in response to IL-6 and OSM (161). Importantly, a study by Shen et al. showed that mice with only one wild-type STAT3 allele (WT/-) develop normally, but ~75% of mice with one STAT3 mutant allele, where Ser\textsuperscript{727} was mutated to alanine (S727A/-), die perinatally (162). Embryonic fibroblasts from the S727A/- mice exhibited a reduced transcriptional
response to OSM, demonstrating that STAT3 transcriptional activity is critical for proper development and is likely modulated by Ser\textsuperscript{727} phosphorylation \textit{in vivo}. Mechanistically, there is ample evidence to suggest that Ser\textsuperscript{727} phosphorylation acts as a phosphoserine binding site for transcriptional coactivators such as p300 (161, 163).

More recently, several other posttranslational modifications have been discovered on STAT3 including arginine and lysine methylation, lysine acetylation, and threonine phosphorylation (Figure 6). Yang et al. reported that IL-6 induces STAT3 Lys\textsuperscript{140} methylation, and that this modification increases or decreases STAT3 transcriptional activity in a gene-specific manner (164). The protein arginine methyltransferase 2 (PRMT2) methylates STAT3 at Arg\textsuperscript{31}, and this modification is a negative regulator of leptin signaling (165). Various sites of lysine acetylation of STAT3 have also been reported (166–169), and it is believed that acetylation of Lys\textsuperscript{685} by p300 regulates STAT3 dimerization (167). STAT3 Thr\textsuperscript{714} phosphorylation has been identified in several phosphoproteomic studies (170–172), but site-specific studies have not been pursued and proximal kinases for this modification are unknown.
STAT3 Domains and Modifications

Figure 6. Map of STAT3 posttranslational modifications. STAT3 domains are color-coded and labeled below the map (TAD, transactivation domain). STAT3 is posttranslationally modified at multiple sites. These modifications include arginine methylation (meR), lysine methylation (meK), acetyl-lysine (aK), phosphotyrosine (pY), phosphothreonine (pT), and phosphoserine (pS).

Taken together, these results suggest that numerous STAT3 PTMs cooperate to regulate STAT3 transcriptional activity. Although it is clear that regulation of STAT3 function is more complex than simply regulating Tyr\textsuperscript{705} phosphorylation, the overall model of Tyr\textsuperscript{705} phosphorylation and dimerization as the major driver of STAT3 activity has persisted. More recently, however, alternative mechanisms of STAT3 activation that do not require Tyr\textsuperscript{705} phosphorylation have been discovered and characterized.

1.4.3 Alternative Mechanisms of STAT3 Activation

Recent progress in the STAT3 field has made it increasingly clear that Tyr\textsuperscript{705} phosphorylation is not an absolute requirement for STAT3-mediated gene expression. In fact, multiple laboratories have reported STAT3 functions that occur in the absence of Tyr\textsuperscript{705} phosphorylation including cooperation with the NF-κB subunit p65 (173), phosphorylation-independent dimerization and DNA-binding (174–176), and regulation of cellular respiration in mitochondria (177, 178). These results demonstrate the
complexity of STAT3 regulation and suggest that a wide range of previously unreported noncanonical STAT3 functions may exist.

STAT3 positively regulates the expression of its own gene by binding to its promoter and increasing STAT3 transcription (179, 180). While stimulus-dependent increases in Tyr\textsuperscript{705} phosphorylation return to basal levels relatively quickly, the elevated levels of tyrosine-unphosphorylated STAT3 (U-STAT3) may persist for hours after cytokine stimulation (181). U-STAT3 competes with IκB for p65-binding in the cytoplasm, and high levels of U-STAT3 eventually lead to p65-STAT3 complex formation. This protein complex drives gene expression by binding the promoters of genes with κB elements, including \textit{RANTES} (181). Others have shown that U-STAT3 and NF-κB cooperate to increase expression of serum amyloid A (SAA) after cells are simultaneously treated with IL-1 and IL-6 (173). These reports demonstrate that U-STAT3 drives gene expression in a manner that is distinct from canonical IL-6/JAK-dependent mechanisms. Importantly, these mechanisms highlight the interaction of STAT3 and NF-κB signaling, and reveal an underlying combinatorial complexity of processes that control STAT3 activation.

U-STAT3 has also been shown to dimerize and bind DNA in the absence of NF-κB subunits. Braunstein et al. first made the unexpected observation that STAT1 and STAT3 form stable homodimers in the absence of tyrosine phosphorylation (174). Although the U-STAT dimers were stable in unstimulated cells, they did not possess DNA-binding activity, suggesting that an additional regulatory mechanism must exist to modulate STAT3 DNA-binding. More recently, Timofeeva et al. used atomic force microscopy to examine the binding of U-STAT3 to GAS elements (175). They found that
U-STAT3 binds to GAS elements as dimers or monomers. Further, it was observed that U-STAT3 binds to AT-rich DNA elements as well as cruciform structures in DNA, suggesting that numerous DNA elements may be targets of U-STAT3 signaling. More recently, a crystal structure of U-STAT3 binding to DNA has been published, demonstrating that U-STAT3 dimers bind to GAS elements in a structurally similar manner to Tyr$^{705}$-phosphorylated STAT3 (176).

These alternative, Tyr$^{705}$-independent mechanisms of STAT3 activation led Yang and Stark to speculate (179) that ancestral STAT3 functions may have existed without tyrosine phosphorylation, and that ligand-dependent STAT3 activation is a more recently evolved process. If this is indeed the case, it stands to reason that numerous alternative mechanisms of ligand-dependent STAT3 activation may have evolved in parallel with JAK-STAT3 signaling.

1.4.4 Dysregulation of STAT3 Signaling in Disease

STAT3 controls the expression of genes regulating cell growth, survival, and wound healing (182–185). Additionally, STAT3 is aberrantly activated in a majority of human cancers and drives pathological angiogenesis by positively regulating VEGF-A expression (185). Importantly, there are several reports that demonstrate STAT3 plays a role in cancer progression in the absence of Tyr$^{705}$ phosphorylation (186–188). Collectively, these reports suggest that STAT3 is a viable target for anti-cancer therapy, and that multiple, independent mechanisms of STAT3 activation should be considered when designing STAT3-targeted therapies.
The importance of STAT3 activation in cancer was first realized when it was observed that v-Src overexpression causes constitutive phosphorylation of STAT3 at Tyr\(^{705}\) (189). Subsequent studies found that STAT3 is required for v-Src induced transformation of fibroblasts (190, 191). In a series of experiments, it became clear that STAT3 is a central mediator of cellular transformation, as overexpression of various oncogenes leads to constitutive STAT3 activation (192), and numerous signaling pathways cooperate to maximize the full transformative potential of STAT3 (193–196).

These initial experiments focused on Tyr\(^{705}\) phosphorylation as an indicator of STAT3 activity in cancer. This was a natural endpoint for analyzing STAT3 activation, as the JAK-STAT pathway was relatively well characterized at the time (143). Importantly, subsequent work found that oncogenic kinases that are frequently mutated in human malignancies, including EGFR, JAK2, and ALK induce STAT3 Tyr\(^{705}\) phosphorylation, STAT3 target gene expression, and cancer cell proliferation (197–202). Genomic alterations of EGFR, including the constitutively active EGFR\(_{vIII}\) mutants and an EGFR-SEPT14 gene fusion, also cause STAT3 activation via Tyr\(^{705}\) phosphorylation and drive oncogenesis, proliferation, and cellular invasion in various forms of cancer (203–207). Furthermore, STAT3 Tyr\(^{705}\) is found in a subset of patients with clear cell and papillary renal cell carcinomas (208), and inhibition of STAT3 activity significantly slows growth of xenograft renal tumors in a mouse model (209). These results demonstrate that STAT3 activation via Tyr\(^{705}\) phosphorylation positively regulates oncogenesis and tumor growth, making it a strong candidate for targeted anti-cancer therapy.
STAT3 has also been shown to regulate target gene expression, cell survival, and proliferation in the absence of Tyr<sup>705</sup> phosphorylation. This observation is consistent with the previously discussed roles of U-STAT3 in transcriptional regulation, as well as the ability of U-STAT3 to dimerize, bind GAS elements, and cooperate with NF-κB family members (174, 175, 179). Liu et al. first showed that Ser<sup>727</sup>-phosphorylated STAT3 drives expression of Mcl1 in macrophages in the absence of detectable Tyr<sup>705</sup> phosphorylation (187). Yang et al. reported that overexpressing a STAT Y705F mutant activated the expression of many target genes including EGFR, MET, and MRAS (210). Ng et al. found that STAT3 that is Ser<sup>727</sup>-phosphorylated, but not Tyr<sup>705</sup>-phosphorylated, regulated NGF-dependent gene expression in PC12 cells (211). Further, U-STAT3 is induced in response to IL-6, binds to NF-κB, and activates a set of genes that are not directly activated by P-STAT3 including RANTES, MET, IL-8, and IL-6 (181). In human prostate cancer cells, a Ser<sup>727</sup> phosphomimetic STAT3 mutant (S727E) is capable of driving cellular growth and tumor formation in mice (188). Further, tumors harboring a S727E/Y705F STAT3 double mutant did not show reduced tumorigenesis or invasiveness, and the degree of Ser<sup>727</sup> phosphorylation correlated with Gleason score of human prostate cancer tissue in the study (188). These results conclusively demonstrated that STAT3 is activated in the absence of Tyr<sup>705</sup> phosphorylation in a manner that drives tumor growth and invasion in vivo. In agreement with these findings, a more recent report demonstrated that STAT3 is constitutively phosphorylated at Ser<sup>727</sup> in patients with chronic lymphocytic leukemia (CLL) and can bind DNA in the absence of Tyr<sup>705</sup> phosphorylation (186). The researchers also found that inhibition of STAT3 in patient CLL cells reduced expression of STAT3 target genes and induced apoptosis, suggesting
that Ser\textsuperscript{727}-phosphorylated STAT3 can be targeted in human malignancies to inhibit disease progression (186).

The canonical pathway of JAK-STAT signaling originally put forth by Darnell, Kerr, and Stark (212) has been shown to participate in numerous cellular processes including immunity, angiogenesis, and oncogenesis (143). However, it has become increasingly clear that there exist alternative mechanisms of STAT3 activation that do not require Tyr\textsuperscript{705} phosphorylation. The full repertoire of molecular mechanisms governing STAT3 function likely includes an astoundingly complex combination of U-STAT3, Ser\textsuperscript{727}-phosphorylated STAT3, cooperation with NF-κB family members, and binding to various DNA elements including GAS, κB, and AT-rich elements to modulate transcription.
CHAPTER II

STAT3-MEDIATED COINCIDENCE DETECTION REGULATES NON-CANONICAL IMMEDIATE EARLY GENE INDUCTION

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2.1 Abstract

Signaling pathways interact with one another to form dynamic networks in which the cellular response to one stimulus may depend on the presence, intensity, timing, or localization of other signals. In rare cases, two stimuli may be simultaneously required for cells to elicit a significant biological output. This phenomenon, generally termed “coincidence detection,” requires a downstream signaling node that functions as a Boolean AND gate to restrict biological output from a network unless multiple stimuli are received within a specific window of time. Simultaneous activation of EGF receptor (EGFR) and a thrombin receptor (protease-activated receptor-1, PAR-1) increases the expression of multiple immediate early genes (IEGs) associated with growth and angiogenesis. Using a bioinformatic comparison of IEG promoter regions, we identified STAT3 as a critical transcription factor for the detection of coincident EGFR/PAR-1 activation. EGFR activation induces classical STAT3 Tyr\textsuperscript{705} phosphorylation but also initiates an inhibitory signal through the PI3K-AKT signaling axis that prevents STAT3 Ser\textsuperscript{727} phosphorylation. Coincident PAR-1 signaling resolves these conflicting, EGF-activated pathways by blocking AKT activation and permitting GSK-3α/β-dependent STAT3 Ser\textsuperscript{727} phosphorylation and STAT3-dependent gene expression. Functionally, combinatorial EGFR/PAR-1 signaling suppresses EGF-induced proliferation and thrombin-induced leukocyte adhesion and triggers a STAT3-dependent increase in endothelial cell migration. This study reveals a novel signaling role for STAT3 in which the simultaneous presence of extracellular EGF and thrombin is detected at the level of STAT3 post-translational modifications. Collectively, our results describe a novel
regulatory mechanism in which combinatorial EGFR/PAR-1 signaling regulates STAT3-dependent IEG induction and endothelial cell migration.

2.2 Introduction

Cell-type and spatiotemporal signaling contexts are critical determining factors for the propagation of signaling cascades from cell surface receptors (213). Rather than having defined pathways of signaling, receptors may activate unique complements of pathways depending on the presence, intensity, timing, or localization of other signals (213, 214). This nuanced view of cellular signaling requires integration of multivariate extracellular information via coordinated crosstalk between signaling pathways. In this manner, signaling interactions enable cells to process disparate extracellular information at downstream signaling nodes and formulate appropriate biological responses based on specific combinations of signaling inputs (121, 215). Crosstalk is an important factor in a number of biological processes including cellular migration (142), proliferation (128), gene expression (84, 216), and calcium influx (215). However, the specific molecular mechanisms by which cells integrate signals from multiple cell surface receptors are poorly understood (213).

In many cases, the output of signaling interactions is merely the sum of individual signaling inputs. Less often, multiple signaling inputs induce moderate synergisms of phosphorylation, calcium influx, or gene expression. In rare cases, however, simultaneous signals may induce marked synergisms, where the response to combined stimuli is far greater than the sum of responses to individual inputs (215). This type of superadditive response can be expressed quantitatively as a synergism ratio (SR)¹ and
calculated by dividing the response to combined stimulation by the sum of individual responses. As SR increases, signaling interactions begin to function as cellular coincidence detectors, where simultaneous stimuli are required for cells to elicit a significant biological response. Coincidence detection may occur by several mechanisms including multiple phosphorylation events (217), cooperative binding of regulatory molecules (218), multivalent binding of modular protein domains (219), two-state allosteric regulation (220), protein scaffolding (221), and simultaneous alterations in relative activity between antagonistic enzymes (e.g. phosphatase/kinase) (216, 222).

Much research has focused on signaling interactions between G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) (223). The classical model of RTK/GPCR crosstalk involves GPCR-mediated transactivation of RTKs via metalloprotease-mediated cleavage of membrane-bound growth-factor precursors and subsequent autocrine activation of RTKs (100). Although this model of RTK/GPCR interactions constitutes a crosstalk between cell surface receptors, it only describes a form of linear information transfer where an RTK is essentially a downstream effector of GPCR signaling. As such, this model does not address mechanisms by which intracellular pathways integrate extracellular information following coincident activation of RTKs and GPCRs.

Previous work in our lab focused on a RTK/GPCR interaction in endothelial cells (EC) involving the epidermal growth factor (EGF) receptor and the thrombin receptor, protease-activated receptor-1 (PAR-1) (84). EC are situated in direct contact with the bloodstream and are constantly exposed to a wide variety of stimuli including growth factors, hormones, biomechanical forces, microbial pathogens, and inflammatory agents.
Responses to these stimuli are mediated, at least in part, by the induction of immediate early genes (IEGs). As a family of transcription factors, cytokines, phosphatases, and other enzymes, IEGs are important determinants of delayed gene expression and phenotypic outcomes (224, 225). We previously reported that simultaneous exposure of EC to EGF and thrombin caused a synergistic induction of the IEG mitogen-activated protein kinase phosphatase-1 (MKP-1) and enhanced migration of EC in vitro (84). EGF is a well-known stimulator of cell growth and induces EC proliferation, migration, and angiogenesis (226). Thrombin is a serine protease that regulates blood coagulation, but also acts as a signaling molecule by cleaving the NH₂-terminus of protease-activated receptors (PARs). We previously showed that synergistic induction of MKP-1 by EGF and thrombin was mediated specifically by PAR-1 activation (84). An increased understanding of signaling interactions between EGFR and PAR-1 may yield valuable insight into the mechanisms by which EC signaling networks integrate multivariate extracellular information in pathological microenvironments.

In this report, we describe a detailed, novel molecular mechanism of EGFR/PAR-1 crosstalk. We show that STAT3 is a critical point of convergence of signals from EGFR and PAR-1, and functions as a cellular coincidence detector to enhance IEG expression following simultaneous EGFR/PAR-1 activation. Importantly, combinatorial receptor signaling coincides with a suppression of EGF-induced proliferation and thrombin-induced leukocyte adhesion, and causes a STAT3-dependent enhancement of EC migration. Combinatorial activation of STAT3 is therefore a critical event for increasing the magnitude of IEG expression, and plays an important role in determining the ultimate phenotypic response of EC during simultaneous exposure to disparate signaling inputs.
2.3 Materials And Methods

Cell-Culture, Transfections, and Treatments. Human EC were isolated by trypsinization of umbilical veins as previously described (67). EC were plated on fibronectin coated cell-culture dishes and maintained in MCDB/F12 media containing 15% FBS, .009% heparin, and .015% endothelial cell growth supplement. All experiments were carried out using cells between the third and fifth passage. EC were transfected using Targefect reagents (Targeting Systems) according to the manufacturer’s protocol. Unless otherwise indicated, EC were serum-starved for 2 hours prior to treatment with EGF (16 ng/ml), TRAP (100 µM), or thrombin (5U/ml).

