THE ROLE OF JAK2 TYROSINE KINASE IN REGULATING ANGIOTENSIN II-MEDIATED CELLULAR TRANSCRIPTION

By

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This dissertation is dedicated to my parents, for their constant love, support, and wisdom.

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Jak2 tyrosine kinase is activated by angiotensin II (AngII) via the AT_1 receptor. This activation has been implicated in the progression of various cardiovascular disease states. Unfortunately, the precise downstream targets of Jak2, when activated via the AT_1 receptor, remain elusive.

Here, we used gene-profiling technology to determine AngII-inducible genes that require Jak2 for their regulation. Specifically, microarray experiments compared *jak2*-/- cells with wild type cells and identified over 400 AngII-inducible genes as being differentially expressed as a function of Jak2. Two specific gene targets that were identified and then further investigated in this study were the serum glucocorticoid-regulated kinase and the inositol 1,4,5 trisphosphate (IP_3) receptor.

Serum glucocorticoid-regulated kinase (*sgk1)* maintains proper Na^+ homeostasis in the kidney, and therefore is an important regulator of blood pressure. Here, the data show that *sgk1* mRNA and protein levels are increased by AngII treatment in Jak2-expressing

cells. Conversely, we did not observe a significant increase of *sgk1* expression in Jak2-deficient cells. Furthermore, when the *sgk1* promoter was transfected into cells, only cells expressing Jak2 protein had an increase in *sgk1* promoter activity when treated with AngII. We hypothesize that upon activation via the AT_1 receptor, Jak2 initiates a Jak/STAT signaling cascade that results in transcription of *sgk1*. This hypothesis was supported by evidence of STAT proteins binding within the promoter region of *sgk1*.

The IP_3 receptor was another gene identified by the microarray as regulated by Jak2. As opposed to the transcriptional effects of Jak2 on *sgk1* mRNA regulation, these studies suggest that Jak2 is regulating the IP_3 receptor protein through direct signaling cascades within the cytosol. Specifically, the data show that Jak2 activation protects the IP_3 receptor from rapid AngII-induced ubiquitination. Conversely, the loss of a functional Jak2, either by pharmacological inhibition or through the stable expression of a Jak2 dominant negative mutant, causes rapid AngII-induced degradation in vascular smooth muscle cells within 1 hour.

In conclusion, these studies identified many important targets of Jak2 in response to activation by AngII. Identifying the downstream signaling mechanisms of Jak2 may better elucidate its physiological and pathophysiological effects within the cardiovascular system.

CHAPTER 1
INTRODUCTION

When first discovered in the early 1990's, members of the Jak tyrosine kinase family were given the nickname of "Just Another Kinase." Now, nearly 15 years after their identification, it is clear that the Jaks have surpassed expectations, and are anything but "just another kinase". For example, of the four members belonging to the Jak tyrosine kinase family, Jak2 alone is essential for normal development and function. In addition, studies have demonstrated a potential role for Jak2 in the progression of various cancers and cardiovascular pathologies. This chapter will serve as an introduction into the background and functions of Jak2. Furthermore, the specific relationship of angiotensin II (AngII) and Jak2 will be explored as an attempt to link the signaling cascades of Jak2 to vascular diseases associated to the renin-angiotensin system. Lastly, the later chapters of this work identify two novel downstream targets of Jak2 as a function of AngII signaling. As such, the function and regulation of these two genes, the inositol 1,4,5 trisphosphate (IP_3) receptor and the serum glucocorticoid kinase 1 (SGK1), will be introduced.

Jak2 Tyrosine Kinase

History

Tyk2 was the first member of the *Janus* tyrosine kinase family (a.k.a. Just Another Kinase Family) to be discovered (Firmbach-Kraft *et al.,* 1990). It was cloned and identified in 1990 and by 1994 the three other members of the *Janus* family were found (Jak1, Jak2, and Jak3) (Wilks *et al.,* 1991; Harpur *et al.,* 1992; Duhe *et al.,* 1995;

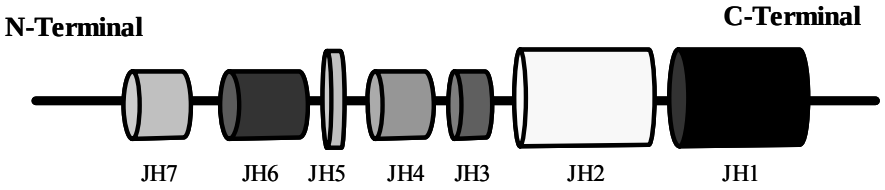
Kawamura *et al.,* 1994; Takahashi and Shirasawa, 1994). The Jaks were unique in that all four members of the family shared a highly conserved C-terminal tyrosine kinase domain that was immediately adjacent to a "kinase-like" or "pseudokinase" domain. These contrasting tandem domains were reminiscent of Janus, the Roman God of Two Faces who is the namesake of the Jaks.

Interest in Jak2 specifically was heightened when Jak2 was discovered to be a critical mediator of cytokine-dependent signaling. Concurrent studies found that Jak2 was activated in response to erythropoietin and growth hormone binding to their obligatory receptors (Argetsinger *et al.,* 1993; Witthuhn *et al.,* 1993). In the years to follow, many additional cytokines and growth factors were associated with the activation of Jak2 (Silvennoinen *et al.,* 1993; Rui *et al.,* 1994; Narazaki *et al.,* 1994; Watling *et al.,* 1993). As the activators of Jak2 were being discovered, simultaneous work identified a correlation between the cytokine-induced activation of Jak2 and increased gene transcription. These studies were the first to identify cytokine-responsive transcription factors, which were termed the Signal Transducers and Activators of Transcription (STAT) proteins (Schindler *et al.,* 1992; Shuai *et al.,* 1992). These latent transcription factors were found to mediate gene transcription when phosphorylated by active Jak2 in the cytoplasm. Thus, within two years of their identification, the basic signaling mechanisms of the Jak/STAT signaling paradigm were uncovered.

Structure

Structurally, the members of the *Janus* Tyrosine Kinase family are highly homologous and relatively large in size, having a mass of roughly 130 kDa. To date, no three-dimensional structure has been obtained but much has been elucidated about Jak2's

structure through analysis of the primary nucleotide and amino acid sequence. Specifically, Jak2 is ubiquitously expressed throughout most tissues and is highly conserved amongst species. Similar to other Jaks, Jak2 contains seven highly conserved Jak homology (JH) domains, termed JH1 through JH7 (Figure 1-1). The carboxyl half of the protein is composed of the JH1 and JH2 regions, which encode the kinase and pseudokinase domains, respectively. The kinase activation loop, which is known to be required for ligand-dependent activation of the Jaks, resides within the JH1 domain. For a long time, the function of the pseudokinase domain remained unresolved. Recent work, however, suggests that specific regions of JH2 interact with JH1 to negatively regulate kinase activity (Saharinen *et al.,* 2000; Saharinen and Silvennoinen, 2002).