Generation of EGR1 Promoter Luciferase Reporter and Luciferase Assay. A 2.1 kb fragment of the EGR1 promoter was PCR amplified (See Table VIII for primers) from human genomic DNA. The fragment spanned from -1958 bp upstream of the EGR1 transcription initiation site to +160 bp into the first exon (Figure 61), and was cloned into pGL3-Basic firefly luciferase reporter between the MluI and BglII restriction sites. Site directed mutagenesis of the EGR1 promoter distal GAS element (TTCCCGGAA → gcgCCGGAA) and proximal GAS element (TTCCCGGAA → gcgCCCGA) was performed using the GeneArt site-directed mutagenesis kit (Invitrogen) according to the manufacturer’s instructions. Luciferase assays were performed by transfecting EC with EGR1-Promoter reporter 24 hours prior to treatment. Cells were lysed with passive lysis buffer (Promega) and luciferase activity was assayed using the luciferase assay system (Promega) according to the manufacturer’s instructions.
**SDS-Page and Western Blotting.** Total cell lysate from approximately $10^5$ EC was resolved using Bis-Tris buffered SDS-PAGE gels ranging from 8-12% depending on the protein of interest. Gels were soaked in protein transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS) and transferred to a PVDF membrane using a BioRad semi-dry transfer cell. After transfer, PVDF membranes were washed briefly in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Tween-20) and then blocked for 2 hours in 5% Bovine Serum Albumin in TBST. After blocking, primary antibodies were diluted 1:1000 in 5% BSA in TBST and incubated overnight at 4°C. Membranes were washed and then incubated with horseradish peroxidase (HRP) conjugated secondary antibody for one hour and HRP signals were detected by chemiluminescence.

**Quantitative Real-Time PCR.** Total RNA was isolated using Qiagen RNeasy spin columns. First strand cDNA was synthesized using TaqMan reverse transcription reagents (Roche). cDNA reactions were diluted 6-fold in deionized water and used as a qRT-PCR template. Reactions were performed using Sybr Green Master Mix (Applied Biosystems). Gene induction was calculated relative to untreated controls using the $2^{-\Delta\Delta Ct}$ method.

**Immunoprecipitation.** EC were washed once with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 5 mM Na$_3$VO$_4$) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche). Lysis was allowed to proceed for 30 minutes at 4°C under gentle agitation. Lysates were cleared by high-speed centrifugation and primary
antibodies were added for overnight incubation. Antibody complexes were precipitated with protein A/G-agarose beads, washed three times with ice cold RIPA, and denatured with 3x Laemmli buffer for SDS-PAGE.

**Quantitative mass spectrometry.** After immunoprecipitation with an anti-STAT3 antibody, an 86 kDa band was cut from Coomassie-stained gel, digested with trypsin, and analyzed by capillary column LC- tandem MS to identify phosphopeptides. The LC-MS system was a Finnigan LTQ linear ion trap mass spectrometer system. The HPLC column was a self-packed 9 cm x 75 μm id Phenomenex Jupiter C18 reversed-phase capillary chromatography column. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. The data were analyzed by using all CID spectra collected in the experiment to search the NCBI non-redundant database with the search program Mascot using a human taxonomy filter. All matching spectra were verified by manual interpretation. The interpretation process was aided by additional searches using the programs Sequest and Blast as needed.

**Phospho-kinase protein array.** The Human Phospho-kinase Antibody Array was purchased from R&D Systems. 500μg of HUVEC protein was incubated with the array membranes overnight at 4°C under gentle agitation. Membranes were washed and then incubated with a phospho-antibody detection cocktail for two hours at room temperature. Membranes were washed and then incubated with a streptavidin-HRP containing solution
for 30 minutes at room temperature. After extensive washing, phosphorylation was detected by chemiluminescence and relative changes were quantified using ImageJ software.

**STAT3 DNA-binding assay.** STAT3 DNA binding activity was measured using the TransAM transcription factor ELISA kit from Active Motif according to the manufacturer’s instructions. Briefly, stimulus-treated EC were harvested in hypotonic lysis buffer (25 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$) and lysed at 4°C for 30 minutes to release cytosolic proteins. Cytosolic fractions were collected, EC pellet was washed once with hypotonic lysis buffer, and nuclear proteins were extracted with high-salt lysis buffer (25 mM HEPES pH 7.5, 420 mM KCl, 1.5 mM MgCl$_2$, .2 mM EDTA, 10% glycerol) to collect the nuclear fraction. Nuclear extract was diluted in DNA binding buffer according to the manufacturer’s instructions. 10 µg of nuclear extract was added to each well of a 96-well plate that was coated with oligonucleotides containing consensus GAS elements (5’-TTCCCGGAA-3’), and incubated at room temperature for 1 hour. Unbound nuclear proteins were washed away, and DNA-bound STAT3 was detected with an anti-STAT3 antibody, followed by colorimetry using TMB as a chromogenic substrate.

**Immune-complex kinase assay.** An AKT kinase assay kit (#9840) was purchased from Cell Signaling Technology. Briefly, EC were treated with agonists for 10 minutes and lysed with a proprietary lysis buffer containing protease and phosphatase inhibitors. Insoluble cellular material was removed by centrifugation (16,000xg) and the
supernatant was used for immunoprecipitation. Total AKT was precipitated with pan-AKT antibody (Cell Signaling, #4691). A portion of the immunoprecipitate was diluted 400-fold for the *in vitro* kinase assay. Recombinant GST-tagged GSK3α was used as an AKT substrate and incubated in the presence of ATP for 30 minutes. Reaction was stopped with 4x SDS loading buffer and GSK3α was resolved by SDS-PAGE and blotted with an anti-phospho- GSK3α antibody (#9327).

**BrdU proliferation assay.** BrdU incorporation was measured using a BrdU proliferation kit (Cell Signaling Technology #6813) according to the manufacturer’s instructions. EC were serum-starved overnight and then treated with agonists for 8 hours. BrdU labeling was performed between 8-12 hours without removing treatment media.

**Endothelial wound healing.** HUVEC were seeded in 6-well plates in full growth medium. Cells were washed twice with serum-free MCDB and serum-starved for 2 hours. Linear wounds were created using a 200 µl pipette tip, cells were washed once with MCDB, and phase contrast images were taken to capture wound size prior to treatment. Cells were then stimulated with agonists and allowed to migrate for 16 hours. Wounds were imaged again and the percent of scratch closure was calculated using ImageJ software and comparing the wound size of specific fields before and after migration.

**Monocyte adhesion assay.** HUVEC were serum-starved for two hours and treated with agonists for 6 hours. U937 monocytes were maintained in RPMI in 10% FBS. U937 cells were washed once and labeled with 2 µM Calcein-AM (Invitrogen) in
MCDB for 15 minutes. U937 cells were washed twice before adding $2 \times 10^6$ monocytes to each well of activated EC after removing EC treatment media. Binding was allowed to proceed for 30 minutes, after which adhered monocytes were counted by fluorescent microscopy of EC-bound monocytes.

**Statistical analyses.** Unless otherwise indicated, data are expressed as means +/- SEM. Differences between groups were analyzed by analysis of variance (ANOVA). Bonferroni post-hoc tests were performed to evaluate pairwise differences between groups. Synergism Ratio ($\text{SR}_{\text{EGF+TRAP}}$) = $\frac{\text{Response}_{\text{EGF+TRAP}}}{(\text{Response}_{\text{EGF}} + \text{Response}_{\text{TRAP}})}$, where the response is the stimulus-inducible value. To calculate the presence of a synergism, a one-sample t-test was performed by comparing the mean SR to a hypothetical value of 1.0. Significance is expressed as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

**List of Reagents.** A comprehensive list of reagents, kits, antibodies, and primers used in this study can be found in the appendix in Tables II-VIII.
2.4 Results

Synergistic induction of multiple IEGs following simultaneous EGFR and PAR-1 activation.

To investigate the effect of simultaneous EGFR/PAR-1 activation on IEG induction, we treated EC with EGF, thrombin receptor agonist peptide (TRAP), or both for one hour (30 minutes for c-Fos) and measured IEG mRNA expression. We calculated a SR by dividing the response to combined stimulation with EGF plus TRAP by the sum of individual responses to EGF or TRAP alone. The transcription factors early growth response 1 and 3 (EGR1, EGR3), and c-Fos were induced with SRs of 2.18, 10.1, and 4.11, respectively (Figure 7). The prostanoid synthase cyclooxygenase-2 (COX-2) was induced with a SR of 1.52, and interleukin-8 (IL-8) displayed a SR of 2.20 (Figure 7). Protein levels of EGR1 and MKP-1 were also synergistically increased following dual EGF plus TRAP treatment with SRs of 1.96 +/- 0.09 and 2.95 +/- 0.84, respectively (Figure 7, bottom-right).
Figure 7. Simultaneous EGFR/PAR-1 activation synergistically induces multiple IEGs. EC were treated with EGF (16 ng/ml), TRAP (100 µM), or both for 30 minutes (c-Fos) or 1 hour (EGR1, EGR3, COX-2, IL-8). Cells were lysed, total RNA was isolated, and processed for qRT-PCR analysis. Data represent the mean and SEM from 3 independent experiments. Synergism Ratio $(SR_{EGF+TRAP}) = \frac{Response_{EGF+TRAP}}{Response_{EGF} + Response_{TRAP}}$. Bottom right, immunoblots of EC lysates treated for one hour with the indicated agonists.
We selected EGR1 as an endpoint to investigate the molecular mechanism of EGFR/PAR-1 crosstalk. In the presence of EGF, thrombin or TRAP displayed similar synergistic induction of EGR1 protein (EGF+thrombin SR = 2.57 +/- 0.82; EGF+TRAP SR = 2.57 +/- 0.52) (Figure 8). The kinetics of EGR1 expression were similar under all conditions, peaking at one hour and returning toward basal levels by three hours (Figure 9), suggesting that EGFR/PAR-1 crosstalk controls the magnitude, but not the kinetics, of IEG expression. EGR1 is occasionally expressed in both a high molecular weight (~75 kDa) and low molecular weight (~57 kDa) form (227). We observe the 75 kDa species in all experiments, but the 57 kDa form is rarely induced. Pretreatment of EC with cyclohexamide, an inhibitor of de novo protein synthesis, had no effect on EGR1 mRNA induction or the EGFR/PAR-1 synergism (Figure 10), demonstrating that EGR1 was induced as an IEG.

Figure 8. Thrombin and TRAP induce synergistic EGR1 expression in combination with EGF. Serum-starved EC were treated with EGF (16 ng/ml), TRAP (100 µM), or thrombin (5U/ml) for 1 hour and EGR1 was detected by immunoblot. Densitometry analysis was performed using ImageJ densitometry software and represents the quantification of 3 independent immunoblots.
Figure 9. Timecourse of EGR1 induction. EC were serum-starved for 2 hours and then treated with EGF, TRAP or both for up to 3 hours. EGR1 induction was analyzed by immunoblot.

Figure 10. De novo protein synthesis is not required for synergistic EGR1 induction. EC were serum-starved for 2 hours, and pre-treated with PBS or cyclohexamide (CHX, 1 µg/ml) for 30 minutes. EC were then treated for 60 minutes with EGF (16 ng/ml), TRAP (100 µM), or both, and inducible EGR1 mRNA expression was measured by qRT-PCR analysis.
ERK-independent coincidence detection underlies synergistic EGR1 induction.

Numerous publications have reported that EGR1 induction is mediated by the MAP kinase ERK1/2 in response to various agonists, including EGF (228, 229). To determine specific pathways mediating EGR1 induction by EGF plus TRAP, we pre-treated EC with the MEK1 inhibitor PD98059 to prevent ERK activation. PD98059 treatment almost completely inhibited EGR1 induction in response to individual treatment with EGF or TRAP (Figure 11). However, when cells were treated with EGF and TRAP simultaneously, an ERK-independent portion of EGR1 induction was triggered with a high degree of synergism (SR = 19.88) (Figure 13). In a signal transduction network, a SR of this magnitude suggests the presence of a signaling node that effectively functions as a Boolean AND gate; whereby, a cellular output (EGR1 induction) is elicited only when two stimuli (EGF and TRAP) are simultaneously present. This result revealed two important facts about the mechanism of EGFR/PAR-1 synergism. First, synergistic EGR1 induction occurs via two distinct signaling pathways (ERK-dependent and ERK-independent). Second, at some level, the ERK-independent pathway likely utilizes a mechanism of coincidence detection to induce ERK-independent EGR1 expression only when EGFR and PAR-1 are simultaneously activated.
Figure 11. Synergistic EGR1 induction is ERK-independent. EC were pre-treated for 30 minutes with the MEK1 inhibitor PD98059 (10 µM). EC were treated with EGF (16 ng/ml), TRAP (100 µM), or both for 1 hour and EGR1 was measured by immunoblot. Graph represents the mean +/- SEM from 4 independent experiments.

The ERK-independent pathway was sensitive to the EGFR kinase inhibitor AG1478 (Figure 12), which completely inhibited EGF-induced EGR1 and prevented a synergism during simultaneous EGF plus TRAP treatment (SR = 1.15 +/- 0.23) (Figure 12). This result revealed that in addition to the canonical pathway of ERK activation, EGFR kinase activity is also required to initiate the alternative, synergistic pathway of EGR1 induction. Therefore, combinatorial EGFR/PAR-1 signaling must be integrated at some downstream signaling node that functions as an AND gate to induce EGR1 following coincident receptor activation (Figure 13).
Figure 12. Synergistic pathway of EGR1 induction requires EGFR kinase activity. EC were pre-treated for 30 minutes with the EGFR kinase inhibitor AG1478 (1 μM). Treatments and immunoblots were performed as in (Figure 11). Graph represents the mean +/- SEM from 3 independent experiments.

Figure 13. ERK-independent pathway functions as a cellular coincidence detector. SRs from individual experiments were averaged to calculate a mean SR and SEM. Data represent the synergism ratio of EGR1 induction when the indicated agonist (x-axis) was used to stimulate EC in the presence of EGF.
STAT3 is required for detection of coincident EGFR/PAR-1 activation.

To identify candidate transcription factors involved in ERK-independent EGR1 induction, we examined the promoter regions of the six gene targets of the EGFR/PAR-1 synergism for common transcription factor binding sites. Using a combination of literature-based searches and web-based bioinformatics tools, (P-match and TFSEARCH) we compiled a list of potential transcription factor binding sites for each of the six IEGs (Figure 14). Several common transcription factors were identified, including ELK-1, the downstream target of the ERK1/2 signaling pathway that mediates EGR1 induction (230, 231), and CREB, most commonly activated by protein kinase A (PKA). PKA inhibition did not affect synergistic induction of EGR1 following EGF plus TRAP treatment (Figure 15), so we did not pursue further experiments on CREB signaling. Notably, every synergistically-induced gene had at least one potential binding site for a STAT transcription factor. Further examination revealed that these candidate STAT binding sites were consensus STAT3 binding elements (Gamma-IFN-activated sequences, GAS elements) (See appendix, Table II). Each of the six IEGs contained a putative STAT3 binding site between ~1.4-1.8 kb upstream to the transcription initiation site (Figure 16), raising the possibility that STAT3 was a component of the ERK-independent coincidence detector.
Figure 14. *In silico* analysis of DNA elements in the *EGR1* promoter. Promoter regions from synergistically induced IEGs were scanned for candidate transcription factor binding sites (up to 2 kb from the transcription initiation start site). Two freely available, web-based searches were used: P-match ([www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi](http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi)) and TFSEARCH ([www.cbrc.jp/research/db/TFSEARCH.html](http://www.cbrc.jp/research/db/TFSEARCH.html)), and matches were cross-checked between programs. Conflicting results were evaluated by literature-based searches, and if a conflict could not be resolved, the candidate binding site was included in the comparison to increase the number of possible matches. It is important to note that the main purpose of this bioinformatic approach was to generate hypotheses. Therefore, the figure does not constitute a comprehensive list of possible transcription factor sites.
**Figure 15. Protein kinase A activity is not required for synergistic EGR1 induction.** Serum-starved EC were pre-treated for 30 minutes with the Protein Kinase A inhibitor KT5720 (1 μM). Cells were treated with EGF, TRAP, or both for 1 hour and EGR1 was detected by immunoblot.

**Figure 16. Candidate STAT3 binding sites are present in all six synergistically induced genes.** Illustration showing the location of candidate STAT3 binding elements within the promoter regions of six synergistically-induced IEGs.
siRNA-mediated knockdown of STAT3 completely inhibited the synergistic induction of EGR1 protein and mRNA (Figure 17) without affecting EGR1 levels induced by EGF or TRAP alone. STAT3 knockdown significantly reduced the SR of inducible EGR1 protein (2.86 to 0.89) and mRNA (2.09 to 1.14) (Figure 18), suggesting a complete loss of function of EGFR/PAR-1 crosstalk. Similarly, the induction of another synergistically induced IEG, MKP-1, was inhibited when STAT3 protein was depleted by RNAi (Figure 19). These results provide convincing evidence that STAT3 is a critical transcription factor mediating the EGFR/PAR-1 synergism, and they implicate STAT3 in the mechanism of coincidence detection observed in experiments with PD98059 (Figure 11, 13).

![Figure 17. STAT3 is required for synergistic induction of EGR1.](image)

Left) EC were transfected with non-targeting or STAT3-targeting siRNA. 48 hours post-transfection, EC were treated with EGF, TRAP, or both for 1 hour and EGR1 was measured by immunoblot. (Center) Densitometry analysis of 4 independent immunoblots. (Right) STAT3 was depleted by RNAi, and EGR1 mRNA was measured by qRT-PCR. Data are averaged from 3 experiments and expressed as fold induction relative to untreated controls and normalized to levels of GAPDH mRNA.
Figure 18. Synergism ratio of inducible EGR1 in the presence or absence of STAT3. SRs for experiments using STAT3 siRNA. Dashed line at y = 1 represents an additive response.