Domain	Amino Acid
JH7	38-122
JH6	144-284
JH5	288-309
JH4	322-440
JH3	451-538
JH2	543-824
JH1	836-1123

Figure 1-1 Summary of the Jak2 structural domains. Shown are the seven Jak homology (JH) domains and their relative positions within Jak2. The corresponding amino acid sequence of each domain is also shown.

The amino half of Jak2 contains domains JH3 through JH7. Although the Jak family members are thought to lack a canonical Src Homology 2 (SH2) domain, it was noted that in Tyk2, the second half of the JH4 domain plus the whole of the JH3 domain weakly resembled an SH2 domain (Bernards, 1991). Upon the cloning of the murine

Jak2 cDNA, it was similarly noted that the sequence GLYVLRWS bore weak homology to the core sequence element of SH2 domains (FLVRES) (Harpur *et al.,* 1992). However, studies have reported conflicting findings as to whether this domain is truly functional (Higgins *et al.,* 1996; Kampa and Burnside, 2000; Giordanetto and Kroemer, 2002). Clearly, additional studies are required in order to elucidate what role, if any, this SH2-like domain has within Jak2.

Immediately N-terminal to the putative SH2 domain lies the FERM domain which spans from the middle of the JH4 domain through the JH7 domain. This domain is involved in mediating stable interactions with other cellular proteins (Girault *et al.,* 1998; Yonemura *et al.,* 1998). Furthermore, the amino terminal region of Jak2, especially the JH6 and JH7 domains, has been shown to be crucial for Jak2/cell surface receptor interactions (Frank *et al.,* 1994; Tanner *et al.,* 1995; Zhao *et al*., 1995).

In summary, the early collective molecular dissection of Jak2 suggested that it possessed the appropriate structural features to bind other cellular proteins and phosphorylate those proteins on tyrosine residues.

Jak/STAT Signaling Paradigm

While traditionally the Jak/STAT signaling pathway has been activated via cytokines and growth factors, Jaks are also activated by numerous seven transmembrane receptors, such as the AT_1 receptor.

Cytokine and growth factor receptors

The signaling mechanisms surrounding Jak2 activation and the subsequent regulation of gene transcription have been extensively studied. A summary of the Jak/STAT pathway is depicted in Figure1-2A. As is typically done in literature reviews, this overview uses the activation of Jak2 via a cytokine receptor as an example of how

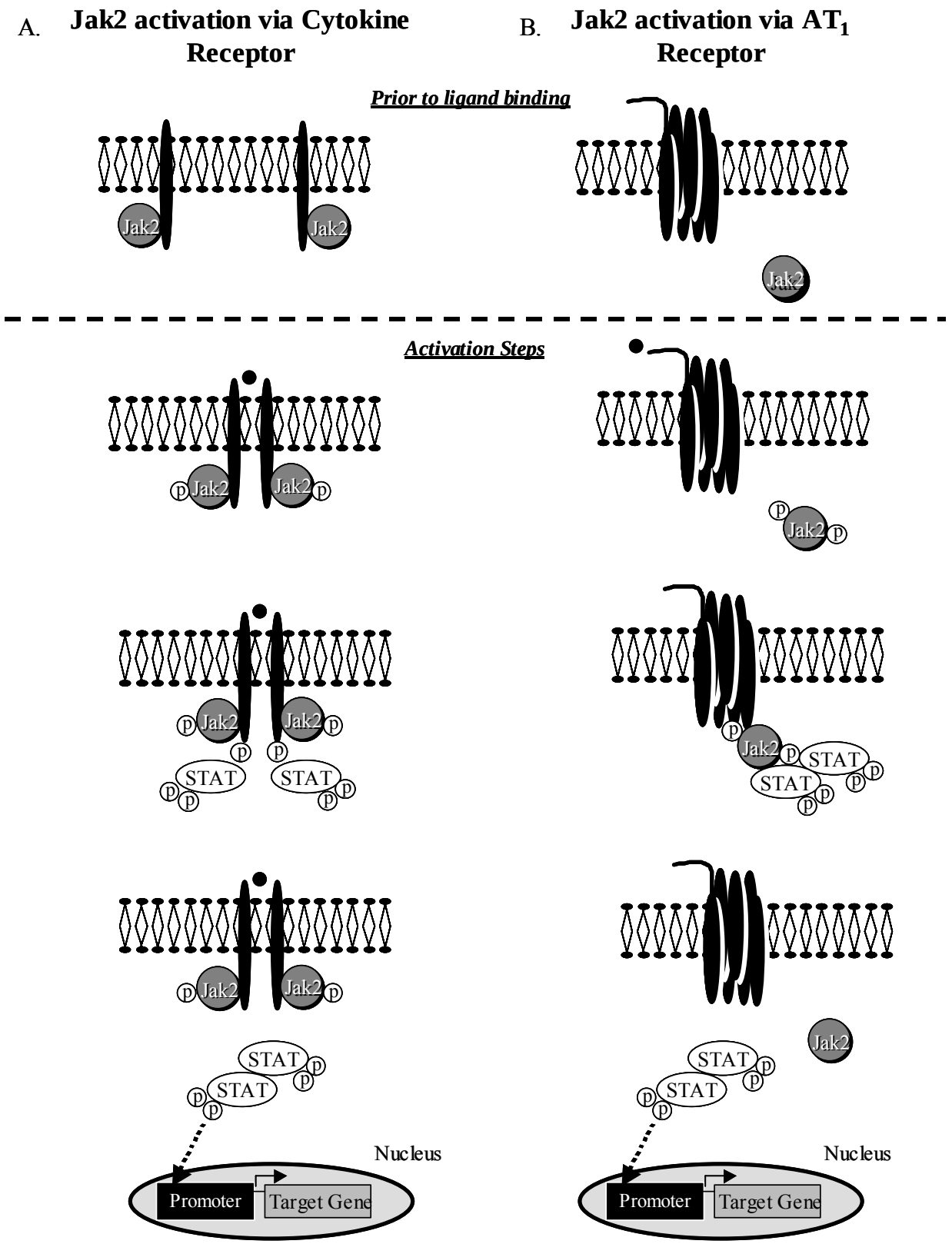


Figure 1-2 Jak/STAT signaling paradigm A) Activation of Jak2 via a cytokine receptor. B) Activation of Jak2 via the AT_1 receptor.

Jak2 acts in a receptor-based signaling paradigm. The first event in the Jak/STAT pathway is ligand binding to its cell surface receptor, resulting in receptor dimerization. The dimerization event triggers a phosphorylation cascade by the receptor that results in the activation of constitutively bound Jak2 molecules. These activated Jak2 molecules subsequently phosphorylate tyrosine residues on the cytosolic tail of the receptor, thereby creating docking sites for the STAT proteins. In turn, STATs are phosphorylated on tyrosine residues by Jak2 and then released from the complex. Phosphorylated homo- or heterodimeric STATs translocate into the nucleus, where they bind to STAT recognition sequences, such as GAS elements, and initiate transcription of specific downstream target genes.

Seven transmembrane spanning receptors

In addition to cytokine receptors, Jak2 is also activated by various seven transmembrane spanning receptors, such as the AT_1 receptor (Marrero *et al.,* 1995). Figure 1-2B illustrates the general differences in activation of Jak2 for a cytokine receptor versus the AT_1 receptor. For the most part, activation of Jak2 via the AT_1 receptor propagates a similar signaling cascade as when Jak2 is activated by cytokines. However, there do exist a number of important differences in the method of Jak2 activation depending on the receptor that is propagating the extracellular signal. First, it appears that Jak2 molecules are not constitutively associated with the AT_1 receptor in the absence of activation, but rather reside unbound within the cytoplasm. Furthermore, Jak2 must be catalytically active and autophosphorylate prior to recruitment to and association with the AT_1 receptor (Ali *et al.,* 1998). To date, the intermediate mechanisms responsible for activation of the Jak2 molecules within the cytoplasm, as well as the mechanisms for recruitment to the receptor, remain to be elucidated. Once activated and

bound to the receptor, Jak2 then acts as a molecular bridge between the receptor and the STAT proteins. Catalytically active Jak2 binds the SH2 domain of the STATs and thereby recruits the STATs to the receptor complex (Ali *et al.,* 2000). From this point, the remainder of the Jak/STAT pathway remains similar to activation via cytokine receptors, resulting in STAT nuclear translocation and transcriptional regulation within the nucleus.

Downstream Targets of Jak2

Jak2 plays an expansive role in the regulation of gene transcription. Given its ability to be activated by such a wide array of ligands, it is not surprising that Jak2 is responsible for the transcriptional regulation of many diverse genes.

Previous work has identified the gene targets of Jak2 in response to activation by one specific agonist, growth hormone (GH). Using a combination of both mutated GH receptors and cells lacking Jak2, it was shown that Jak2 is necessary for GH-dependent STAT activation (Smit *et al.,* 1996; Smit *et al.,* 1997; Han *et al.,* 1996; Hackett *et al.,* 1995). To date**,** GH is known to cause activation of STATs 1, 3, 5A, and 5B in a variety of cell types (Smit *et al.,* 1996). Gene targets of GH-activated STATs 5A and 5B include *spi* 2.1, genes in the *CYP 2/3* families, the acid labile subunit of the circulating insulin-like growth factor-binding protein complex, *insulin 1*, and *igf-1* genes (Ooi *et al.,* 1998; Bergad *et al.,* 1995; Galsgaard *et al.,* 1996; Subramanian *et al.,* 1995; Menton *et al.,* 1999; Waxman *et al.,* 1995; Gebert *et al.,* 1997). Furthermore, STATs 1 and 3 bind to the Sis-Inducible Element (SIE) of the *c-fos* promoter to activate additional genes (Meyer *et al*., 1994; Campbell *et al*., 1995). As clearly demonstrated by GH, Jak2 can mediate a wide array of genes in response to just one ligand. Other Jak2-activating ligands can similarly activate different STATs in the cytosol, leading to a plethora of transcriptional targets.

In addition to mediating transcriptional events through the activation of STAT's, Jak2 can also phosphorylate other cytosolic proteins. For example, when activated via the AT_1 receptor, Jak2 forms a complex with the Src family tyrosine kinase, Fyn (Sayeski *et al.,* 1999a). Following the formation of the complex, Jak2 activates Fyn, thereby resulting in a conformational change within Fyn that permits its kinase domain to become assessable to substrate. To date however, the functional consequence of Jak2-dependent activation of Fyn has not been elucidated.

Fyn is not alone. Many other cytosolic proteins have been found to form a complex with activated Jak2. Some of the molecules that are recruited to Jak2 signaling complexes include c-Src, Grb2, PI3 kinase, Yes, Raf-1, Shc, Syp, and FAK (Sayeski *et al.,* 1999b; Chauhan *et al.,* 1995; Fuhrer and Yang, 1996; Xia *et al.,* 1996; Vanderkuur *et al.,* 1995; Fuhrer *et al.,* 1995; Zhu *et al.,* 1998). In conclusion, there exist various levels of crosstalk between Jak2 and other signaling pathways. Studies have shown that Jak2 coordinates a complicated array of signaling cascades within a cell. Although the precise functional consequences of these signaling complexes have not been elucidated, it is logical to assume that each interaction has a specific biological purpose.

Jak2 and Cardiovascular Disease

Jak2 has been implicated in diverse cardiovascular pathologies, including hypertrophy, heart failure, and ischemia/reperfusion injury. The precise roles of the Jak/STAT pathway in the heart remain elusive and, to some degree, contradictory. For example, some studies have found that the Jak/STAT pathway increases apoptosis within the heart, thereby leading to detrimental effects (Mascareno *et al.,* 2001; Stephanou *et al.,* 2000). However, other studies have conflicted these findings by suggesting a cardioprotective function of Jak2 (Bolli *et al.,* 2001; Hattori *et al.,* 2001; Negoro *et al.,*

2000; Xuan *et al.,* 2001). Needless to say, the association of Jak2 to various cardiovascular insults remains an ongoing area of research.

Cardiac hypertrophy, which is defined as an abnormal increase in cardiac muscle mass, is a major cause of morbidity and mortality worldwide. While cardiac hypertrophy alone has no symptoms, if left untreated it can lead to a number of serious cardiovascular diseases, such as heart failure and myocardial ischemia. The mechanisms governing the development of cardiac hypertrophy are not completely understood. Evidence has accumulated over the years indicating that cardiac hypertrophy is induced by pressure overload (Mann *et al.,* 1989; Sadoshima *et al.,* 1992; Baker *et al.,* 1990) and/or secretion of numerous humoral factors such as Ang II (Schunkert *et al.,* 1995; Sadoshima *et al.,* 1993). Interestingly, many of the stimuli known to initiate hypertrophy have also been shown to activate Jak2. For example, acute pressure overload in rats increases Jak2 tyrosine phosphorylation levels through the paracrine/autocrine secretion of AngII as well as via members of the interleukin-6 family of cytokines (Pan *et al.,* 1997; Pan *et al.,* 1999).