Figure 19. STAT3 is required for MKP-1 induction in response to EGF plus TRAP. EC were transfected with non-targeting or STAT3-targeting siRNA. 48 hours post-transfection, EC were treated with EGF (16 ng/mL), TRA (100 µM), or both for 1 hour and MKP-1 was measured by immunoblot.
STAT3 activity is primarily regulated by two post-translational modifications (232). Phosphorylation of STAT3 at Tyr$^{705}$ leads to dimerization and permits DNA-binding (123). STAT3 Ser$^{727}$ phosphorylation, a modification within the COOH-terminal transactivation domain (TAD), facilitates physical interactions between STAT3 and histone acetyltransferases (163, 233), and is required for full transcriptional activity of STAT3 (159). We hypothesized that if STAT3 is the node of integration for detecting simultaneous EGFR/PAR-1 activation, then there should be some unique regulation of STAT3 during co-treatment with EGF plus TRAP. EGF treatment per se weakly induced Tyr$^{705}$ phosphorylation (Figure 20), and this modification was not significantly affected by co-treatment with TRAP. Strikingly, STAT3 Ser$^{727}$ phosphorylation did not occur in response to either EGF or TRAP alone, but was significantly increased when cells were simultaneously stimulated with EGF plus TRAP (Figure 20), suggesting that Ser$^{727}$ phosphorylation is a mediator of coincidence detection following EGFR/PAR-1 activation. To verify the synergistic phosphorylation of Ser$^{727}$, STAT3 was immunoprecipitated from EC following agonist exposure, and Ser$^{727}$ phosphorylation was measured by quantitative mass spectrometry (Figure 21). Again, neither EGF nor TRAP induced a significant increase in Ser$^{727}$ phosphorylation. However, Ser$^{727}$ phosphorylation was markedly increased following co-treatment with EGF plus TRAP, suggesting that STAT3 Ser$^{727}$ is a point of convergence for pathways of EGFR/PAR-1 crosstalk.
Figure 20. STAT3 Ser\textsuperscript{727} phosphorylation is strongly induced by EGF plus TRAP. (Left) EC were stimulated for 5 minutes with EGF (50 ng/ml), TRAP (100 µM), or both. STAT3 phosphorylations were detected by immunoblotting with antibodies specific for Tyr\textsuperscript{705} and Ser\textsuperscript{727} phosphorylation. (Center) Quantification of STAT3 Tyr\textsuperscript{705} phosphorylation from 4 independent immunoblots. (Right) Quantification of STAT3 Ser\textsuperscript{727} phosphorylation.

Figure 21. Quantitative mass spectrometry analysis of STAT3 Ser\textsuperscript{727} phosphorylation. HUVEC were treated for 5 minutes with EGF (16, ng/ml), TRAP (100 µM), or both. Phosphorylation was quantified by comparing the relative abundance of STAT3 Ser\textsuperscript{727} phosphopeptides to unmodified STAT3 peptides. Data are averaged from 2 independent experiments.
Ser\textsuperscript{727}-phosphorylated STAT3 positively regulates synergistic EGR1 induction via a distal GAS element.

We cloned a ~2kb fragment of the human EGR1 promoter region upstream of a luciferase reporter to investigate the role of STAT3 Ser\textsuperscript{727} phosphorylation in synergistic EGR1 induction (Figure 22, 23). Previous studies of the EGR1 promoter demonstrated that the ELK-1 and SRF binding sites within the proximal promoter region are critical for promoter activation in response to various stimuli including thrombin and EGF (231, 234–237). EGR1 promoter activity was synergistically increased in cells transfected with WT-STAT3 in response to EGF plus TRAP. (Figure 24). A STAT3 S727A mutation significantly decreased EGF plus TRAP induced EGR1 promoter activity and prevented a synergism during co-treatment (Figure 24), suggesting that STAT3 Ser\textsuperscript{727} is critical for promoter activation during EGFR/PAR-1 crosstalk.

![Map of EGR1 promoter](image)

**Figure 22. Map of EGR1 promoter.** Illustration showing location of transcription factor binding sites within the human *EGR1* promoter. Detailed sequence information is depicted in Figure 23.
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Figure 23. Sequence of cloned EGR1 promotor. The figure displays the full cloned sequence of human EGR1 promotor used in luciferase reporter assays. A partial list of consensus binding sites have been highlighted and color-coded. Primers used to clone this DNA fragment from human genomic DNA are listed in Table VIII.

Figure 24. STAT3 Ser\textsuperscript{727} is required for synergistic activation of EGR1 promotor. (Left) HUVEC were co-transfected with EGR1-promoter luciferase reporter and WT-STAT3 or STAT3-S727A. 24 hours post-transfection, EC were serum-starved for 4 hours prior to stimulation with EGF, TRAP, or both for 4 hours. Luciferase activity was measured using a luminometer. (Right) SRs for luciferase reporter experiments using STAT3-WT and STAT3 S727A. Dashed line at y = 1 represents an additive response.
Mutation of the distal GAS element, but not the proximal GAS element, significantly inhibited EGF plus TRAP induced promoter activity (Figure 25). Because MAPK signaling pathways are active in this context, residual promoter activity of the distal GAS mutant is likely due to EGF-induced activation of serum-response elements (SREs) within the proximal promoter (234, 238). Next, we measured the DNA-binding activity of STAT3 in response to EGF plus TRAP treatment using a DNA-binding ELISA assay. Surprisingly, we found that untreated STAT3 exhibited DNA-binding activity (Figure 26). Stimulation of EC with EGF or TRAP for 15 minutes did not affect STAT3 DNA binding, but co-treatment induced a ~2-fold increase in STAT3-specific DNA binding activity. Together, these results demonstrate that simultaneous activation of EGFR and PAR-1 causes synergistic activation of the EGR1 promoter via a distal GAS element in a manner that requires STAT3 Ser\textsuperscript{727} phosphorylation.

Figure 25. Distal GAS element in EGR1 promoter is required for maximal promoter activity. EC were transfected with EGR1-promoter luciferase reporter or constructs containing mutations in the distal GAS element (TTCCCGGAA → gcgCCGGAA) or proximal GAS element (TTCCCCGAA → gcgCCCGAA). 24 hours post-transfection, inducible luciferase activity was measured as in Figure 24.
Figure 26. STAT3 DNA-binding activity in HUVEC. DNA-binding activity of STAT3 was measured using a TransAM ELISA from ActiveMotif. EC were stimulated with indicated treatments for 15 minutes, and nuclear extracts were incubated at room temperature in a 96-well microplate coated with oligonucleotides containing consensus GAS-elements. Nuclear extract was washed away and DNA-bound STAT3 was detected using an anti-STAT3 antibody and colorimetry using TMB as a chromogenic substrate. Individual extracts were processed in duplicate wells and data represent the mean and SEM of 2 independent experiments.

Coincident EGFR/PAR-1 signaling prevents EGF-induced AKT activation.

To identify signaling pathways involved in combinatorial regulation of STAT3 Ser<sup>727</sup> phosphorylation, we used a phospho-kinase signaling array (R&D Systems) to examine the relative activation of various cell signaling intermediates following individual or combined stimulation with EGF and thrombin. The protein array was composed of nitrocellulose membranes spotted with antibodies for cell signaling mediators, and phosphorylation was detected by chemiluminescence with anti-phosphotyrosine, threonine, and serine antibodies (Figure 27).
Figure 27. Thrombin treatment prevents EGF-induced AKT phosphorylation. (Left) Phospho-kinase array (R&D Systems, Catalog # ARY003) was performed according to the manufacturer’s instructions. Briefly, EC were treated with EGF, thrombin, or both for 10 minutes. EC were lysed with a proprietary lysis buffer and lysates were incubated with antibody-spotted nitrocellulose membranes. Relative phosphorylation was detected with anti-phospho-Tyr, Thr, and Ser secondary antibodies. (Right) Heatmap of data from protein array was created after quantification using ImageJ software. Raw data is available in Table III in the appendix. Heatmap was generated using R-project statistical graphing software, and expressed as fold induction relative to untreated controls for each antibody.
The results of the phosphokinase array experiment are summarized in the heatmap (Figure 27), and detailed in Table III in the appendix. As expected, EGF treatment induced the phosphorylation of numerous signaling molecules including ERK1/2, MSK1/2, eNOS, AKT, and GSK-3α/β. TRAP treatment also induced phosphorylations of known, thrombin-regulated proteins (c-Src, CREB). The most dramatic feature of the co-treatment condition was a striking inhibition of AKT phosphorylation at Ser\textsuperscript{473} relative to cells treated with EGF alone (Figure 27, box, bottom; heatmap, top row). Further analysis revealed that phosphorylation of known AKT substrates, including eNOS and GSK-3α/β (Figure 27, box, top), was also reduced during simultaneous EGF plus TRAP treatment (Appendix, Table VIII). This observation raised the possibility that EGF plus TRAP treatment modulates AKT-mediated signaling pathways by inhibiting AKT activity. Results of the protein array were confirmed by immunoblotting to detect phosphorylation of AKT. EGF treatment strongly induced phosphorylation of AKT Ser\textsuperscript{473}, and this increase was significantly inhibited by simultaneous EGF plus TRAP treatment (Figure 28). The inhibition appeared to be specific to AKT signaling, as ERK1/2 phosphorylation was not reduced during EGF plus TRAP treatment (Figure 27, 28).
Figure 28. PAR-1 activation prevents EGF-induced AKT phosphorylation. EC were treated as in Figure 27 with TRAP substituted for thrombin, and levels of phosphorylated ERK and AKT were detected by immunoblot. (Right) Quantification of 5 immunoblots of experiments performed as in the left panel.

EGFR does not have direct binding sites for the SH2 domain of PI3K, but activates the PI3K-AKT signaling axis via adaptors proteins such as GAB1 (239–241). Phosphorylation of EGFR at Y1068 and Y1086 are the predominant GAB1-binding sites for activation of PI3K (241). We investigated whether PAR-1 activation could modulate EGF-induced phosphorylation at these sites to prevent AKT phosphorylation in response to EGF. Co-treatment with EGF and TRAP did not significantly affect total tyrosine phosphorylation of EGFR, pY1068, or pY1086 (Figure 29), suggesting that PAR-1 activation inhibits EGF-induced AKT phosphorylation through a downstream signaling mechanism.
Figure 29. Analysis of EGFR phosphorylation in response to EGF plus TRAP. EC were treated with EGF (50 ng/ml), TRAP (100 µM), or both for 5 minutes. Cells were lysed in ice-cold RIPA buffer and EGFR was immunoprecipitated with anti-EGFR antibody overnight. Immunoprecipitate was resolved by SDS-PAGE, and total or site-specific tyrosine phosphorylation was analyzed by immunoblot.

Next, we systematically inhibited PAR-1-coupled Ga proteins to identify the Ga member responsible for inhibition of EGF-induced AKT activation. Individual knockdown of Gaq, Ga12, and Ga13, or inhibition of Gai by pertussis toxin did not significantly affect the ability of PAR-1 to inhibit AKT activation in response to EGF (Figure 30). However, dual knockdown of Ga12 and Ga13 caused an increase in AKT phosphorylation in response to EGF plus TRAP treatment (Figure 31). Together, these data suggest that PAR-1 activation inhibits EGF-induced AKT Ser\textsuperscript{473} phosphorylation in a Ga12/13-dependent manner without any detectable changes in EGFR phosphorylation.
Figure 30. Role of Gα subunits in PAR-1-mediated inhibition of AKT phosphorylation. (Top-left) Confluent EC were pre-treated with pertussis toxin (250 ng/ml) or PBS for 30 minutes, after which they were treated with EGF, TRAP, or both for 5 minutes. AKT Ser^{473} phosphorylation was analyzed by immunoblot. (Top-right) EC were transfected with non-targeting or Gαq-targeting siRNA. 48 hours post-transfection, EC were treated with EGF (16 ng/mL), TRAP (100 µM), or both for 5 minutes and AKT Ser^{473} phosphorylation was analyzed by immunoblot. (Bottom-left) EC transfected with Gα12 siRNA, (Bottom-right) EC transfected with Gα13 siRNA, and treated as in the top panels.

Figure 31. Gα12/13 are required for PAR-1-dependent inhibition of AKT phosphorylation. EC were transfected with non-targeting or Gα12/13-targeting siRNA. 48 hours post-transfection, EC were treated with EGF (16 ng/mL), TRAP (100 µM), or both for 5 minutes and AKT Ser^{473} phosphorylation was analyzed by immunoblot.
Inhibition of AKT is required for STAT3 Ser\textsuperscript{727} phosphorylation and enhanced EGR1 expression.

To test whether the inhibition of AKT signaling was involved in enhancing EGR1 expression, we did a side-by-side comparison of EGF plus TRAP-treated cells and cells treated with EGF plus the PI3K inhibitor, LY294002. EGF treatment strongly induced AKT Ser\textsuperscript{473} phosphorylation at 5 minutes, and both EGF plus TRAP and EGF plus LY294002-treated cells showed little to no AKT phosphorylation relative to EGF-treated cells (Figure 32). In both cases, the inhibition of AKT phosphorylation at 5 minutes preceded an increase in EGR1 induction at 60 minutes (Figure 32) relative to cells treated with EGF alone. EGF plus TRAP and EGF plus LY294002 induced EGR1 to similar levels relative to EGF-treated cells (Figure 32), suggesting that modulation of EGF-induced AKT signaling by PAR-1 is a critical step in STAT3-dependent EGR1 induction. Further, EGF-induced EGR1 promoter activity was significantly increased in EC expressing a DN-AKT construct relative to a WT-AKT expressing cells (Figure 33).
Figure 32. Inhibition of PI3K increases EGF-induced EGR1 expression. EGF, TRAP, and EGF plus TRAP-induced AKT phosphorylation and EGR1 induction were compared to EGF signaling in the presence of a PI3K inhibitor. EC were serum-starved for 90 minutes and then incubated with DMSO or LY294002 for an additional 15 minutes before treatment with indicated agonists. AKT phosphorylation and EGR1 induction were determined by immunoblot. (Right) Quantification of 2 independent experiments performed as in the left panels.

Figure 33. AKT activity suppresses EGR1 promoter activity in response to EGF. HUVEC were co-transfected with EGR1-promoter luciferase reporter and WT-AKT or DN-AKT. 24 hours post-transfection, EC were serum-starved for 4 hours prior to stimulation with EGF, TRAP, or both for 4 hours. Luciferase activity was measured using a luminometer and data represent 4 independent experiments.
To investigate whether the inhibition of AKT kinase activity was required for STAT3 Ser\textsuperscript{727} phosphorylation, EC were incubated for 15 minutes with either DMSO or the PI3K inhibitor LY294002, after which they were treated with agonists for five minutes, and STAT3 was immunoprecipitated to assess Ser\textsuperscript{727} phosphorylation by immunoblot. Similar to previous results (Figure 20, 21), individual EGF treatment did not induce Ser\textsuperscript{727} phosphorylation. However, in the presence of a PI3K inhibitor, Ser\textsuperscript{727} phosphorylation was EGF-inducible (Figure 34), demonstrating that inhibition of AKT activity is a prerequisite for EGF-induced Ser\textsuperscript{727} phosphorylation. Further, this result demonstrates that the TRAP-mediated inhibition of EGF-activated AKT is likely the mechanism by which STAT3 Ser\textsuperscript{727} phosphorylation occurs during coincident EGFR/PAR-1 activation.

![Image](image.png)

**Figure 34.** STAT3 Ser\textsuperscript{727} phosphorylation is EGF-inducible during PI3K inhibition. HUVEC were transfected with recombinant V5-tagged STAT3. Cells were pre-treated with DMSO or LY294002 for 15 minutes before treatment with EGF for 5 minutes. Recombinant STAT3 was immunoprecipitated using anti-V5 antibody and STAT3 Ser\textsuperscript{727} phosphorylation was analyzed by immunoblot.
We next examined the effect of combinatorial EGFR/PAR-1 signaling on AKT kinase activity. Two phosphorylation sites are involved in AKT activation, Thr^{308} and Ser^{473} (242), and phosphorylation at both sites was reduced by EGF plus TRAP co-treatment (Figure 35). Although the mechanism by which this inhibition occurs is unclear, this result suggests that it is upstream to AKT, rather than a regulation of AKT itself. Next, AKT activity was measured via an immune-complex kinase assay (Figure 35). AKT that was immunoprecipitated from EGF-treated cells strongly phosphorylated a GST-GSK-3α fusion protein, while TRAP-treated AKT showed little to no activity (Figure 35). EGF plus TRAP treatment significantly reduced the ability of AKT to phosphorylate the GST-GSK-3α fusion protein compared to EGF-treated AKT, indicating a significant reduction in AKT kinase activity during co-stimulation with EGF plus TRAP (Figure 35). Additionally, EGF-induced GSK-3α/β phosphorylation was completely inhibited by pre-treatment with a PI3K inhibitor (Figure 36), indicating that EGF-induced AKT activity is likely the primary mediator of EGF-induced GSK-3α/β phosphorylation in EC.
**Figure 35. EGF plus TRAP treatment decreases AKT kinase activity.** Serum-starved EC were treated with agonists for 5 minutes. Cells were lysed, and total AKT was immunoprecipitated using a pan-AKT antibody (Cell Signaling, #4691). A portion of the lysate (~10%) was diluted 400-fold in kinase assay buffer and incubated with recombinant GST-GSK3-α fusion protein. The majority of lysate was used for immunoblot analysis to determine levels of AKT phosphorylation. Densitometry values (right) represent the mean +/- SEM of 3 immune-complex kinase assays.