Jak2 has also been associated with cardiac damage typically found in diabetic patients. Diabetes and abnormal glucose tolerance typically lead to diabetic cardiomyopathy, a condition characterized by severe left ventricular hypertrophy (Galderisi *et al.,* 1991; Devereux *et al.,* 2000). As a result, diabetic patients often suffer from heart failure (Kannel *et al.,* 1974). Recent studies suggest that Jak2 contributes to the hypertrophy of ventricular myocytes in response to high glucose levels. Mechanistically, the hyperglycemia enhances AngII generation in myocytes, thereby causing phosphorylation of Jak2. Therefore, these studies suggest that activated Jak2

may contribute to the deleterious growth effects of the heart that are associated with abnormal glucose levels (Modesti *et al.,* 2005).

Additionally, studies have identified a potential role for Jak2 in cardiac injury during ischemia-reperfusion (Mascareno *et al.,* 2001). The first evidence of the Jak/STAT pathway being activated in response to an ischemic event was the identification of STAT3 phosphorylation at 1-24 hours following coronary occlusion in rats. Furthermore, when the Jak2 pharmacological inhibitor, AG490, was administered, the STAT3 phosphorylation was suppressed, indicating a critical role for Jak2 (Negoro *et al.,* 2000). Since then, additional studies have emerged showing that treatment with AG490 leads to a dramatic reduction in cardiac infarct size and a reduction in apoptotic cell death of cardiomyocytes following ischemia-reperfusion in isolated perfused rat hearts (Mascareno *et al.,* 2001). Despite the evidence supporting Jak2's activation in response to ischemia/reperfusion, there remains no elucidation of the functional significance of the activation.

While much still remains to be elucidated about Jak2's functional effects in various cardiovascular insults, it is clear that Jak2 and its downstream targets are activated in a number of disease states. Interestingly, many of the cardiovascular pathologies associated with Jak2 have previously been linked to AngII, suggesting that AngII may signal through Jak2 to elicit its deleterious effects in the cardiovasculature. If so, identification of the downstream targets of Jak2, in response to AngII, may have therapeutic merit in the future.

The Renin Angiotensin System

Angiotensin II

The renin angiotensin system (RAS) is a critical regulator of blood pressure, electrolyte balance, and endocrine function. The majority of these classic actions are mediated through angiotensin II (AngII), the principal effector peptide of the RAS.

The RAS was first discovered over a century ago by Tigerstedt and Bergman, when they identified a pressor compound produced by the kidneys they named renin. Renin is a well-defined aspartyl protease that triggers the conversion of angiotensinogen to the decapeptide angiotensin I. AngII, an octapeptide, is then generated by the cleavage of 2 amino acids from angiotensin I by angiotensin converting enzyme (ACE).

AngII, a potent vasoconstrictor, elicits its effects through G-protein coupled receptors that can be separated into two distinct classes, the type 1 (AT_1) and the type 2 (AT_2) receptors (Timmermans *et al.,* 1993). While both receptors are expressed in cardiovascular tissues, the AT_1 receptor predominates in most organs (Gasc *et al.,* 1994). Generally, the classical effects on vascular tone and fluid homeostasis by the RAS occur via the AT_1 receptor. Conversely, the AT_2 receptor has been found to counterbalance the actions of the AT_1 receptor with respect to blood pressure and cellular proliferation (Horiuchi *et al.,* 1999).

Beyond contributing to maintaining proper blood pressure and electrolyte balance, AngII can additionally lead to the development of various cardiovascular diseases. Amongst these vascular pathologies are primary hypertension, heart failure, neointimal formation, and vascular abnormalities commonly associated with diabetes (Gavras and Gavras, 2002; Kennefick and Anderson, 1997; Barnett, 2001). In recent years, great advancements have been made in controlling the deleterious effects of AngII using

diverse methods such as AT_1 receptor blockade, inhibition of ACE, β-adrenergic receptor blockade, and gene targeting (Timmermans *et al.,* 1999).

AT_1 Receptor

Structure and function

The AT_1 receptor has been extensively studied since its successful cloning in 1991 (Murphy *et al.,* 1991; Sasaki *et al.,* 1991). As is common to all G-protein coupled receptors, the AT_1 receptor is a seven transmembrane spanning receptor. Composed of 359 amino acids, the third intracellular loop is responsible for coupling to G-proteins, and thereby initiates propagation of signaling cascades (Shirai *et al.,* 1995). These classically defined G-protein signaling cascades result in the activation of various intermediate signaling molecules such as adenylate cyclase, phospholipase C and protein kinase C. Interestingly, two AT_1 receptor isoforms exist in rodents, the AT_{1A} and AT_{1B} receptor. These isoforms are highly homologous and are products of separate genes (Iwai *et al.,* 1992). Conversely, only a single isoform of the AT_1 receptor has been confirmed in humans.

Tyrosine kinase signaling cascades

In addition to its classic haemodynamic effects, the AT_1 receptor can also act as a growth factor by activating various tyrosine kinases such as Jak2, Tyk2, c-Src, Fyn, Fak, and Pyk2 (Marrero *et al.,* 1995; Sadoshima and Izumo, 1996; Ishida *et al.,* 1995; Li and Earp, 1997). In pathological conditions, these growth-promoting responses can lead to various vascular diseases, such as neointimal formation.

Currently, Jak2 is the only tyrosine kinase that has been shown to physically associate with the AT_1 receptor directly. As previously described, upon AngII binding to the AT_1 receptor, Jak2 becomes activated within the cytosol. Following this activation,

Jak2 is recruited to the AT_1 receptor and binds to the receptor tail at amino acids 319-322. These four amino acids form the YIPP motif and are necessary for the association of Jak2 to the AT_1 receptor (Ali *et al.,* 1997). Once bound to the receptor, Jak2 acts as a molecular bridge between the receptor and the STAT proteins. Upon the formation of the complex at the AT_1 receptor, Jak2 is able to phosphorylate the STATs. Activated STATs subsequently dimerize and translocate into the nucleus where they regulate transcription of various genes. To date, the downstream target genes of Jak2 activation via the AT_1 receptor remain largely undetermined. Given that Jak2 is activated in a number of AT_1 receptor-induced cardiovascular diseases, we believe that identification of these downstream targets could potentially elucidate the role of Jak2 in the progression of various pathologies.

Inositol 1,4,5 Trisphosphate (IP_3) Receptor

The finely tuned regulation of cytosolic calcium is responsible for many essential biological processes within the cell. The specific control of intracellular calcium is achieved through the involvement and maintenance of various receptors, transporters, pumps, and binding proteins**.** The inositol 1,4,5 trisphosphate (IP_3) receptors are intracellular calcium channels expressed on the membrane of the endoplasmic reticulum (ER). When activated, the IP_3 receptors undergo a conformational change that leads to the release of calcium from internal stores within the ER. The sudden increase in cytosolic calcium within the cell can cause a wide array of biological processes such as muscle contraction, cellular secretion, metabolism, cell growth and differentiation. As such, understanding proper function and regulation of the IP_3 receptors can be very useful in determining their contribution to various pathophysiological conditions.

Structure and Function

Three structurally distinct IP_3 receptor isoforms have been identified and are differentially expressed in a cell specific manner (Nakagawa *et al.,* 1991). Of the three subtypes, Type 1 was the first to be cloned and has the highest expression throughout all cell types studied (Mignery *et al.,* 1989; Furuichi *et al.,* 1989; De Smedt *et al.,* 1994). Types 2 and 3 have lower expression levels overall, typically showing highest expression in many non-neural cell types (De Smedt *et al.,* 1994; Wojcikiewicz, 1995). The widespread expression of these receptors underscores their important role in cellular signaling. However, little is known of the functional differences between the isoforms.

The IP_3 family of receptors exists as tetramers and is composed of 3 main domains; a C-terminal channel region, a large regulatory domain, and an N-terminal IP_3 binding domain (Mignery *et al.,* 1990). The channel region of the IP_3 receptor is characterized by six-membrane-spanning helices with the C-terminus extending into the cytoplasm. When IP_3 binds within the binding domain at the N-terminal end of the receptor, the receptor undergoes a conformational change that regulates the gating of the channel, allowing the rapid release of calcium from internal stores (Mignery *et al.,* 1990; Yoshida and Imai, 1997; Patel *et al.,* 1999).

IP_3 is a second messenger produced through the stimulation of phosholipase C-β (PLC-β)-coupled receptors, such as the AT_1 receptor. Specifically, the binding of ligand to a G-protein-linked receptor activates the plasma-membrane-bound enzyme PLC-β. Activated PLC-β then causes the hydrolysis of the membrane-bound phosphatidylinositol 4,5-bisphosphate, thereby generating two cleaved products: diacylglycerol and IP_3. Following its production, IP_3 leaves the plasma membrane and rapidly diffuses through

the cytosol. Once at the membrane of the ER, IP_3 binds to and opens the IP_3 receptors, resulting in a rapid release of calcium into the cytoplasm (Berridge, 1993).

Regulation of the IP_3 Receptor

Maintaining precise regulation of calcium signaling within a cell is critical for normal cellular functions. Regulation of calcium is maintained via a complex interplay between changes in IP_3 concentration and the various levels of IP_3 receptor expression on the membrane of the ER. Amongst the various regulatory processes that mediate receptor expression is phosphorylation. The IP_3 receptor is phosphorylated by multiple kinases including cyclic-AMP-dependent protein kinase (PKA), protein kinase C (PKC), and Fyn tyrosine kinase (Ferris *et al.,* 1991a, 1991b; Jayaraman *et al.,* 1996; Harnick *et al.,* 1995).

Fyn is a member of the Src-family of tyrosine kinases. Studies investigating calcium signaling during lymphocyte activation identified the IP_3 receptor as a target of phosphorylation by Fyn in T lymphocytes. These initial studies identified that Fyn activated the IP_3-gated calcium channel *in vitro*. Furthermore, it was determined that *fyn*-/- mice demonstrate a significant reduction in tyrosine phosphorylation of the IP_3 receptor during T cell activation (Jayaraman *et al.,* 1996). Recently, studies determined that the precise residue within the receptor that is phosphorylated by Fyn is tyrosine 353 (Y^{353}), which is found within the IP_3 binding domain of the receptor. Furthermore, evidence suggests that the phosphorylation of Y^{353} via Fyn increases the binding affinity of IP_3 to its receptor at low concentrations of IP_3. However, the effect of Y^{353} phosphorylation in response to ligand treatment (i.e., high IP_3 levels) has not yet been defined (Cui *et al.,* 2004).

In addition to phosphorylation, the IP_3 receptor is also regulated through degradation events that reduce expression of the receptor from the membrane of the ER

following agonist treatment. The IP_3 receptor is degraded in WB liver cells in response to AngII. This degradation event occurs at a minimum of six hours with maximal degradation at 24 hours (Bokkala and Joseph, 1997). To date, AngII-mediated degradation of the IP_3 receptor has not been shown in smooth muscle cells (Taylor *et al.,* 1996; Sipma *et al.,* 1998).

Serum- and Glucocorticoid-Regulated Kinase 1 (SGK1)

Aldosterone is a key regulator of Na^+ balance and thereby plays a large role in the regulation of blood pressure. Aldosterone mediates sodium reabsorption by increasing the activity of the epithelial Na^+ channel (ENaC) in the aldosterone-sensitive distal nephron (ASDN). Aldosterone is unable to regulate ENaC directly however, and thereby elicits its effects through transcriptional events. An exciting development in the elucidation of aldosterone targets was the identification of serum-glucocorticoid-induced kinase 1 (*sgk1*) (Webster *et al.,* 1993a). Not long after its identification as a glucocorticoid-induced gene, *sgk1* was recognized to be an aldosterone-induced early response gene and has provided researchers the link between aldosterone action and ENaC regulation (Chen *et al.,* 1999, Naray-Fejes-Toth *et al.,* 1999).

Background and Function

sgk1 was initially identified as a gene whose transcription was rapidly induced by glucocorticoids in rat mammary tumor cells (Webster et al , 1993a). Therefore, unlike other kinases that are constitutively present in cells and are activated by post-translational mechanisms, *sgk1* and its other family members are rapidly transactivated in response to specific hormonal and environmental stimuli. *sgk1* induction therefore requires new transcription. *sgk1* is a member of the AGC subfamily of serine/threonine protein kinases. To date, three isoforms of *sgk* have been identified, *sgk1, sgk2*, and *sgk3*;

however, only sgk*1* is responsive to aldosterone or glucocorticoids at the transcriptional level. Transcriptional regulation of *sgk1* via aldosterone occurs through a classic canonical pentadecamer, *cis*-acting steroid response element found in the promoter region of *sgk1* (Webster *et al.,* 1993b). Of the members of the AGC subfamily, SGK1 most resembles protein kinase B (PKB), although it lacks the characteristic PtdIns(3,4,5)P_3-binding pleckstrin homology (PH) domain. The catalytic domain of SGK1 is 54% identical to that of PKB and although it lacks the PH domain, SGK1 retains the same residues in PKB that are phosphorylated by protein kinase 1 (PDK1) and protein kinase 2 (PDK2). Studies have shown that following transcription, SGK1 is activated by PDK1 at Ser^{422} and/or Thr^{256} depending on cell type (Kobayashi and Cohen, 1999).