**Figure 36. GSK-3α/β Ser^{21}/Ser^{9} phosphorylation is downstream of PI3K.** EC were pre-treated with DMSO or LY294002 (10µM) for 15 minutes before treatment with EGF (16 ng/ml) for 5 minutes. GSK-3α/β phosphorylation was analyzed by immunoblot.
AKT-mediated phosphorylation of GSK-3α at Ser\textsuperscript{21} and GSK-3β at Ser\textsuperscript{9} leads to autoinhibition and reduced activity toward downstream substrates (243–245). We hypothesized that a reduction in the inhibitory phosphorylation of GSK-3α/β during EGF plus TRAP treatment could allow GSK-3α/β to participate in STAT3 Ser\textsuperscript{727} phosphorylation. The compound CHIR99021, a potent and highly specific inhibitor of GSK-3α/β (246), reduced Ser\textsuperscript{727} phosphorylation in response to EGF plus TRAP (Figure 37, 38, 39), but roscovitine, a cyclin-dependent kinase inhibitor, and rapamycin, an inhibitor of mTOR signaling, had no effect (Figure 38, 39). These results demonstrate that GSK-3α/β kinase activity is required for EGF plus TRAP induced Ser\textsuperscript{727} phosphorylation, thus placing GSK-3α/β in the STAT3-dependent, non-canonical pathway of EGR1 induction. Simultaneous knockdown of GSK-3α/β by RNAi significantly reduced EGF plus TRAP-induced EGR1 expression, while individual knockdown of GSK-3α or GSK-3β did not significantly affect EGR1 induction (Figure 40).

**Figure 37.** GSK-3α/β kinase activity positively regulates STAT3 Ser\textsuperscript{727} phosphorylation. EC were pre-treated for 30 minutes with the GSK-3α/β inhibitor CHIR99021 (1 μM) and then stimulated for 5 minutes with EGF, TRAP, or both. STAT3 phosphorylations were detected by immunoblot.
Next, we investigated whether GSK-3α/β activity was required for ERK-independent EGR1 expression. The MEK1 inhibitor PD98059 inhibited ~50% of EGF plus TRAP-induced EGR1 and CHIR99021 inhibited EGR1 induction to an approximately equal extent (Figure 41). Simultaneous inhibition of MEK1 and GSK-3α/β completely inhibited EGR1 induction by EGF plus TRAP. Similar results were seen for the IEG MKP-1, as inhibition of both GSK-3α/β and ERK1/2 was required to completely inhibit MKP-1 protein induction in response to EGF plus TRAP (Figure 41). This result shows that, like STAT3, GSK-3α/β function in an ERK-independent pathway that is independently capable of inducing EGR1 expression in response to simultaneous EGF plus TRAP treatment. Further, given that GSK-3α/β is required for increased STAT3 Ser\textsuperscript{727} phosphorylation during EGF plus TRAP treatment (Figure 37, 38, 39), it is reasonable to conclude that altered GSK-3α/β activity, due to a reduction in AKT-mediated inhibitory phosphorylation (Figure 28, 35), is a critical mediator of EGFR/PAR-1 crosstalk and STAT3-dependent gene expression.
Figure 38. Effects of Serine/Threonine kinase inhibitors on STAT3 Ser$^{727}$ phosphorylation. EC were pre-treated for 30 minutes with the GSK-3α/β inhibitor CHIR99021 (10 µM), roscovitine (20 µM), or rapamycin (10 nM), and then stimulated for 5 minutes with EGF and TRAP. STAT3 phosphorylations were detected by immunoblot. Graph represents the mean +/- SEM from 3 independent experiments.

Figure 39. Effects of serine/threonine kinase inhibitors on STAT3 Ser$^{727}$ phosphorylation measured by quantitative mass spectrometry. EC were pre-treated with the serine-threonine kinase inhibitors CHIR99021 (1 µM), roscovitine (20 µM), and rapamycin (10 nM). STAT3 was immunoprecipitated from EGF or EGF plus TRAP treated ECs and immunoprecipitates were resolved by SDS-PAGE. Gels were stained with coomassie blue solution, an ~86 kDa band was cut from the gel and Ser$^{727}$ phosphorylation was analyzed by quantitative mass spectrometry.
Figure 40. GSK-3α and GSK-3β mediate EGR1 induction in response to EGF plus TRAP.
EC were transfected with control siRNA or siRNA targeting GSK-3α, GSK-3β, or both. 48 hours post-transfection, cells were treated with EGF, TRAP, or EGF plus TRAP for 60 minutes, and EGR1 induction was measured by immunoblot. Data represent the mean +/- SEM of 3 independent experiments.

Figure 41. GSK-3α/β-dependent EGR1 induction is ERK-independent. EC were pre-treated for 30 minutes with the GSK-3α/β inhibitor CHIR99021 (10 μM), PD98059 (10 μM), or both and then stimulated for 60 minutes with EGF and TRAP. EGR1 and MKP-1 were detected by immunoblot.
Coincident EGFR/PAR-1 signaling enhances EC migration in a STAT3-dependent manner.

EGFR and STAT3 are well-known regulators of cell growth and proliferation. To investigate how the EGFR/PAR-1 crosstalk affects EC proliferation, serum-starved EC were stimulated with EGF, TRAP, or both for 8 hours, and BrdU incorporation was measured between 8-12 hours after agonist exposure (Figure 42). EGF treatment increased BrdU incorporation to approximately 2.5 fold compared to non-stimulated cells. TRAP treatment had little to no effect on BrdU incorporation. Interestingly, simultaneous stimulation of EC with EGF and TRAP caused a ~50% reduction in BrdU incorporation relative to EGF treatment alone. siRNA-mediated knockdown of STAT3 further reduced EGF plus TRAP-induced BrdU incorporation, suggesting that STAT3 is critical for EGF-induced DNA synthesis in EC during simultaneous EGFR/PAR-1 activation (Figure 42).
Figure 42. **STAT3 positively regulates EC proliferation.** EC were serum-starved for 16 hours and then treated with agonists in serum-free medium for 8 hours. BrdU was added to wells for 4 hours and cells were fixed in a DNA-denaturing proprietary lysis buffer. BrdU incorporation was detected by an anti-BrdU antibody and colorimetry using HRP-conjugated secondary antibody and 3,3',5,5'-tetramethylbenzadine as a chromogenic substrate. Data represent the mean +/- SEM for 4 independent experiments.

We previously reported that thrombin induces the expression of leukocyte adhesion molecules on EC and increases monocyte-EC interactions *in vitro* (67, 68, 83). To investigate the effect that combinatorial signaling has on thrombin-induced leukocyte-EC interactions, we performed an *in vitro* monocyte adhesion assay on EGF, thrombin, or EGF plus thrombin-stimulated EC. We observed very few monocyte-EC interactions under both untreated and EGF-treated conditions (Figure 43). Thrombin induced a significant increase in monocyte adhesion to the EC monolayer (Figure 43), and this effect was almost completely inhibited when EC were simultaneously exposed to both EGF and thrombin (Figure 43), suggesting that coincident EGFR signaling can at least partially inhibit thrombin-induced inflammatory signaling. These results provide
evidence that the known phenotypic responses of EGF-induced proliferation and thrombin-induced inflammation are both suppressed during combinatorial EGFR/PAR-1 signaling.

Figure 43. EGFR signaling suppresses thrombin-induced leukocyte-EC interactions. An EC monolayer was treated with indicated agonists for 6 hours, after which agonists were washed away with serum-free media. Calcein-AM-stained U937 monocytes were added to the stimulated EC and allowed to adhere for 30 minutes. Non-adhered monocytes were washed away and adhered monocytes were imaged and quantified per field with ImageJ software. Data represent the mean +/- SEM from 3 independent experiments.

Previous work from our laboratory demonstrated that simultaneous stimulation of EC with EGF and TRAP caused a significant increase in EC transwell migration in vitro (84). We observed a similar synergistic increase in migration when an EC monolayer was mechanically wounded and then treated with EGF, TRAP, or both for 16 hours (Figure 44). To investigate a potential role for STAT3 in mediating enhanced EC migration, we depleted STAT3 by RNAi and performed in vitro wound healing assays and measured the degree of scratch closure. Scratch closure was enhanced in cells stimulated with both
EGF and TRAP compared to either EGF or TRAP alone (Figure 44). siRNA-mediated knockdown of STAT3 slightly reduced EGF-induced wound-healing, and completely inhibited enhanced wound-healing by EGF plus TRAP treatment (Figure 44). These results suggest that the combinatorial activation of STAT3 causes increased EC migration, and this increase coincides with a suppression of proliferation and inflammation elicited by either EGF or thrombin treatments per se.

Figure 44. Combinatorial EGFR/PAR-1 signaling increases EC migration in a STAT3-dependent manner. (Left), An EC monolayer was mechanically wounded with a P200 pipette tip and dislodged cells were washed away with serum-free media. EC were treated with indicated agonists, and cells were imaged immediately after scratching, and 16 hours post-treatment. (Middle), EC wound healing assay was performed. EC were transfected with control or STAT3 siRNA 40 hours prior to the experiment. Dashed white lines indicate the boundaries of the wounds before agonist treatment. (Right), SRs from EC wound healing experiments. Dashed line at y = 1.0 represents an additive response. One-sample t-test was used to assess the presence of a significant synergism compared to y=1 (P<.05), and a student’s t-test was used to compare differences between two groups (P=.053).
2.5 Discussion

There have been considerable discrepancies regarding EGFR expression in EC (226). Several studies indicated that EGFR was expressed on human microvascular EC (MVEC) and that MVEC were responsive to EGF (137, 247), while other reports failed to detect EGFR expression on both MVEC (136) and HUVEC (136–138). Reports from our laboratory and others (84, 141, 248, 249) demonstrate that MVEC and HUVEC do indeed express significant levels of EGFR and are responsive to EGF. Hirata et al. used a mouse corneal micropocket assay to show that EGF is as potent as VEGF in inducing angiogenesis in vivo (140). It has also recently been suggested that VEGF-induced EC migration may be mediated via EGFR transactivation (142). Moreover, although the expression of EGFR on EC has been debated, it is clear that tumor-associated EC express EGFR and are responsive to EGF (247, 250). It is important to address the deficiencies in our knowledge of endothelial EGFR signaling so that we may better understand its role in endothelial physiology, thereby providing a basis for determining the relative contribution of endothelial EGFR signaling to disease states like atherosclerosis and cancer.

It is well-established that PAR-1 and other GPCRs can transactivate RTKs via inside-out growth-factor signaling in a variety of cell-types (100, 129, 223, 251). Through this process, GPCRs can induce proliferative, migratory, or invasive phenotypes in an RTK-dependent manner (128–130). Our results demonstrate that GPCR-mediated transactivation of RTKs may not be identical to RTK activation per se. Inside-out autocrine signaling may coincide with intracellular modulation of RTK-dependent
signaling pathways by GPCR-mediated pathways. While GPCR activation leads to EGFR transactivation in many contexts, our results raise the possibility that the specific combinations of signaling pathways propagated from EGFR are determined by coincident GPCR activation. Therefore, the temporal relationship between GPCR and RTK activation may have an important effect on EGF-induced phenotypic changes such as migration and proliferation.

We have shown that STAT3 is required for a non-canonical, ERK-independent pathway of IEG induction that is triggered by coincident EGFR/PAR-1 activation. EGF induces the classical STAT3 phosphorylation at Tyr<sup>705</sup> (Figure 20), but also initiates an inhibitory signal through the PI3K-AKT signaling axis that phosphorylates GSK-3α/β, prevents STAT3 Ser<sup>727</sup> phosphorylation, and inhibits STAT3-dependent EGR1 induction. PAR-1 signaling resolves the conflicting, EGF-activated pathways by preventing AKT activation, thus permitting GSK-3α/β-dependent STAT3 Ser<sup>727</sup> phosphorylation and ERK-independent EGR1 induction (Figure 45). Similar to our results, others have shown that coincidence detection in a network-based setting can occur by the introduction of an activating signal while simultaneously suppressing an inhibitory signal (216). Collectively, our results describe a novel mechanism by which EGFR and PAR-1 cooperate to form a network-based coincidence detector that regulates STAT3-dependent gene expression.
Figure 45. STAT3-mediated coincidence detection regulates a non-canonical pathway of IEG induction. Either EGF or TRAP is sufficient to induce EGR1 expression via the canonical ERK1/2 pathway. EGF induces the activating STAT3 phosphorylation at Tyr\(^{705}\), but prevents STAT3 Ser\(^{727}\) phosphorylation by strongly activating AKT. Activated AKT phosphorylates and inhibits GSK-3\(\alpha/\beta\). Coincident PAR-1 signaling inhibits EGF-induced AKT activation, thereby reducing AKT-mediated phosphorylation and inhibition of GSK-3\(\alpha/\beta\), and permitting GSK-3\(\alpha/\beta\)-dependent STAT3 Ser\(^{727}\) phosphorylation. Simultaneous EGFR and PAR-1 activation causes increased phosphorylation of both STAT3 Tyr\(^{705}\) and Ser\(^{727}\) and triggers STAT3-dependent IEG induction.

The EGFR/PAR-1 signaling interaction regulates the magnitude of IEG induction without any detectable change in the kinetics of expression. We show that maximal EGR1 induction can be separated into discrete portions, with ERK1/2 and STAT3 mediating independent pathways. It has been shown that alterations in IEG expression, both in magnitude and duration, can profoundly impact cellular physiology (224, 225, 252). In particular, one recent study showed that the magnitude of EGR1 induction is critical for digitizing inconsistent or weak growth-factor signals into all-or-nothing phenotypic outputs (224). As Zwang et al. showed, the degree of EGR1 induction may be
a critical determinant of cell-cycle progression, where EGR1 expression must exceed a certain threshold in order for cells to pass a restriction point and initiate DNA synthesis.

GSK-3α/β are multifunctional serine/threonine kinases that are constitutively active under basal conditions, and undergo rapid inhibition via serine phosphorylation following growth-factor stimulation (245, 253). Our data demonstrate that GSK-3α/β activity is critical for a higher-order function of crosstalk by regulating the central node of coincidence detection between EGFR and PAR-1. Several studies have demonstrated that GSK-3α/β is critical for crosstalk during cytokine signaling (254–256). Beurel and Jope showed that inhibitors of GSK-3β signaling reduced STAT3 Tyr705 phosphorylation in response to interferon-γ and LPS in cell culture and in mouse brain tissue (254, 257). Interestingly, their signaling studies in Raw 264.7 macrophages showed that STAT3 Ser727 phosphorylation is unaffected by GSK-3α/β inhibition, in contrast to our results, and indicative of differential regulation of STAT3 by GSK-3α/β depending on the signaling context. To our knowledge, our results are the first example of GSK-3α/β mediating growth-factor activation of STAT3, and may represent a novel opportunity for therapeutic inhibition of STAT3 downstream of oncogenic RTKs.

It is particularly interesting to note that although the observed regulation of STAT3 Ser727 phosphorylation resembles a strong synergism (Figure 20, 21, 37), STAT3-mediated EGR1 expression appears to be almost perfectly gated by combinatorial EGFR/PAR-1 signaling (Figure 11, 17, 41). In other words, there appears to be a low level of noise at the signaling node (STAT3) that is not reflected in the cellular output.
(STAT3-dependent EGR1 induction). The exact mechanism by which this noise is filtered remains unclear. However, it is intriguing to consider the possibility that other transcription factors may also be required for the STAT3-dependent EGR1 expression. In our comparison of promoter elements of synergistically induced genes (Figure 14), we identified several transcription factors that may be common to these genes, including NFκB family members and CREB. Le Goff et al. reported that CREB1-dependent expression of the ABCA1 gene required a nearby STAT element (258), suggesting that cooperation between STATs and CREB may facilitate enhanced gene expression. Our results clearly show that STAT3 is required for EGR1 induction, but they do not rule out the possibility that other factors are required to act in concert with STAT3 signaling to induce EGR1. We speculate that EC may utilize multiple synergistically activated transcription factors, working in parallel but required in combination, to filter noise at signaling nodes and increase the stringency with which STAT3-dependent EGR1 induction is gated by combinatorial signaling.