While the activated form of SGK1 has been found to interact with the α-and β-subunits of ENaC *in vitro* (Wang *et al.,* 2001), this association does not appear to result in phosphorylation of ENaC. Instead, SGK1 mediates ENaC function by phosphorylation of an intermediate target termed Nedd4-2 (Debonneville *et al.,* 2001; Snyder *et al.,* 2002). Nedd4-2 is an ubiquitin ligase that binds proline-rich motifs (PY) located in the carboxy terminus of the three ENaC subunits (Kamynina and Staub, 2002). In its unphosphorylated form, Nedd4-2 catalyzes the ubiquitination of residues in the amino terminus of the subunits, thereby providing a signal for the endocytosis of the channel (Staub *et al.,* 1997). When Nedd4-2 is phosphorylated via SGK1, the affinity of Nedd4-2 for the PY motifs is diminished leading to a decrease in the endocytosis of ENaC (Debonneville *et al.,* 2001). The disassociation of Nedd4-2 from ENaC results in an increase in both the activity of the channel as well as the number of channels on the surface of the plasma membrane (Alvarez de la Rosa *et al.,* 1999).

Transcriptional Regulation of *sgk1*

In addition to glucocorticoids and aldosterone, many other agonists, acting through a variety of signal transduction pathways, have been shown to induce *sgk1* gene transcription in cells and tissues. For example, increased transcription of *sgk1* in response to osmotic shock appears to be mediated by stress-activated protein kinase 2 (p38) (Bell *et al.*, 2000; Waldegger *et al.*, 2000). Additionally studies have shown *sgk1* transcription can also be regulated by follicle stimulating hormone (FSH) in ovarian cells through signaling cascades that involve p38 as well as PI3K and cAMP (Gonzalez-Robayna *et al.*, 2000). Overall, however, very little is currently known about the specific signaling pathways that mediate the transcriptional regulation of *sgk1*. Table 1-1 shows a comprehensive list of agonists that have been shown to increase transcriptional activation of *sgk1*.

To date, the molecular mechanisms that regulate transcription of the *sgk1* promoter are largely undetermined. Nevertheless, studies have identified a number of interesting response elements within the promoter region. For example, a DNA binding site for the p53 tumor suppressor protein has been identified within a 35-base pair region of the promoter. Studies show this region is sufficient to permit p53-dependent transactivation on a heterologous promoter (Maiyar *et al.*, 1997). Additionally, a 20-base pair G/C-region between –63 and –43 of the *sgk1* promoter has been identified and confers sensitivity to FSH and forskolin. Specifically, this region binds the transcription factors Sp1 and Sp3 and this binding is abolished following mutation of two base pairs within this region (Alliston *et al.*, 1997). Interestingly, this same Sp1 binding region within the *sgk1* promoter confers sensitivity to high osmolarity, which, as mentioned previously, is mediated by p38 (Bell *et al.*, 2000).

Despite its many activators, *sgk1* remains best known for its physiological contributions to Na^+ retention in response to aldosterone secretion. Although clearly an important contributor of blood pressure regulation, current studies have not determined SGK1 to be directly regulated by AngII, which is regarded as a critical mediator of blood

Table 1-1: Activators of *sgk1* gene transcription

Activators	Tissues/cells	References
Conditions		
Brain Injury	Brain	Hollister *et al.*, 1997; Imaizumi *et al.*, 1994
Hypertonic stress	Hepatcytes	Waldegger *et al.*, 1997
	Xenopus Collecting Duct Cells	Rozansky *et al.*, 2002
High Glucose	Kidney, fibroblasts	Lang *et al.*, 2000; Kumar *et al.*, 1999
Increased [Ca^{2+}]	CHO-IR cells	Imai et al., 2003
Agonists		
Serum	Mammary tumor cells	Webster *et al.*, 1993a
	Fibroblasts	Webster *et al.*, 1993b
Glucocorticoids	Mammary tumor cells	Webster *et al.*, 1993a; Maiyar *et al.*, 1996; Maiyar *et al.*, 1997
	Fibroblasts	Webster *et al.*, 1997b
	Rat kidney and distal colon	Brennan and Fuller, 2000
Mineralcorticoids	Renal epithelial cells	Chen *et al.*, 1999; Neray-Fejes-Toth *et al.*, 1999; Shigaev *et al.*, 2000; Cowling et al., 2000a
FSH	Ovarian granulosa cells	Alliston *et al.*, 1997; Gonzalez-Robayna *et al.*, 2000
LH	Ovarian granulose cells	Lang *et al.*, 2000
VIP	Shark rectal gland	Waldegger et al., 1998
Carbachol	Shark rectal gland	Waldegger et al., 1998
TGF-β	Macrophages	Waldegger *et al.*, 1999
	HepG2 liver cells	Waldegger *et al.*, 1999
Thrombin	Kidney	Kumar *et al.*, 1999
Lipopolysaccharides	Granulocytes	Cowling *et al.*, 2000b
fMLP	Granulocytes	Cowling *et al.*, 2000b
TNF-α	Granulocytes	Cowling *et al.*, 2000b
GMCSF	Peripheral blood granulocytes	Cowling *et al.*, 2000b
PPARgamma	Renal cortical collecting ducts	Hong *et al.*, 2003
Endothelin-1	Smooth muscle cells	Wolf *et al.*, 2004

FSH- follicle stimulating hormone, LH- luteinizing hormone, VIP- vasoactive intestinal polypeptide, TGF-β- transforming growth factor-β, , fMLP-formyl methionyl leucyl phenylalanine, TNF-α– tumor necrosis factor-α, GMCSF- granulocyte-macrophage colony-stimulating factor.

pressure. The current studies investigate the direct relationship, if any, between *sgk1* and AngII. As such, the role of SGK1 in regulating blood pressure, as well as other biological processes, may be expanded.

Summary and Rationale

Jak2 is an important mediator of gene transcription within the cell. Beyond mediating normal physiological functions in cells, Jak2 can also contribute to numerous pathologies, including various cancers and cardiovascular diseases. In addition to its traditional effects on transcriptional regulation, Jak2 has been found to mediate phosphorylation effects on various cytosolic proteins. While the precise consequences of these post-translational effects remain uncertain, these signaling events are believed to have important biological merit.

The goal of the study presented in the following chapters is to elucidate the regulatory effects of Jak2 within the nucleus as well as clarify its role in initiating previously undefined signaling cascades within the cytosol. Specifically, this study's focus is to identify the downstream targets of Jak2 in response to AngII. By elucidating the signaling cascades and downstream targets of Jak2 in response to AngII, it may be possible to better understand the physiological and pathophysiological effects of Jak2 within the cardiovascular system.

CHAPTER 2
JAK2 TYROSINE KINASE IS A KEY MEDIATOR OF LIGAND-INDEPENDENT GENE EXPRESSION

Introduction

Jak2 tyrosine kinase is a key mediator of gene transcription. It is activated by a variety of cytokine, growth factor, and seven transmembrane spanning receptors, resulting in signaling cascades that facilitate the activation of various downstream target genes (Buggy, 1998; Gadina *et al.,* 2001; Ju *et al*., 2000; Lukasova *et al.,* 2003; Marrero *et al.,* 1995; Park *et al.,* 1996; Peeler *et al.,* 1996; Sasaguri *et al.,* 2000). Upon binding of ligand, Jak2 mediates gene transcription through the activation of cytosolic transcription factors, termed STAT proteins. Thus, to date, Jak2 is regarded as an important regulator of ligand-dependent gene activation.

Early studies have dissected the cellular and biochemical mechanisms that lead to the activation of Jak2. Specifically, when agonists of Jak2 bind to their obligatory receptors, Jak2 molecules undergo a juxtapoistioning that permits the transphosphorylation of one another. Some of this transphosphorylation occurs on tyrosine residues within the activation loop, leading to full kinase activation. Jak2 has three tyrosine residues within the activation loop located at positions 1007, 1008 and 1021. The tyrosine residue at position at 1007 has been shown to be critical for activation, since it is phosphorylated in response to the various agonists of Jak2 (Feng *et al.,* 1997). Furthermore, mutagenesis studies have determined that *ligand-induced*

activation and signaling is lost when this tyrosine residue is substituted with phenylalanine (Feng *et al.,* 1997).

Previous studies demonstrated that mice lacking a functional Jak2 allele die during early embryonic development (Neubauer *et al.,* 1998; Parganas *et al.,* 1998). These knockout mice are deficient in mandatory cytokine signaling as well as severely anemic, demonstrating a complete lack of erythropoiesis. The lethal effects associated with Jak2 knockout mice indicate the important physiological role Jak2 has in normal embryonic development.

In this study, microarray technology was used to identify and characterize Jak2-dependent genes that are differentially expressed as a function of the presence, or absence, of Jak2 in cells. By using cells that lack Jak2 expression, we were able to determine the contribution of Jak2 in regulating cellular transcription. Gene profiling experiments identified 621 genes that had a greater than 2-fold change in expression as a function of basally expressed Jak2. Surprisingly, this differential expression pattern did not require the addition of exogenous ligand to activate a cell surface receptor, but merely a basal level of Jak2 kinase function within the cell, as measured by a combination of Northern blot analysis, RT-PCR and luciferase reporter assays. Cellular transcription was even further increased in Jak2-containing cells when treated with a ligand, indicating that these cells were capable of initiating proper Jak/STAT signaling cascades in a ligand-dependent manner.

Additionally, we found that the large number of genes activated by the basal level of Jak2 represent a wide range of ontological functions including transcription factors, signaling molecules, and cell surface receptors. Interestingly, of the 621 genes identified

in this study, 56 have already been shown to be cytokine responsive, thereby suggesting that these genes are true targets of Jak2 action. Lastly, we found that a Jak2 mutant containing a tyrosine to phenylalanine substitution mutation at position 1007 maintained a basal level of transcription that was consistent to wild type controls, suggesting that the basal regulation of transcription is completely independent of active activation loop phosphorylation.

As such, this work demonstrates for the first time that, in addition to being a key mediator of ligand-activated gene transcription, Jak2 is also a critical mediator of basal level gene expression. Additionally, the large numbers of genes found to be dependent upon Jak2 for their transcriptional regulation indicate the critical and encompassing role that Jak2 has in transcriptional processes within the cell.

Materials and Methods

Creation of Stable Cell Lines/ Cell Culture

Creation of the Jak2 null cell line, termed γ2A, has already been described (Kohlhuber *et al.,* 1997). Briefly, the γ2A cells are a human fibroblast cell line that is devoid of Jak2 protein. Using this background, the cells were stably transfected with either a Jak2 expression plasmid and a Zeocin selectable vector (γ2A/Jak2 cells) or the Zeocin selectable marker alone (γ2A). Cells transfected with the Zeocin selectable marker alone (γ2A), were used as controls. Both cells lines were also stably transfected with an AT_1 receptor to establish its expression on the plasma membrane. The AT_1 receptor used for transfection had an HA-tag inserted just after the initiation methionine. Two days after transfection, cells were transferred to medium supplemented with 250 μg/ml Zeocin. Two weeks later, individual colonies were ring cloned as previously

described (Sayeski *et al.,* 1999a). AT_1 receptor-binding assays were conducted on the stable cell lines using ^{125} I –labeled AngII and respective γ2A-derived clones that had nearly identical binding parameters were identified. γ2A (clone #4) had a K_d of 0.44 nM and a B_{max} of 201 fmol/mg protein while γ2A/Jak2 (clone #1) had a K_d of 0.41 nM and a B_{max} of 226 fmol/mg protein (Sandberg *et al.,* 2004). The relative Jak2 expression of each clone was then determined using Western blot analysis as described in the Results.

The rat aortic smooth muscle (RASM) cells stably over-expressing either a Jak2 dominant negative allele (RASM DN) or the neomycin resistant cassette (RASM WT) have been described previously (Sayeski *et al.,* 1999a). The γ2A cells stably expressing either the growth hormone receptor alone (γ2A/GHR) or the growth hormone receptor along with wild type Jak2 (γ2A/GHR/Jak2) have also been described (He *et al.,* 2003).

Cells were grown in DMEM +10% FBS at 37ºC in 5%CO_2 humidified atmosphere. All cells were made quiescent by washing them extensively with phosphate-buffered saline and then placing them in serum free media for either 20 (γ2A cells) or 48 (RASM cells) hrs, prior to use.