We have shown that STAT3 essentially functions as a cellular coincidence detector, triggering EGR1 induction only when EGFR and PAR-1 are simultaneously activated. Coincidence detection, in this context, reflects an extreme synergism where the response to multiple agonists is far greater than responses to individual agonists. A large-scale analysis of crosstalk in Raw 264.7 macrophages found that although non-additive events (positive or negative) are relatively common, the prevalence of marked superadditive responses to pair-wise combinations of agonists is rare (215). In fact, less than 1.5% of all ligand-pairs in the study demonstrated a significant superadditive response, and the authors noted that although many ligands fail to induce cytokine
production by themselves, many ligands display at least one non-additive interaction when analyzed in combination with another input (215). Thrombin is capable of activating a variety of intracellular signaling pathways via activation of PAR-1. However, in many cases, thrombin’s regulation of signaling pathways (e.g. ERK) and cellular outcomes (e.g. gene expression, migration, angiogenesis) appears to be much weaker than more potent agonists like EGF and VEGF (84, 106). Our results show for the first time that perhaps one of the strongest responses elicited by PAR-1 signaling is a modulation of EGF-induced PI3K-AKT signaling and a triggering of STAT3-dependent gene expression. We therefore propose that in a combinatorial signaling environment, where growth-factors likely predominate as the most potent determinants of phenotypic outcomes, GPCRs like PAR-1 may act as modulators of growth-factor induced signaling by determining the specificity of RTK-dependent responses.
CHAPTER III

SIGNAL INTEGRATION AND GENE INDUCTION BY A FUNCTIONALLY DISTINCT STAT3 PHOSPHOFORM

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3.1 Abstract

Aberrant activation of the ubiquitous transcription factor STAT3 is a major driver of solid tumor progression and pathological angiogenesis. STAT3 activity is regulated by numerous posttranslational modifications (PTMs), including Tyr$^{705}$ phosphorylation, which is widely used as an indicator of canonical STAT3 function. Herein, we report a noncanonical mechanism of STAT3 activation that occurs independent of Tyr$^{705}$ phosphorylation. Using quantitative liquid chromatography-tandem mass spectrometry, we have discovered and characterized a novel STAT3 phosphoform that is simultaneously phosphorylated at Thr$^{714}$ and Ser$^{727}$ by GSK-3α/β. Both Thr$^{714}$ and Ser$^{727}$ are required for STAT3-dependent gene induction in response to simultaneous activation of EGF receptor (EGFR) and protease-activated receptor-1 (PAR-1) in endothelial cells. In this combinatorial signaling context, preventing formation of doubly phosphorylated STAT3, by depleting GSK-3α/β, is sufficient to disrupt signal integration and inhibit STAT3-dependent gene expression. Levels of doubly phosphorylated STAT3, but not Tyr$^{705}$-phosphorylated STAT3, are remarkably elevated in clear cell renal cell carcinoma relative to adjacent normal tissue, suggesting that the GSK-3α/β-STAT3 pathway is active in this disease. Collectively, our results describe a functionally distinct, noncanonical STAT3 phosphoform that positively regulates target gene expression in a combinatorial signaling context, and identify GSK-3α/β-STAT3 signaling as a potential therapeutic target in renal cell carcinoma.
3.2 Introduction

The signal transducer and activators of transcription (STATs) are a family of seven transcription factors that regulate numerous physiological and pathophysiological processes including immunity, angiogenesis, cellular survival, metastasis, and oncogenesis (143, 182). STAT3 is aberrantly activated in a vast majority of human cancers and is a downstream target of several oncogenic tyrosine kinases including EGF receptor (EGFR), JAKs, and Src family kinases (SFKs) (122, 259, 260). Consequently, much research has focused on understanding the role of STAT3 in malignancies, and studies are ongoing to determine the efficacy of STAT3 inhibition to treat human cancers (126, 261). It is therefore critical to identify and characterize novel mechanisms of STAT3 activation in order to elucidate unexplored opportunities to inhibit its function.

A wide range of stimuli including growth factors, oncogenic kinases, and cytokines can activate STAT3 (262). These stimuli modulate STAT3 function by regulating a diverse set of PTMs including tyrosine and serine phosphorylation, lysine acetylation, and lysine and arginine methylation (123, 159, 160, 164–168). Activation of receptor and non-receptor tyrosine kinases stimulates STAT3 Tyr705 phosphorylation to induce dimerization and increase STAT3 DNA binding activity (123, 157, 262, 263). Phosphorylation of Ser727 is mediated by various serine kinases (e.g. MAPKs, CDKs, PKCs), and this modification increases STAT3 transcriptional activity by facilitating protein-protein interactions with transcriptional coactivators (159, 163, 262, 264–266). Acetylation of several lysine residues, most notably Lys685, has also been reported to regulate STAT3 dimer formation and transcriptional activity (166–169). STAT3 is
methylated at Lys\textsuperscript{140} in response to IL-6, and this modification can inhibit or enhance
STAT3-dependent transcription in a gene-specific manner (164). STAT3 has also been
reported to be methylated at Arg\textsuperscript{31} by PRMT2 to negatively regulate leptin signaling
(165).

The abundance and diversity of STAT3 PTMs suggest that numerous distinctly
modified STAT3 forms (mod-forms) may be simultaneously present in a given cellular
context. Indeed, there potentially exist $2^n$ STAT3 mod-forms, where $n$ is the number of
modified STAT3 sites. As $n$ increases, or as the number of possible PTMs at a single site
increases (e.g. acetylation or methylation of lysine) there is a corresponding exponential
increase in the potential proteomic complexity of STAT3 mod-forms. This mechanism of
proteomic expansion has been suggested to increase the functional repertoire of cellular
proteins, and is likely to confer signal integration potential to STAT3 (267).

We previously reported that STAT3 is a critical signal integrator downstream of
coincident EGFR and protease-activated receptor-1 (PAR-1) signaling in vascular
endothelial cells (EC) (268). In this context, GSK-3\alpha/\beta-dependent phosphorylation of
STAT3 Ser\textsuperscript{727} is required to trigger inducible expression of the transcription factor early
growth response 1 (EGR1). Importantly, STAT3-dependent gene expression is triggered
only when EGFR and PAR-1 are simultaneously activated, suggesting that the temporal
information of coincident EGFR/PAR-1 activation is transduced via GSK-3\alpha/\beta-STAT3
signaling. GSK-3\alpha/\beta are multifunctional serine/threonine kinases that regulate substrates
with multiple phosphorylation sites in a manner that often requires a “priming”
phosphorylation (269). Recently, proteome-wide analyses have identified STAT3 Thr$^{714}$ as a novel phosphorylation site (170–172), but the regulation and function of this modification have not been investigated.

In this report, we provide evidence that GSK-3α/β directly phosphorylate STAT3 to generate a STAT3 phosphoform that is simultaneously modified at Thr$^{714}$ and Ser$^{727}$. Both Thr$^{714}$ and Ser$^{727}$ phosphorylation are required for stimulus-dependent Mcl1 induction in a combinatorial signaling context, suggesting that the doubly modified phosphoform is the mediator of signal integration and gene induction during simultaneous EGFR/PAR-1 activation. Levels of doubly phosphorylated STAT3 are significantly elevated in renal tumors relative to matched normal tissue, suggesting that the GSK-3α/β-STAT3 signaling axis may be active in this disease. In summary, we provide a seminal example of temporal information encoding via multisite STAT3 phosphorylation in the context of combinatorial signaling.

3.3 Materials And Methods

Cell-Culture, Transfections, and Treatments. Human EC were isolated by trypsinization of umbilical veins as previously described (67). EC were plated on fibronectin coated cell-culture dishes and maintained in MCDB/F12 media containing 15% FBS, .009% heparin, and .015% endothelial cell growth supplement. All experiments were carried out using cells between the third and fifth passage. EC were transfected using Targefect reagents (Targeting Systems) according to the manufacturer’s protocol. Unless otherwise indicated, EC were serum-starved for 2 hours prior to
treatment with EGF (16 ng/ml), TRAP (100 µM), or thrombin (5U/ml). siRNA targeting STAT3 (s743), GSK-3α (s6237), and GSK-3β (s6241) were purchased from Life Technologies. STAT3 3’UTR-targeting siRNA was purchased from Dharmacon (#D-003544-19-0005)

**SDS-Page and Western Blotting.** Total cell lysate from approximately 10^5 EC was resolved using Bis-Tris buffered SDS-PAGE gels ranging from 8-12% depending on the protein of interest. Gels were soaked in protein transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS) and transferred to a PVDF membrane using a BioRad semi-dry transfer cell. After transfer, PVDF membranes were washed briefly in TBST (150 mM NaCl, 50 mM Tris-HCl, pH7.5, 0.1% Tween-20) and then blocked for 2 hours in protein free TBST blocking buffer (Pierce). After blocking, primary antibodies were diluted 1:1000 in blocking buffer and incubated overnight at 4°C. Membranes were washed and then incubated with horseradish peroxidase (HRP) conjugated secondary antibody for one hour and HRP signals were detected by chemiluminescence. Antibodies for the following targets were purchased from Cell Signaling Technology (catalog numbers in parentheses): STAT3 (#9139), STAT3 (#4904), pTyr705 STAT3 (# 9131), pTyr705 STAT3 (# 9138), pTyr705 STAT3 (# 9145), PMpSP Motif (#2325), pThr-Pro Motif (#9391), GSK-3α/β (#5676), GAPDH (#5174), Mcl1 (# 5453). Anti-α-Tubulin (#T5168) antibody was purchased from Sigma Aldrich. Anti-pSer^727^ STAT3 antibody (sc-136193) was purchased from Santa Cruz Biotechnology.
**Immunoprecipitation.** EC were washed once with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 5 mM Na$_3$VO$_4$) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche). Lysis was allowed to proceed for 30 minutes at 4°C under gentle agitation. Lysates were cleared by high-speed centrifugation and primary antibodies were added for overnight incubation. Antibody complexes were precipitated with protein A/G-agarose beads, washed three times with ice cold RIPA buffer, and denatured with 2x Laemmli buffer for SDS-PAGE.

**EGR1 Promoter Luciferase Reporter and Luciferase Assay.** Luciferase reporter assays were performed using a 2.1 kb fragment of the EGR1 promoter as previously described (268). Briefly, luciferase assays were performed by transfecting EC with EGR1-Promoter reporter 24 hours prior to treatment. Cells were lysed with passive lysis buffer (Promega) and luciferase activity was assayed using the luciferase assay system (Promega) according to the manufacturer’s instructions.

**Quantitative Mass Spectrometry.** After immunoprecipitation with an anti-STAT3 antibody (CST #9139), an ~86 kDa band was cut from Coomassie-stained gel, digested with trypsin, and analyzed by capillary column LC-MS/MS to identify phosphopeptides. The LC-MS/MS system were a Finnigan LTQ linear ion trap mass spectrometer and a LTQ-Orbitrap Elite system. The HPLC column was a self-packed 9 cm x 75 µm id Phenomenex Jupiter C18 (LTQ) or a Dionex 15 cm x 75 µm id Acclaim Pepmap C18 reverse phase capillary column (Orbitrap). The digest was analyzed in both
a survey manner and a targeted manner. The survey experiments were performed using
the data dependent multitask capability of the instrument acquiring full scan mass spectra
to determine peptide molecular weights and product ion spectra to determine amino acid
sequence in successive instrument scans. These data were analyzed by using all CID
spectra collected in the experiment to search the human reference sequence database with
the search program Mascot. The targeted experiments involve the analysis of specific
STAT3 peptides including the phosphorylated and unmodified forms of the Y705, S727,
and T714 tryptic peptides. The chromatograms for these peptides were plotted based on
known fragmentation patterns and the peak areas of these chromatograms were used to
determine the extent of phosphorylation.

Protein Extraction from Tumor Tissue. Tumor tissue was procured from fresh-
frozen nephrectomies and an experienced kidney pathologist confirmed ccRCC or pRCC
diagnosis by histology. ~100 mg samples of tumor tissue were solubilized in RIPA buffer
supplemented with protease and phosphatase inhibitors using a dounce homogenizer, and
lysates were cleared once by high-speed centrifugation, tumbled end-over end overnight,
cleared again by centrifugation, after which STAT3 was immunoprecipitated using an
anti-STAT3 monoclonal antibody.

Development of Polyclonal Anti-Sera for STAT3 Thr^{714} Phosphorylation. Anti-STAT3 P-Thr^{714} polyclonal anti-sera was generated by immunizing a rabbit with a
phosphorylated peptide corresponding to phosphorylation at that site (KFICVpTPTTC).
In vitro kinase assays. Recombinant STAT3 was purchased from Abcam (ab64310) and active GSK-3β was purchased from New England Biolabs (NEB P6040) and utilized according to the manufacturer’s instructions. Briefly, STAT3 was incubated with GSK-3β in ATP-supplemented kinase buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl2, 5 mM DTT) for 1 hour at 30°C. For immune complex phosphorylation assays, recombinant WT STAT3, T714A STAT3, and S727A STAT3 was expressed in EC by transient transfection of STAT3 cDNAs. Prior to lysis, EC were serum starved for 2 hours to reduce STAT3 basal phosphorylation. EC were lysed and STAT3 was immunopurified using an anti-STAT3 monoclonal antibody and protein A/G beads. Beads were washed with RIPA buffer and resuspended in kinase buffer supplemented with ATP in the absence or presence of GSK-3β for 1 hour at 30°C. Reactions were terminated with 2x SDS buffer and analyzed by SDS-PAGE and immunoblot.

Chromatin Immunoprecipitation. Chromatin immunoprecipitations (ChIP) were performed using a commercially available kit according to the manufacturer’s instructions (Millipore, #17-295). Human EC treated with EGF plus TRAP or IL-6 for 15 minutes were cross-linked by the addition of formaldehyde. Cross-linked EC were lysed in an SDS lysis buffer and sonicated using a sonicator 3000 (Misonix Inc, Farmingdale, NY). Chromatin-protein complexes were immunoprecipitated overnight with an anti-STAT3 antibody, and genomic DNA was purified using DNA PrepEase columns (Affymetrix). Purified genomic DNA was analyzed by quantitative real-time PCR using primers flanking the upstream GAS element in the EGR1 promoter (268); forward primer: 5’-CAGGAGGAGCCTCCCTCCCG-3’; reverse primer: 5’-
CTGGGGCCGAACGCAACAG-3’. Data were normalized relative to the amount of input DNA for each sample and expressed as the fold change relative to the untreated control sample.

**StatisticalAnalyses.** Unless otherwise indicated, data are expressed as mean +/- SEM. Differences between groups were analyzed by analysis of variance (ANOVA). Bonferroni post-hoc tests were performed to calculate multiplicity adjusted p-values and evaluate pairwise differences between groups. Differences between tumor and normal tissue were evaluated by two-tailed paired t-tests. Significance is expressed as: *,p<0.05; **,p<0.01; ***,p<0.001.

### 3.4 Results

**Multiple STAT3 phosphoforms are induced by combinatorial EGFR/PAR-1 signaling in endothelial cells.**

We previously showed that GSK-3α/β participate in STAT3 activation downstream of simultaneous EGFR/PAR-1 signaling by positively regulating STAT3 Ser\(^{727}\) phosphorylation (Figure 46). STAT3 Thr\(^{714}\) is located in an evolutionarily conserved region that includes conservation of proline in the +1 position, raising the possibility that it is a direct substrate of GSK-3α/β. We therefore examined Thr\(^{714}\) and Ser\(^{727}\) phosphorylation in EC by quantitative mass spectrometry (Table I). STAT3 was immunoprecipitated from EC treated with EGF plus thrombin receptor agonist peptide (TRAP), a PAR-1 activator. Immunoprecipitates were resolved by SDS-PAGE and ~86 kDa protein bands were cut from the gel for LC-MS/MS analysis. We readily detected
phosphorylation of STAT3 at Thr\textsuperscript{714} or Ser\textsuperscript{727} (Figure 47). We also identified STAT3 phosphopeptides that were phosphorylated at both Thr\textsuperscript{714} and Ser\textsuperscript{727} (Figure 48). Surprisingly, we failed to detect STAT3 Tyr\textsuperscript{705} phosphopeptides by mass spectrometry under these conditions (Table I).

![Figure 46](image)

**Figure 46. STAT3 is a potential GSK-3α/β substrate.** Coincident PAR-1 activation prevents EGF-induced inhibition of GSK-3α/β by AKT, thus allowing GSK-3α/β to positively regulate Ser\textsuperscript{727} phosphorylation (268). As proline-directed kinases, GSK-3α/β may directly phosphorylate both Thr\textsuperscript{714} and Ser\textsuperscript{727}.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Sequence</th>
<th>Phospho-Site</th>
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</tr>
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<tbody>
<tr>
<td>2362</td>
<td>FIGVIPTTCSNIDMLEmO3PR</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>2466</td>
<td>FIGVIPTTCSNIDLEM03PR</td>
<td>pT714</td>
<td>+</td>
</tr>
<tr>
<td>2406</td>
<td>FIGVIPTTCSNITDLPM03PR</td>
<td>pS727</td>
<td>+</td>
</tr>
<tr>
<td>2486</td>
<td>FIGVIPTTCSNITDLPM03PR</td>
<td>pT714,pS727</td>
<td>+</td>
</tr>
<tr>
<td>2566</td>
<td>FIGVIPTTCSNITDLPEmO3PR</td>
<td>+3PO\textsubscript{3}</td>
<td>—</td>
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<tr>
<td>2502</td>
<td>YQRESQHEPAGSAAPYLNK</td>
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<tr>
<td>2582</td>
<td>YQRESQHEPAGSAAPYLNK+PO\textsubscript{3}</td>
<td>pY705</td>
<td>—</td>
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**Table I. List of peptides targeted by LC-MS/MS.** Serum-starved EC were treated with EGF plus TRAP for 15 min., after which STAT3 was immunoprecipitated, and STAT3 phosphorylation was quantified by selected reaction monitoring targeting the specific STAT3 peptides listed in the table.
Figure 47. Identification of singly phosphorylated STAT3 Thr\textsuperscript{714} and Ser\textsuperscript{727}. The CID spectrum of the singly phosphorylated form of the T710-729 STAT3 peptide, FICVTPTCSNTIDLPMoSPR. This spectra is consistent with the presence of two different forms of the peptide. The y\textsubscript{5} ion at 683.3 Da is consistent with phosphorylation at S727. The presence of an unmodified y\textsubscript{15} ion at 1705 Da and only a modified y\textsubscript{16} ion at 1886 Da indicates that a second isoform with modification at T714 is also present.

Figure 48. Identification of STAT3 phosphorylated at both Thr\textsuperscript{714} and Ser\textsuperscript{727}. The CID spectrum of the doubly phosphorylated form of the T710-729 STAT3 peptide, FICVTPTCSNTIDLPMoPR. The identification of the C-terminal y\textsubscript{5} ion at 683 Da is consistent with phosphorylation at Ser\textsuperscript{727}. In addition, the mass difference between the y\textsubscript{16} and y\textsubscript{15} ions is 181 Da and is consistent with the second site of phosphorylation occurring at Thr\textsuperscript{714}. 
Differential induction of STAT3 phosphoforms by EGFR/PAR-1 signaling.

We next sought to characterize signaling mechanisms regulating the inducible formation of STAT3 phosphoforms. EC were stimulated with EGF plus TRAP for up to one hour, STAT3 was immunoprecipitated and resolved by SDS-PAGE (Figure 49), and the kinetics with which STAT3 phosphoforms are induced were analyzed by LC-MS/MS. Singly phosphorylated Thr$^{714}$ or Ser$^{727}$ phosphoforms are induced as early as 5 minutes following EGF plus TRAP treatment, and reach a maximum at ~15 minutes post-treatment (Figure 50, red and blue). Levels of Thr$^{714}$-phosphorylated STAT3 and Ser$^{727}$-phosphorylated STAT3 begin to decrease after 15 minutes and stabilize at ~50% of their maximum levels between 30-60 minutes after stimulation. Doubly phosphorylated STAT3 exists in much lower abundance than singly modified phosphoforms (Figure 50, green), but demonstrates the highest degree of stimulus-dependent inducibility (Figure 50). Further, the level of doubly phosphorylated STAT3 remains elevated up to 30 minutes after stimulus exposure, after which it returns to ~5 fold of the basal level (Figure 50).

Figure 49. Immunoprecipitation of STAT3 for timecourse LC-MS/MS experiment. STAT3 was immunoprecipitated from the pooled extracts and resolved by SDS-PAGE. An ~86 kDa band corresponding to Coomassie-stained STAT3 was cut from the gel, digested with trypsin, and subjected to LC-MS/MS and selected reaction monitoring (SRM) to quantify specific STAT3 phosphoforms. This method allowed independently treated replicates to yield sufficient endogenous STAT3 for LC-MS/MS analysis.
Figure 50. Quantitative analysis of STAT3 phosphoforms in response to EGF plus TRAP. (Left) Serum-starved EC were treated with EGF and TRAP for the indicated times, after which they were lysed in RIPA buffer, and STAT3 was immunoprecipitated for mass spectrometry analysis as described in materials and methods. 3 independently-treated 100 mm dishes were pooled and used for each point indicated on the graph. % Phosphorylation was calculated as the abundance of a specific phosphoform divided by the abundance of all STAT3 tryptic peptides at that site. (Right) Data presented as fold-change relative to basal levels for each specific STAT3 phosphoform. Curve fit analyses were performed using a two-phase exponential model.

To determine the extent to which STAT3 phosphoforms are regulated by EGFR/PAR-1 crosstalk, EC were treated with EGF, TRAP, or both for 15 minutes (Figure 51) and STAT3 phosphoforms were measured by LC-MS/MS. Singly phosphorylated Thr<sup>714</sup> STAT3 was moderately induced by either EGF or TRAP alone, and combination treatment caused an almost perfectly additive increase in pThr<sup>714</sup> STAT3 abundance (Figure 52). Singly phosphorylated Ser<sup>727</sup> STAT3 was also moderately induced by either EGF or TRAP alone, and was synergistically induced by combination treatment with EGF plus TRAP (Figure 52). Doubly phosphorylated STAT3 was undetectable during unstimulated conditions, and stimulation with either EGF or TRAP caused a minimal increase in the abundance of this phosphoform (Figure 52). Remarkably, combination treatment with EGF plus TRAP increased levels doubly phosphorylated STAT3 in a manner that was strongly synergistic compared to the response with either EGF or TRAP alone (Figure 52).
Figure 51. Immunoprecipitation of STAT3 for LC-MS/MS analysis after combination treatment with EGF and TRAP. Coomassie-stained polyacrylamide gel of STAT3 immunoprecipitates after treatment with EGF (16 ng/ml), TRAP (100 µM), or both for 15 minutes.

Figure 52. Quantitative analysis of STAT3 phosphoform abundance during combinatorial EGFR/PAR-1 signaling. Serum-starved EC treated with EGF, TRAP, or both for 15 minutes and processed for quantitative mass spectrometry of Thr<sup>714</sup> phosphorylation (blue), Ser<sup>727</sup> phosphorylation (red), and doubly phosphorylated STAT3 (green) as in Figure 50. The synergism ratio for individual STAT3 phosphoforms was calculated as: Response<sub>EGF+TRAP</sub>/(Response<sub>EGF</sub>+Response<sub>TRAP</sub>).
EGF plus TRAP-induced STAT3 phosphorylation is distinct from IL-6-induced phosphorylation.

Next, we sought to validate our mass spectrometry results for Thr$^{714}$, Ser$^{727}$, and Tyr$^{705}$ phosphorylation. STAT3 was immunoprecipitated from HUVEC and various phospho-specific antibodies were used to assess the levels of distinct STAT3 phosphorylations. To investigate stimulus-inducible Thr$^{714}$ phosphorylation by immunoblot, a polyclonal STAT3 anti-pThr$^{714}$ antibody was generated by immunizing a rabbit with a phosphopeptide corresponding to the STAT3 residues at that phosphosite. STAT3 Thr$^{714}$ phosphorylation was synergistically induced in response to 15 minute EGF plus TRAP treatment (Figure 53). Ser$^{727}$ phosphorylation was also synergistically induced when STAT3 immunoprecipitates were probed with an anti Pro-Met-pSer-Pro motif (PMpSP) antibody that is designed to recognize phosphorylated serine in the MAP kinase consensus motif corresponding to Ser$^{727}$ (Figure 53). We previously showed that a commercially available pSer$^{727}$ STAT3 antibody recognizes synergistically induced Ser$^{727}$ phosphorylation in a manner consistent with mass spectrometry results after 5 minute stimulation with EGF plus TRAP (268). However, after 15 minute stimulation, the commercial antibody does not reflect the $\sim$8-10 fold induction of Ser$^{727}$ phosphorylation that occurs (as indicated by mass spectrometry and PMpSP antibody), possibly due to the increased abundance of cooperating modifications near that site (Figure 53). Therefore, in subsequent experiments, we used immunoprecipitation and anti-PMpSP immunoblots to assess Ser$^{727}$ phosphorylation.
Figure 53. Antibody standardization for synergistic Thr$^{714}$ and Ser$^{727}$ phosphorylation. Serum-starved EC were treated with EGF, TRAP, or both for 15 minutes, and STAT3 was immunoprecipitated and resolved by SDS-PAGE. STAT3 phosphorylations were assessed by immunoblot. The anti-pThr$^{714}$ antibody was generated by immunizing a rabbit with a phosphorylated peptide corresponding to phosphorylation at that site (KFICVpTPTTC). STAT3 Ser$^{727}$ phosphorylation was detected using a Pro-Met-phospho-Ser-Pro motif antibody and a commercially available antibody from Santa Cruz Biotechnology. Densitometry analysis of Thr$^{714}$ and Ser$^{727}$ phosphorylation was performed from 3 independent experiments.

We next sought to standardize immunoblotting methods for detecting multiple STAT3 phosphorylations. Our mass spectrometry results suggested that phosphorylation of STAT3 Tyr$^{705}$ is low or absent in EC. To validate this result, we performed a dose-response experiment using increasing levels of EGF in the presence of TRAP and analyzed Tyr$^{705}$ phosphorylation by immunoblot using three commercially available antibodies (Figure 54). Tyr$^{705}$ phosphorylation was strongly induced by IL-6, but not by EGF plus TRAP, even at high EGF concentrations. Basal Tyr$^{705}$ phosphorylation was detectable upon overexposure of the film, suggesting that this modification occurs basally at very low abundance in EC. A commercially available pSer$^{727}$ antibody did not detect a strong EGF plus TRAP induced phosphorylation at Ser$^{727}$, suggesting that there is a loss of affinity of the antibody for phosphorylated STAT3 at this timepoint (Figure 54). EGF plus TRAP stimulation increased Thr$^{714}$ phosphorylation and PMpSP phosphorylation (Ser$^{727}$) of STAT3 at all concentrations (Figure 54).
**Figure 54. Dose-response analysis of STAT3 Tyr\(^{705}\) phosphorylation.** Serum-starved EC were treated with EGF at the indicated concentration in the presence of TRAP for 15 minutes. EC were lysed with RIPA buffer and STAT3 was immunoprecipitated and resolved by SDS-PAGE. Three antibodies (Ab.) were used to assess Tyr\(^{705}\) phosphorylation. Ab. 1 is Cell Signaling Technology (CST) #9131, Ab. 2 is CST #9145, and Ab. 3 is CST #9138. The immunoblot with CST #9138 was deliberately overexposed to film to detect basal STAT3 Tyr\(^{705}\) phosphorylation. Thr\(^{714}\) and Ser\(^{727}\) phosphorylation were assessed as in Figure 53. PMpSP antibody is used to target Ser\(^{727}\) phosphorylation, as the pSer\(^{727}\) antibody (Santa Cruz) does not recognize phosphorylated Ser\(^{727}\) in a manner consistent with mass spectrometry data at this timepoint. IL-6 (10 ng/ml) was included as a positive control for Tyr\(^{705}\) phosphorylation.

**GSK-3α/β phosphorylate STAT3 to generate a doubly modified phosphoform.**

We previously reported that GSK-3α/β positively regulated STAT3 Ser\(^{727}\) phosphorylation and STAT3-dependent gene expression in EC (268). Therefore, we investigated the extent to which GSK-3α/β regulates the stimulus-inducible generation of specific STAT3 phosphoforms downstream of EGFR/PAR-1 signaling. Depletion of GSK-3α/β by RNAi caused a 90% decrease in the levels of inducible doubly phosphorylated STAT3, while causing only a partial reduction in single pThr\(^{714}\) or pSer\(^{727}\) STAT3 (Figure 55). This result was verified by immunoblotting STAT3 immunoprecipitates using a STAT3 pThr\(^{714}\) antibody (Figure 56) and by anti-pThr-Pro
and Pro-Met-pSer-Pro motif antibodies to detect the levels of pThr\textsuperscript{714} and pSer\textsuperscript{727}, respectively (Figure 57). In all cases, GSK-3\textalpha/\textbeta depletion caused a reduction in total pThr\textsuperscript{714} and pSer\textsuperscript{727}, demonstrating GSK-3\textalpha/\textbeta positively regulate STAT3 Ser/Thr phosphorylation. Moreover, this result demonstrates that there are both GSK-3\textalpha/\textbeta-dependent and GSK-3\textalpha/\textbeta-independent components of STAT3 Thr\textsuperscript{714} and Ser\textsuperscript{727} phosphorylation, and GSK-3\textalpha/\textbeta are required for generating doubly phosphorylated STAT3 in this context.

Figure 55. GSK-3\textalpha/\textbeta-dependent generation of STAT3 phosphoforms. GSK-3\textalpha/\textbeta were depleted from EC by RNAi. 48 hours after siRNA transfection, EC were stimulated with EGF plus TRAP for 15 minutes and STAT3 phosphoform abundance was analyzed by mass spectrometry. pThr\textsuperscript{714} is indicated by blue bars, pSer\textsuperscript{727} by red bars, and pThr\textsuperscript{714}/pSer\textsuperscript{727} by green bars.
Figure 56. **Immunoblot analysis of GSK-3-dependent STAT3 Thr$^{714}$ phosphorylation.** GSK-3α/β were depleted from EC by RNAi, and the effect on EGF+TRAP-induced STAT3 Thr$^{714}$ phosphorylation was analyzed by immunoblots of STAT3 immunoprecipitates using anti-STAT3 pThr$^{714}$ polyclonal anti-sera.

Figure 57. **Immunoblot analysis of GSK-3-dependent STAT3 serine/threonine phosphorylation.** GSK-3α/β were depleted from EC by RNAi, and the effect on EGF+TRAP-induced STAT3 Thr$^{714}$ phosphorylation was analyzed by immunoblots of STAT3 immunoprecipitates using anti-Thr-Pro and anti-Pro-Met-pSer-Pro motif antibodies. The amino acid sequence under the immunoblot represents STAT3 710-729 and indicates the sites targeted for immunodetection. Densitometry values represent quantification of 3 independent immunoblots.
GSK-3β efficiently phosphorylated STAT3 in vitro at a pThr-Pro site (Figure 58). To test the ability of GSK-3β to phosphorylate STAT3 at Thr$^{714}$ and Ser$^{727}$, we immunopurified WT STAT3, S727A STAT3, and T714A STAT3 for use as in vitro GSK-3β substrates. GSK-3β efficiently phosphorylated both Thr$^{714}$ and Ser$^{727}$ (Figure 59). Mutation of Ser$^{727}$ to alanine dramatically reduced the ability of GSK-3β to phosphorylate Thr$^{714}$. In contrast, mutation of Thr$^{714}$ to alanine did not affect Ser$^{727}$ phosphorylation by GSK-3β (Figure 59), suggesting that Ser$^{727}$ may regulate priming or processive phosphorylation of STAT3 Thr$^{714}$ by GSK-3α/β.

![Figure 58. GSK-3β directly phosphorylates STAT3.](image)

*Figure 58. GSK-3β directly phosphorylates STAT3. In vitro kinase assay using recombinant GSK-3β (New England Biolabs) and STAT3 (Abcam) substrate was performed for 1 hour at 30°C, and threonine phosphorylation was detected using a pThr-Pro motif antibody.*
Figure 59. Phosphorylation of STAT3 Thr$^{714}$ by GSK-3β requires Ser$^{727}$. Endogenous STAT3 was depleted by RNAi targeting the 3'UTR of STAT3, and recombinant WT-STAT3, S727A STAT3, or T714A STAT3 was immunopurified from serum-starved EC and used as an in vitro substrate for GSK-3β. The amino acid sequence under the immunoblot represents STAT3 710-729 and indicates the sites targeted for immunodetection.

STAT3 phosphorylation at Thr$^{714}$ by GSK-3α/β positively regulates target gene expression.

We previously showed that simultaneous EGFR/PAR-1 activation causes synergistic induction of EGR1 in a manner that requires STAT3 Ser$^{727}$ phosphorylation (268). To identify established STAT3 targets that are regulated by EGFR/PAR-1 signaling, we examined Bcl2 family members that are known to require STAT3 Ser$^{727}$ phosphorylation, including Mcl1 (187). Indeed, we found that EGF plus TRAP maximally induced Mcl1 at two hours post-treatment (Figure 60), and this induction was synergistic compared to treatment with either EGF or TRAP alone (Figure 61).
Figure 60. Timecourse of Mcl1 induction in response to EGF plus TRAP. Serum-starved EC were treated with EGF plus TRAP over a timecourse of up to 8 hours and inducible Mcl1 expression was analyzed by immunoblot.

Figure 61. Synergistic induction of Mcl1 in response to EGF plus TRAP. Serum starved EC were treated for 2 hours with EGF, TRAP, or both and Mcl1 expression was analyzed by immunoblot. Densitometry analysis (right) represents the quantification of 3 independent immunoblots.

Depletion of STAT3 by RNAi completely inhibited synergistic induction of Mcl1 (Figure 62), demonstrating that STAT3 is critical for EGFR/PAR-1 crosstalk, consistent with our previously published results for EGR1 induction (268). Inducible expression of Mcl1 was reduced ~50% when GSK-3α/β were depleted by RNAi, consistent with our hypothesis that GSK-3α/β positively regulate STAT3 activity (Figure 63).
Figure 62. **Synergistic induction of Mcl1 requires STAT3.** STAT3 was depleted by RNAi and EC were treated with EGF, TRAP, or both for two hours and Mcl1 expression was analyzed by immunoblot.

Figure 63. **GSK-3α/β are required for Mcl1 induction by EGF plus TRAP.** GSK-3α/β were depleted from EC by RNAi, and the effect on Mcl1 induction was analyzed by immunoblot. Densitometry values represent the quantification of 4 independent experiments.
Further, WT STAT3, but not T714A, S727A, or T714A/S727A STAT3 mutants, rescued EGF plus TRAP induced Mcl1 expression when endogenous STAT3 was depleted by RNAi (Figure 64). In contrast, a STAT3 Y705F mutant lacking the canonical phosphorylation site rescued Mcl1 induction to a similar extent as WT STAT3, suggesting that combinatorial activation of STAT3 by EGFR/PAR-1 is Tyr\textsuperscript{705}-independent (Figure 65).

**Figure 64. STAT3 Thr\textsuperscript{714} and Ser\textsuperscript{727} are required for Mcl1 induction in response to EGF plus TRAP.** Endogenous STAT3 was depleted by RNAi with siRNA targeting 3’UTR, and STAT3 levels were reconstituted by transient expression of recombinant WT, T714A, S727A, or T714A/S727A STAT3, and the effect on Mcl1 expression was analyzed by immunoblot. Densitometry values represent quantification of 6 independent experiments for the GFP/control, GFP/siSTAT3, WT/siSTAT3, and T714A/siSTAT3 and 3 experiments for S727A and T714A/S727A STAT3. Statistical analyses were performed using a one-way ANOVA. Multiplicity adjusted p-values were used to determine significant differences from WT STAT3 rescue.
Simultaneous EGFR/PAR-1 signaling induces STAT3 binding to a distal GAS element in the EGR1 promoter.

We next sought to compare combinatorial activation of STAT3 by EGF plus TRAP to canonical activation of STAT3 by IFNγ or IL-6. Simultaneous EGFR/PAR-1 activation strongly induced STAT3 Thr-Pro and Thr\textsuperscript{714} phosphorylation at 15 minutes (Figure 66). A PMpSP motif antibody (Ser\textsuperscript{727}) detected phosphorylated STAT3 Ser\textsuperscript{727} in a manner largely consistent with data from mass spectrometry experiments (Figure 66). Neither IL-6 nor IFNγ induced a significant degree of STAT3 threonine phosphorylation. IL-6 and IFN-γ, but not EGF plus TRAP, strongly induced STAT3 Tyr\textsuperscript{705} phosphorylation, suggesting that STAT3-dependent gene expression can be triggered by EGF plus TRAP in the absence of Tyr\textsuperscript{705} phosphorylation. Further, we previously showed that an upstream GAS element in the EGR1 promoter was critical for EGF plus TRAP induced promoter activation (268). EGF plus TRAP, but not IL-6, induced STAT3 binding to this upstream promoter region (Figure 67).
Figure 66. EGF plus TRAP induced STAT3 phosphorylation is distinct from IL-6 or IFNγ-induced phosphorylation. Serum-starved EC were treated with EGF plus TRAP, IFNγ (10 ng/ml), or IL-6 (10 ng/ml) for 15 min, after which STAT3 was immunoprecipitated and phosphorylations were analyzed by immunoblot.

Figure 67. EGF plus TRAP treatment induces STAT3 binding to the EGR1 promoter. EC were stimulated for 15 min with EGF plus TRAP or IL-6, after which STAT3 was immunoprecipitated from cross-linked cell extracts. DNA was isolated from immunoprecipitates and EGR1 promoter was PCR amplified using forward and reverse primers flanking the upstream GAS element of the EGR1 promoter. Binding of STAT3 to EGR1 promoter was verified by DNA sequencing of PCR amplicons.
Additionally, when EC were cotransfected with an *EGR1* promoter reporter, *EGR1* promoter activation was significantly reduced by cotransfection with T714A STAT3 relative to WT STAT3 (Figure 68). Coupled with our previous results, these data demonstrate that both Thr<sup>714</sup> and Ser<sup>727</sup> are required for maximal *EGR1* promoter activation (268). Overexpression of a phosphomimetic T714D STAT3 mutant was not sufficient to increase basal *EGR1* promoter activity relative to WT STAT3 (Figure 68), suggesting that other modifications and signaling pathways are required. To test the requirement of Tyr<sup>705</sup> in *EGR1* promoter activation, we overexpressed WT STAT3 or Y705F STAT3 in STAT3-depleted EC and measured *EGR1* promoter activity in response to EGF plus TRAP. EGF plus TRAP induced promoter activation was similar for WT STAT3 and Y705F STAT3 (Figure 69).

![Figure 68](image)

**Figure 68. STAT3 Thr<sup>714</sup> phosphorylation is required but not sufficient for *EGR1* promoter activation.** (Left) EC were co-transfected with an *EGR1* promoter reporter and WT-STAT3 or T714A STAT3. EGF+TRAP induced luciferase activity was measured after 4 hour treatment using a luminometer. Data represent the values from 5 independent experiments. Endogenous STAT3 was depleted by siRNA targeting the 3’UTR of STAT3 in all conditions. (Right) EC were co-transfected with an *EGR1* promoter reporter and WT-STAT3 or T714D STAT3. Basal luciferase activity was measured after 8 serum-starvation using a luminometer. Data represent the mean +/- SEM from 3 independent experiments.
Doubly phosphorylated STAT3 is elevated in human renal tumors.

Preclinical studies of renal cell carcinoma (RCC) have validated both STAT3 and GSK-3α/β as targetable molecules for the therapeutic inhibition of RCC progression (209, 270–272). Additionally, aberrant nuclear accumulation of GSK-3β has been reported to occur in >90% of human RCC cases. Based on these studies and our discovery of a direct link between GSK-3α/β and STAT3 Thr\(^{714}\) phosphorylation, we hypothesized that doubly phosphorylated STAT3 may be elevated in renal tumors. We analyzed STAT3 PTMs in a cohort of 4 clear cell RCC (ccRCC) and 4 papillary RCC (pRCC) patients to determine the relative degree of STAT3 Thr\(^{714}\) phosphorylation in these cases. Thr\(^{714}\) phosphorylation was detectable in all patients by immunoblot, but was remarkably elevated in 2/4 ccRCC cases and 1/4 pRCC cases (Figure 70). STAT3 Ser\(^{727}\)
phosphorylation (anti-PMpSP) was elevated in 4/4 ccRCC cases and 1/4 pRCC cases. STAT3 pTyr$^{705}$ was elevated in 4/4 ccRCC patients and 1/4 pRCC patients.

**Figure 70. Immunoblot analysis of STAT3 phosphorylation in renal cell carcinoma.** Human renal tumor samples (clear cell and papillary) were isolated from tissue after nephrectomy. Tumor tissue was solubilized in RIPA buffer, cleared by high-speed centrifugation, and STAT3 was immunoprecipitated overnight. Half of the immunoprecipitate was analyzed by immunoblot to confirm the presence of STAT3 and its phosphorylations. Recombinant (Rec.) STAT3 was included as a non-phosphorylated reference.
The same cases were analyzed by LC-MS/MS to measure the abundance of STAT3 phosphoforms (Figure 71). STAT3 phosphoform abundance was generally elevated in ccRCC vs. pRCC and levels of pSer727 and pTyr705 were significantly elevated in ccRCC (Figure 71).

![Figure 71. Quantitative LC-MS/MS analysis of STAT3 phosphoform abundance in renal cell carcinoma.](image)

Based on these results, we procured 10 additional cases of ccRCC with matched normal tissue to determine the extent to which STAT3 phosphoform abundance differed in tumor tissue relative to adjacent normal tissue. Singly phosphorylated STAT3 at Thr714 or Ser727 was significantly elevated in tumor tissue relative to adjacent normal tissue (Figure 72). Doubly phosphorylated STAT3 was also significantly more abundant in tumor tissue, indicating that the GSK-3α/β-STAT3 signaling may be active in ccRCC (Figure 72). In contrast, pTyr705 STAT3 displayed a high degree of variability and was not significantly different between normal and tumor tissue (Figure 72).
Figure 72. Quantitative analysis of STAT3 phosphoform abundance in renal tumors vs. normal tissue. 10 cases of ccRCC and patient-matched normal tissue were examined by microscopy to confirm diagnosis and estimate the number of tumor nuclei per field. Tumor tissue and matched tissue controls were solubilized as in Figure 70, and STAT3 was immunoprecipitated to analyze phosphoform abundance by LC-MS/MS. Two-tailed paired t-tests were used to evaluate statistical significance.

To examine the relative contribution of cancer cells vs. stromal cells to tumor-associated STAT3 phosphorylation, we examined the extent to which STAT3 phosphoforms correlated with the percentage of tumor nuclei in ccRCC cases. pThr$^{714}$ and doubly phosphorylated STAT3 exhibited a positive correlative trend vs. the percentage of tumor nuclei in each sample, but this relationship was not statistically significant (Figure 73). Neither pSer$^{727}$ nor pTyr$^{705}$ correlated with the percentage of tumor nuclei (Figure 73). These data clearly demonstrate that Thr$^{714}$ and Ser$^{727}$-phosphorylated STAT3 are elevated in renal tumor tissue, suggesting that GSK-3α/β-STAT3 signaling is a novel targetable signaling axis in ccRCC.
Figure 73. Correlation analysis of STAT3 phosphoform abundance vs. percentage of tumor nuclei in tumor samples. An experienced kidney pathologist estimated the percentage of tumor nuclei per field. Pearson correlation coefficients were calculated based on percentage of tumor nuclei (x-axis) vs. phosphoform abundance (y-axis). Pearson r-values and two-tailed p-values for correlation with tumor nuclei were: p Thr\(^{714}\) (r = .37, p = .29); p Ser\(^{727}\) (r = -.42, p = .23); Double p STAT3 (r = .44, p = .20); p Tyr\(^{705}\) (r = -.38, p = .28).

3.5 Discussion

The transcriptional activity of STAT3 is modulated by reversible PTMs that regulate dimerization, DNA-binding, and protein-protein interactions. Activated STAT3, in turn, transcriptionally activates genes that promote growth and survival, and is thought to be a major driver of solid tumor progression. We now report that STAT3 Thr\(^{714}\) is phosphorylated in addition to Ser\(^{727}\) in response to EGF signaling during coincident GPCR activation, and both of these modifications are critical for STAT3-dependent expression of Mcl1 and EGR1 (Figure 64, 68). Therefore, the data herein strongly suggest that EGR1 and Mcl1 induction is driven by a low abundant but dynamically regulated STAT3 phosphoform that is simultaneously modified at Thr\(^{714}\) and Ser\(^{727}\), likely in the absence of Tyr\(^{705}\) phosphorylation (Table I, Figure 48, 54, 66). Further, our data demonstrate that GSK-3α/β exclusively mediate the generation of doubly phosphorylated STAT3 to induce Mcl1 and EGR1 during coincident EGFR/PAR-1 activation (Figure 55, 59). The additive response of p Thr\(^{714}\) STAT3 to EGF plus TRAP (Figure 52), and the observation that T714A does not affect Ser\(^{727}\) phosphorylation...
(Figure 59), suggests that there is little to no conversion of pThr$^{714}$ STAT3 to doubly phosphorylated STAT3 in this context (Figure 74). We therefore propose that doubly phosphorylated STAT3 mediates a noncanonical mechanism of STAT3 activation that is triggered specifically by combinatorial EGFR/PAR-1 signaling and is aberrantly regulated in human renal tumors (Figure 74).

Figure 74. Model of GSK-3α/β-dependent STAT3 phosphorylation. Model showing the inducible flux of STAT3 phosphoforms stimulated with different combinations of EGF plus TRAP. Simultaneous EGFR/PAR-1 activation is required to drive significant formation of doubly phosphorylated STAT3 and STAT3-dependent gene expression in this context. Furthermore, GSK-3α/β are required for nearly all of the doubly phosphorylated STAT3 generated following EGFR/PAR-1 activation (red panel). During individual activation of EGFR or PAR-1, or in the absence of GSK-3α/β, STAT3 is inducibly phosphorylated at Thr$^{714}$ or Ser$^{727}$ (blue panel). Numerous Ser$^{727}$ kinases are known, but there likely exists additional proline-directed Thr$^{714}$ kinases that catalyze single phosphorylation of Thr$^{714}$ in the absence of GSK-3α/β and Ser$^{727}$ phosphorylation.
STAT3 Thr$^{714}$ phosphorylation adds to the high frequency of PTMs within a relatively small region of the STAT3 COOH terminus. Between Lys$^{679}$ and Ser$^{727}$, researchers have identified acetylation of Lys$^{679}$, Lys$^{685}$, Lys$^{707}$, Lys$^{709}$ (166–169), and phosphorylation at Tyr$^{705}$, Thr$^{714}$, and Ser$^{727}$ (123, 159). The mechanisms by which canonical STAT3 modifications (pTyr$^{705}$ and pSer$^{727}$) regulate STAT3 function are relatively clear. However, the exact mechanisms by which lysine acetylation/methylation and threonine phosphorylation regulate transcriptional activity of STAT3 are not well understood, and more work is required to understand the structural basis for promoter-specific functions of individual STAT3 PTMs. In any event, the diversity and abundance of STAT3 PTMs that is now reported, coupled with the established functions of unphosphorylated STAT3 (U-STAT3) (174, 175, 181), demonstrate that Tyr$^{705}$ phosphorylation is neither necessary nor sufficient for STAT3 activation in a number of signaling contexts (175, 179, 181, 186, 188, 210). Further, STAT1/3 phosphorylations at Tyr$^{701/705}$ and Ser$^{727}$ are often mediated by the same kinases, as the sequences surrounding these residues are highly similar. However, the amino acids surrounding Ser$^{708}$ in STAT1 and Thr$^{714}$ in STAT3 are unique, suggesting a possible basis for the differential activation of STAT1/3 via regulation of distinct proximal kinases. In support of this notion, others have shown that IKKe phosphorylates STAT1 Ser$^{708}$ to regulate anti-viral immunity (273, 274), and we now present evidence that STAT3 Thr$^{714}$ is mediated by GSK-3α/β to positively regulate Mcl1 and EGR1 expression.

It is known that STAT3 can dimerize in the absence of Tyr$^{705}$ phosphorylation (174). Furthermore, recent studies using both atomic force microscopy and x-ray
crystallography have directly visualized U-STAT3 binding to GAS elements as an unphosphorylated dimer (175, 176). Our results suggest that STAT3 binds to an upstream GAS element in the EGR1 promoter in the absence of Tyr$^{705}$ phosphorylation. Interestingly, others have shown that oncostatin M induces STAT3 Tyr$^{705}$ phosphorylation and STAT3 binding to the EGR1 promoter within 30 minutes of stimulation (275). However, the same study also showed that IFN$\gamma$ strongly induces STAT3 Tyr$^{705}$ phosphorylation but does not elicit STAT3 binding to the EGR1 promoter, consistent with our results for IL-6 (Figure 66, 67). Although direct promoter binding of Tyr$^{705}$-phosphorylated STAT3 was not tested in that study, our results raise the possibility that canonical and noncanonical mechanisms of STAT3 activation converge on common GAS elements to regulate transcription. This is in agreement with structural studies that suggest U-STAT3 and Tyr$^{705}$-phosphorylated STAT3 bind to the same DNA elements (176, 276).

The identification of doubly phosphorylated STAT3 in human RCC indicates that the GSK-3α/β-STAT3 axis may be active in a subset of human tumors. The multi-kinase inhibitors sorafenib and sunitinib are approved for use in RCC patients, and it is thought that their major mechanism of action is through the inhibition of angiogenesis (277, 278). Sorafenib and sunitinib effectively prolong progression free survival (279, 280), but resistance to these drugs invariably limits their efficacy, and mechanisms of resistance are poorly defined (281). It was recently reported that sorafenib treatment increased GSK-3β activity in RCC cells, and that combination therapy of sorafenib plus GSK-3α/β inhibitors synergistically inhibited xenograft tumor growth in a mouse model of RCC.
(272). Additionally, aberrant nuclear accumulation of GSK-3β is reported to occur in >90% of RCC cases (271), and an immunohistochemistry analysis of renal tumors showed that tyrosine phosphorylated nuclear STAT3 is present in approximately 60% of ccRCC and pRCC cases (208). Importantly, a STAT3 inhibitor reduced tumor-associated angiogenesis in a mouse model of RCC, and sunitinib-induced RCC cell apoptosis is thought to be mediated in part via inactivation of STAT3 (209, 270).

The previous studies of STAT3 in RCC emphasized the role of pTyr705 as an indicator of STAT3 activation in renal tumors. Our results suggest that Tyr705 phosphorylation is not significantly elevated in ccRCC, and Thr714 and Ser727 phosphorylation may be reliable predictors of GSK-3α/β-STAT3 signaling in this disease. Similar results for STAT3 have been reported in peripheral blood cells of patients with chronic lymphocytic leukemia (CLL). Haza-Halavy et al. reported that STAT3 is constitutively phosphorylated at Ser727, but not Tyr705, in this disease (186). Furthermore, Ser727-phosphorylated STAT3 that was isolated from peripheral blood cells of CLL patients bound DNA in the absence of Tyr705 phosphorylation (186). STAT3 also facilitates Ras-dependent malignant transformation in a Tyr705-independent manner by localizing to mitochondria and regulating the activity of the electron transport chain (282). Yet another study used a phosphomimetic STAT3 mutant in which Ser727 was substituted with glutamate (S727E) to show that Ser727 phosphorylation increases prostate cancer cell tumorigenicity and invasive capacity (188). Importantly, a STAT3 double mutant (Y705F/S727E) behaved similarly to the S727E condition, suggesting that the tumorigenic effects of STAT3 Ser727 phosphorylation are Tyr705-independent in a prostate
cancer cell line (188). Our own results demonstrate that singly phosphorylated Ser\textsuperscript{727} STAT3 is more abundant than doubly phosphorylated Thr\textsuperscript{714}/Ser\textsuperscript{727} STAT3 in EC and ccRCC. One possible explanation for this difference is that Ser\textsuperscript{727}-phosphorylated STAT3 exists in higher abundance due to its dual role as a transcriptional regulator and modulator of oxidative respiration.

Our results describe a novel mechanism of signal integration in which combinatorial EGFR/PAR-1 signaling regulates a GSK-3α/β-STAT3 signaling axis. Direct, multisite phosphorylation of STAT3 by GSK-3α/β therefore serves as the molecular conduit through which temporal information of coincident receptor activation is transduced to regulate gene expression in a strict combinatorial manner. This is the first report that Thr\textsuperscript{714} phosphorylation regulates STAT3 function, and the data herein reveal a new opportunity for the therapeutic inhibition of STAT3 transcriptional activity downstream of an oncogenic receptor tyrosine kinase.
CHAPTER IV

GENERAL DISCUSSION AND FUTURE DIRECTIONS

We have discovered a novel mechanism of STAT3 activation that is mediated by direct phosphorylation of STAT3 at Ser\textsuperscript{727} and Thr\textsuperscript{714} by GSK-3α/β. This mechanism is triggered in endothelial cells when EGFR and PAR-1 are simultaneously activated. Further, it appears that the temporal spacing of EGFR and PAR-1 activation is “sensed,” in part, via the opposed actions of EGFR and PAR-1 on the PI3K-AKT signaling axis. EGFR signaling strongly activates AKT to inhibit GSK-3α/β-STAT3 signaling, but concurrent PAR-1 activation prevents AKT-dependent inhibition of GSK-3α/β, thereby permitting GSK-3α/β-mediated phosphorylation of STAT3 and STAT3-dependent gene expression. Overall, our results reveal that biologically consequential signaling axes may not be evident from simply studying “one ligand, one receptor” pathways, and careful
consideration should be paid to the nuances of signaling interactions elicited by simultaneously exposing cells to disparate extracellular stimuli.

Figure 75. Combinatorial activation of STAT3 by EGF and thrombin. Simultaneous activation of EGFR/PAR-1 is required for STAT3-dependent immediate early gene expression. EGFR signaling activates the PI3K-AKT signaling axis causing AKT-dependent phosphorylation and inhibition of GSK-3α/β. Coincident PAR-1 signaling inhibits AKT activation in a Gα12/13-dependent manner, preventing GSK-3α/β phosphorylation. GSK-3α/β phosphorylate STAT3 at Thr\(^{714}\) and Ser\(^{727}\) to generate a doubly modified STAT3 phosphoform. Phosphorylation of both Thr\(^{714}\) and Ser\(^{727}\) are required for induction of Mcl1 and EGR1 in this context. Furthermore, STAT3 Tyr\(^{705}\) phosphorylation is low or absent in response to concentrations of EGF that elicit gene induction, and is not required for STAT3-dependent gene induction in response to EGF plus TRAP.

The expression of EGFR on endothelial cells has been disputed, as discussed in chapter II. We invariably observe EGFR expression in HUVEC, and the data herein demonstrate conclusively that HUVEC respond to EGF. The expression of EGFR in primary endothelial cells may vary between culture conditions after isolation and passage. However, given that others and we have demonstrated EGFR expression in HUVEC, and others have shown that EGFR is expressed in MVEC, the role of
endothelial EGFR warrants further investigation to more fully characterize its physiological significance.

Anti-VEGF therapy is commonly used to inhibit pathological angiogenesis. Drugs such as bevacizumab (Avastin ®) and aflibercept (Eyelea ™) act as VEGF-A scavengers, and are used to inhibit tumor-associated angiogenesis and to treat age-related macular degeneration (283). Although it is clear that VEGF is a potent stimulator of angiogenesis, it is possible, if not likely, that EGF acts as a functionally redundant growth factor in angiogenic microenvironments, thereby bypassing the requirement of VEGF for neovascularization. Indeed, EGF stimulates angiogenesis to the same degree as VEGF in the mouse cornea, suggesting that endothelial EGFR signaling may play an important role in this process (140). It is well established that anti-EGFR drugs inhibit tumor associated angiogenesis, but the scientific consensus has been that this effect is an indirect consequence of decreased VEGF production in cancer cells which causes endothelial cell apoptosis within the tumor (284–288). Recent results from our laboratory and others’ suggest that anti-EGFR therapies likely have direct effects on endothelial cell function (84, 140, 141, 289), and may exert anti-angiogenic responses independent of the inhibitors’ actions on tumor cells (140).

GPCR-RTK signaling interactions have long been studied in the context of “inside-out” transactivation (Figure 5). Our results do not conflict with these reports, but they raise important questions regarding the specificity of RTK signaling following direct activation vs. transactivation. Coincident GPCR signaling from receptors like PAR-1 may
modulate RTK-dependent pathways such that transactivated RTK signaling is distinct from direct activation of the RTK per se. In the case of EGFR and PAR-1, PAR-1 activation drastically inhibits the ability of EGFR to activate AKT (Figure 28). Others have reported a similar phenomenon in the context of insulin receptor (IR) crosstalk with angiotensin II-activated signaling pathways (290). Maeno et al. reported that activation of PKC by angiotensin II led to phosphorylation and inhibition of PI3K, thus preventing insulin-dependent activation of AKT in endothelial cells (290). Our results suggest that PAR-1 coupling to Gα12/13 is critical for inhibition of EGF-induced AKT activation, and others have shown that angiotensin II also couples to Gα12 and Gα13 (291). It is therefore interesting to consider the possibility that PKC activation downstream of PAR-1 and Gα12/13 may be responsible for modulating PI3K-AKT activation in the context of both EGFR/PAR-1 and IR/angiotensin II crosstalk.

The activation of GSK-3α/β-STAT3 signaling requires the simultaneous presence of extracellular EGF and thrombin. EGF and thrombin likely cooperate in the microenvironment of diseases such as atherosclerosis and advanced cancers. For example, it is known that infiltrating immune cells deliver significant amounts of EGF and other growth factors to the hypoxic microenvironment of solid tumors (292, 293). Macrophages are believed to promote tumor progression and metastasis in part via the synthesis and secretion of growth factors including EGF (294). Thrombin and EGF-family ligands are also found in atherosclerotic lesions in humans (295, 296), and receptors for both thrombin and EGF are expressed on multiple cell types associated with atherogenesis including smooth muscle cells, endothelial cells, and macrophages.
Furthermore, efficient wound healing following tissue injury requires localized inflammation, cellular migration, and proliferation. Both thrombin and EGF family ligands are generated at sites of injury (297, 298), and neovascularization is a critical step for the regeneration of functional tissues (299). It is possible that EGFR/PAR-1 crosstalk controls endothelial activation following tissue injury to enhance the rate of wound healing in a STAT3-dependent manner. Future studies in our laboratory will therefore investigate the role of GSK-3α/β-STAT3 signaling in a mouse model of wound healing, and evaluate the extent to which administration of EGF and TRAP may therapeutically enhance wound closure.

We have focused on the cooperation of EGFR and PAR-1 to trigger noncanonical STAT3 activity. However, it is important to note that our results may not be limited to these specific receptors. GPCRs are the most abundant transmembrane receptor in the human genome (86), and many GPCRs couple to similar sets of heterotrimeric G-proteins. It is possible that other receptors also synergize with EGFR by activating STAT3 in a GSK-3α/β-dependent manner. In support of this idea, we previously showed that LPA also induces synergistic expression of MKP-1 in endothelial cells treated with EGF (84). Future work in our laboratory will therefore investigate other potential GPCRs that activate STAT3 in cooperation with EGFR. Further, we will investigate the specific structural and functional properties of GPCRs that confer the ability to modulate EGF-activated PI3K-AKT and STAT3 pathways.
Our results have revealed a mechanism of STAT3 activation that likely occurs in the absence of STAT3 Tyr\textsuperscript{705} phosphorylation. Others have shown numerous ways in which STAT3 functions in the absence of tyrosine phosphorylation including DNA-binding, dimerization, and activating gene transcription (175, 176, 179). We have shown that STAT3 binds to an upstream GAS element in the EGR1 promoter, but we have also discovered that GSK-3α/β is a targetable kinase to inhibit STAT3-dependent gene expression in this context. It is important to note that although our initial results suggest a moderate increase in STAT3 Tyr\textsuperscript{705} phosphorylation in response to EGF, these experiments were performed with high concentrations of EGF (50 ng/ml). At lower concentrations (16 ng/ml), EGF treatment still synergizes with PAR-1 and induces STAT3 Thr\textsuperscript{714} and Ser\textsuperscript{727} phosphorylation in a GSK-3α/β-dependent manner. However, STAT3 Tyr\textsuperscript{705} phosphorylation is undetectable by mass spectrometry at this lower EGF concentration, and western blot analysis of STAT3 immunoprecipitates shows no EGF plus TRAP-dependent increase in STAT3 Tyr\textsuperscript{705} phosphorylation (Figure 54). Furthermore, a STAT3 Y705F mutant induced Mcl1 expression and EGR1 promoter activation to the same extent as WT STAT3. Collectively, these results suggest that the GSK-3α/β-STAT3 signaling pathway can be triggered at EGF concentrations that do not induce STAT3 Tyr\textsuperscript{705} phosphorylation, demonstrating that activation of STAT3-dependent transcription is likely Tyr\textsuperscript{705}-independent in this context.

A major implication of our results is that targeting GSK-3α/β may selectively inhibit the actions of doubly phosphorylated STAT3. This may be desirable in pathological conditions when GSK-3α/β-STAT3 signaling is dysregulated. We do not yet
know the extent to which this pathway functions *in vivo*, but the observation that doubly phosphorylated STAT3 is significantly elevated in clear cell renal cell carcinoma suggests that GSK-3α/β-STAT3 signaling may be aberrantly activated in the microenvironment of renal tumors. This is particularly important because previous studies have shown that both GSK-3α/β and STAT3 positively regulate ccRCC progression (208, 209, 270–272). The GSK-3α/β-STAT3 pathway that we have described is unique in the sense that its actions generate a specific STAT3 phosphoform that appears to be distinct from canonical pathways. We therefore speculate that the levels of doubly phosphorylated STAT3 could be used to infer the relative activity of this GSK-3α/β-STAT3 signaling pathway in solid tumors. Future work in our laboratory will therefore aim to characterize the role of endothelial STAT3 in animal models of vascular diseases and cancer. This work will serve as the basis for evaluating the roles of doubly phosphorylated STAT3 in human physiology and disease.

Higher order information processing mechanisms like “coincidence detection” are critical processes that allow cells to regulate discrete biological responses under a wide range of environmental conditions (300). Our results show that the temporal spacing of EGFR and PAR-1 activation has profound effects on endothelial cell phenotypes up to 16 hours after stimulus exposure. Remarkably, the information of simultaneous receptor activation is processed rapidly, integrated via opposed actions of EGFR and PAR-1 on the PI3K-AKT signaling axis, and ultimately encoded in a distinct STAT3 phosphoform that increases the expression of STAT3 target genes in endothelial cells.
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Beeson, K., Busam, D., Carver, a, Center, a, Cheng, M. L., Curry, L., Danaher, S., 
Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., 
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D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., 
Koduru, S., Love, a, Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, 
I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., 
Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhfel, B., 
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Tint, N. N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., 
Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J. F., Guigó, 
R., Campbell, M. J., Sjolander, K. V, Karlak, B., Kejariwal, a, Mi, H., Lazareva, 
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Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., 
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Y. H., Coyne, M., Dahlke, C., Mays, a, Dombroski, M., Donnelly, M., Ely, D., 
Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, a, 
Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., 
Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kashka, J., Kagan, L., Kraft, C., 
Levitsky, a, Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., 
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APPENDIX
A. SUPPLEMENTAL LIST OF REAGENTS FOR CHAPTER II

Table II. Candidate STAT3 binding sites in the EGR1 promoter.

STAT3 Consensus Sequence:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location†</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>EGR1</td>
<td>1605-1597</td>
<td>TTCCCGGAA</td>
</tr>
<tr>
<td>EGR1</td>
<td>239-231</td>
<td>TTCCCCGAA</td>
</tr>
<tr>
<td>EGR3</td>
<td>1525-1517</td>
<td>TTCCCCGAA</td>
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<tr>
<td>MKP-1</td>
<td>1495-1486</td>
<td>TTCTAGAA</td>
</tr>
<tr>
<td>c-Fos</td>
<td>1801-1793</td>
<td>TTCCACGAA</td>
</tr>
<tr>
<td>c-Fos</td>
<td>289-298</td>
<td>TTCCCGTCAA</td>
</tr>
<tr>
<td>COX-2</td>
<td>1386-1378</td>
<td>TTCTGTAA</td>
</tr>
<tr>
<td>COX-2</td>
<td>754-746</td>
<td>TTCCAAGAA</td>
</tr>
<tr>
<td>IL-8</td>
<td>1740-1732</td>
<td>TTCTGTAA</td>
</tr>
<tr>
<td>IL-8</td>
<td>484-476</td>
<td>TTCTAGAA</td>
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†Basepairs upstream from transcription initiation site.
Table III. Phosphokinase signaling array densitometry data.

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<tr>
<th>Kinase</th>
<th>EGF</th>
<th>TRAP</th>
<th>EGF+TRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt (S473)</td>
<td>7.50</td>
<td>1.00</td>
<td>2.30</td>
</tr>
<tr>
<td>Akt (T308)</td>
<td>1.20</td>
<td>1.25</td>
<td>1.55</td>
</tr>
<tr>
<td>AMPK alpha1 (T172)</td>
<td>0.73</td>
<td>1.39</td>
<td>1.20</td>
</tr>
<tr>
<td>AMPK alpha2 (T172)</td>
<td>1.02</td>
<td>1.20</td>
<td>1.39</td>
</tr>
<tr>
<td>beta-Catenin</td>
<td>0.22</td>
<td>1.12</td>
<td>0.48</td>
</tr>
<tr>
<td>Chk-2 (T68)</td>
<td>1.01</td>
<td>1.31</td>
<td>1.45</td>
</tr>
<tr>
<td>c-Jun (S93)</td>
<td>2.28</td>
<td>1.63</td>
<td>1.62</td>
</tr>
<tr>
<td>CREB (S133)</td>
<td>7.83</td>
<td>7.67</td>
<td>11.02</td>
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<tr>
<td>eNOS (S177)</td>
<td>1.69</td>
<td>0.84</td>
<td>0.92</td>
</tr>
<tr>
<td>ERK1/2 (T202Y204, T185Y187)</td>
<td>11.21</td>
<td>1.44</td>
<td>9.56</td>
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<tr>
<td>FAK (Y397)</td>
<td>0.94</td>
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<td>1.77</td>
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<tr>
<td>Fgr (Y412)</td>
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<td>1.46</td>
<td>1.64</td>
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<tr>
<td>Fyn (Y420)</td>
<td>1.31</td>
<td>1.85</td>
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<tr>
<td>GSK-3 alpha/beta (S21/S9)</td>
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<td>1.00</td>
<td>0.57</td>
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<tr>
<td>Hck (Y411)</td>
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<td>1.61</td>
<td>1.88</td>
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<td>HSP27 (S78/S82)</td>
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<td>1.92</td>
<td>2.23</td>
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<td>JNK pan (T183Y185, T221Y223)</td>
<td>2.61</td>
<td>1.11</td>
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<tr>
<td>Lck (Y394)</td>
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<td>1.58</td>
<td>1.11</td>
</tr>
<tr>
<td>Lyn (Y397)</td>
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<td>1.97</td>
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<tr>
<td>MEK1/2 (S218/S222, S222/S226)</td>
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<td>2.18</td>
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<td>p27 (T157)</td>
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<td>3.03</td>
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<td>1.82</td>
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<td>p53 (S46)</td>
<td>1.13</td>
<td>1.46</td>
<td>1.73</td>
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<tr>
<td>p70 S6 Kinase (T229)</td>
<td>1.45</td>
<td>1.24</td>
<td>1.69</td>
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<tr>
<td>p70 S6 Kinase (T389)</td>
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<td>5.37</td>
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<td>1.12</td>
<td>2.24</td>
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<td>Paxillin (Y118)</td>
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<td>2.09</td>
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<td>Pyk2 (Y402)</td>
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<td>1.91</td>
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<td>RSK1/2 (S221)</td>
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<td>3.18</td>
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Table IV. List of antibodies used in Chapter II experiments.

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<td>phospho-STAT3 S727</td>
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<td>phospho-STAT3 S727</td>
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<td>sc-136193</td>
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Table V. Real-time PCR primers used for gene expression studies in Chapter II.

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<th>mRNA</th>
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<td>EGR1</td>
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<td>c-Fos</td>
<td>CGGACCTGGCGTGCTCAGT</td>
<td>CGGGCTGACCAGGCAGCT</td>
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<td>GAPDH</td>
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Table VI. List of small molecule inhibitors used in Chapter II.

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Table VII. Commercial reagents and kits used in Chapter II experiments.

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<td>TRAP-6 Peptide</td>
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<td>Gαq siRNA</td>
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<td>Gα12 siRNA</td>
<td>Ambion</td>
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<td>Gα13 siRNA</td>
<td>Ambion</td>
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<td>GSK-3β siRNA</td>
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<tr>
<td>Luciferase Assay System</td>
<td>Promega</td>
<td>E1501</td>
</tr>
<tr>
<td>TransAM STAT3 DNA Binding Assay</td>
<td>Active Motif</td>
<td>45196</td>
</tr>
</tbody>
</table>
Table VIII. List of primers used for EGR1 promoter cloning and site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GGTACCACGCGTACAACCTCGGTAGACAGTGGG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GGTACCAGATCTGGGAACACTGAGAAGCTG</td>
</tr>
<tr>
<td>Distal Mutant Forward</td>
<td>GGAGCCTTCCCAGCGCCAGCCCCAATCCCTGTTCC</td>
</tr>
<tr>
<td>Distal Mutant Reverse</td>
<td>GGAACAGGGATTCCGGCGCCGGGAAGGCTCC</td>
</tr>
<tr>
<td>Proximal Mutant Forward</td>
<td>GAGCTCTAGGCGCCGCAGCTTGGGCCTGGGATGC</td>
</tr>
<tr>
<td>Proximal Mutant Reverse</td>
<td>GCATCCAGGCAGCAGGTGTGGGGCCTAGAGGTC</td>
</tr>
</tbody>
</table>