Immunoprecipitation/ Western Blot/ Analysis

Immunoprecipitation/Western blot analyses were performed to determine Jak2 expression and phosphorylation in the γ2A cells. Briefly, to prepare γ2A and γ2A/Jak2 protein lysates, cells were washed with two volumes of ice-cold PBS containing 1 mM Na_3VO_4 and lysed in 800 μL of ice-cold RIPA buffer (20mM Tris [pH 7.5], 10% glycerol, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mM EDTA, 50 mM NaF, 10mM $Na_4P_20_7$, 4 mM benzamidine, 1 mM phenylmethylsulfonyl fluride, 1 mM Na_3VO_4, and 10 μg/mL aprotinin). The samples were then sonicated and incubated on

ice for 30 min. Samples were subsequently spun at 13,200 rpm for 5 min at 4°C, and supernatants were normalized for protein content using the Bio-Rad D_c assay. Normalized lysates (approx. 400 μg/ml) were then either directly resuspended in SDS sample buffer and separated by SDS-PAGE for Western blot analysis or immunoprecipitated.

Immunoprecipitations were performed for 4 hrs at 4°C with 2 μg of monoclonal anti-phosphotyrosine antibody (BD Transduction Laboratories, clone PY20) and 20 μL of Protein A/G Plus agarose beads (Santa Cruz Biotechnology). After centrifugation, protein complexes were washed 3 times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) and resuspended in SDS sample buffer. Bound proteins were boiled, separated by SDS-PAGE, and transferred onto nitrocellulose membranes.

For determination of Jak2 protein expression, whole cell lystates were Western blotted with polyclonal anti-Jak2 antibody (Upstate Biotechnology) in 5% milk/TBST. Membranes were subsequently stripped and re-probed with polyclonal anti-STAT1 antibody (Santa Cruz Biotechnology) to confirm equal protein loading of all samples. To determine Jak2 phosphorylation levels, immunoprecipitated lysates were Western blotted with polyclonal anti-Jak2 antibody (Upstate Biotechnology) in 5% milk/TBST. Proteins were visualized using enhanced chemiluminescence (ECL) following the manufacturers instructions (Amersham).

Preparation of Total and Poly $(A)^+$ mRNA

Cells were serum starved for 20 hrs and total RNA was then isolated using the acid guanidine thiocyanate/phenol/chloroform method of extraction (Chomczynski and Sacchi, 1987). Briefly, γ2A and γ2A/Jak2 cells were serum starved for 20 hours and then

washed with 2 volumes of ice-cold phosphate-buffered saline and lysed in 3 mL of 4M guanidine thiocyanate (GnSCN). Genomic DNA was then sheared using a 20G needle fitted to a 10 cc syringe until viscosity of the samples was reduced. Cell homogenates were then transferred to a fresh tube and 0.1-vol 2M NaOAc, pH 4.0, 1.0-vol aqueous phenol, and 0.2-vol chloroform/isoamyl alcohol were added sequentially. Following a 15-minute incubation on ice, samples were spun at 1,500 x g for 20 minutes at 15ºC. The aqueous layer was subsequently precipitated and the pellet was resuspended in 0.5mL 4M GnSCN. RNA was precipitated and resuspended in a final volume of 200 μL in DEPC-treated water and quantitated. Three confluent 100-mm culture dishes of γ2A or γ2A/Jak2 cells were pooled together in order to avoid artifact that was unique to any one individual plate.

Poly $(A)^+$ mRNA was isolated from both the γ2A and γ2A/Jak2 cells using the Amersham Pharmacia Quick Prep mRNA Purification Kit. Three plates for each condition were again pooled in order to reduce the possibility of any artifact. Total and poly $(A)^+$ mRNA was then used for Affymetrix analysis and/or Northern blot analysis as described below.

Probe Preparation and Affymetrix Chip Hybridization

cRNA probes were prepared for hybridization to microarrays following the manufacturer's instructions (Affymetrix GeneChip® Expresssion Analysis Manual). Briefly, double stranded DNA was prepared from 10 μg of total RNA isolated from both cell lines using the Superscript® Double Stranded cDNA Synthesis kit (Invitrogen). Newly synthesized double stranded DNA was subsequently cleaned using Phase Lock Gels (PLG)-Phenol/Chloroform Extraction. 5 μL of double stranded DNA was then

Biotin-labeled following the Enzo Bioarray High Yield RNA Transcript Labeling Kit protocol (Affymetrix). Biotinylated cRNA was subsequently cleaned using a Qiagen RNeasy column and quantitated. 20 μg of unadjusted cRNA was then fragmented and hybridized to Affymetrix Test3 chips in order to verify the quality of each preparation. Samples having similar metrix values were then hybridized to U95A gene chips at the University of Florida ICBR MicroArray Core Laboratory.

Microarray Data Analysis

The data was analyzed using the Affymetrix Software Package, Microarray Suite Version 4.0. Probe intensities for both cellular conditions were compared and reported in both tabular and graphical formats. The data was deposited in the Gene Expression Omnibus (GEO) repository under accession # GSM16418.

Northern Analysis

Northern Blot analysis was performed as previously described (Sayeski and Kudlow, 1996). Briefly, 25 μg of total or 4 μg of poly $(A)^+$ mRNA was separated on a 1% agarose-6% formaldehyde-containing gel. RNA samples were transferred onto nylon membranes and then hybridized to ^{32}P-labeled cDNA probes. Probes were labeled using the Random Primers DNA Labeling System Kit (Invitrogen). The cDNA's encoding for Pak1(Sells *et al.,* 1997), 4-1BBL (Wen *et al.,* 2002), USA-CyP (Horowitz *et al.,* 2002) and EphB6 (Matsuoka *et al.,* 1997) have been described.

Quantitative RT-PCR

The two-step quantitative RT-PCR method was also used to confirm the differential expression results generated by the microarray experiments. Specifically, total RNA was extracted from either the γ2A or the RASM-derived cell lines and subsequently reverse transcribed using the SuperScript II RNase H^- Reverse Transcriptase Kit (Invitrogen).

Primers were designed for each gene using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/). PCR reactions were prepared using the SYBR Green PCR Core kit (Applied Biosystems) and performed on the GeneAmp ® 5700 Sequence Detector machine (Applied Biosystems). 18s primers were used as a standard internal reference and analyses were accomplished by calculating the $2^{-\blacktriangle\blacktriangle Ct}$ values for each gene (Giulietti *et al.,* 2001; Livak and Schmittgen, 2001).

Luciferase Assay

Cells were transfected with a luciferase reporter construct containing four tandem repeats of the GAS element, upstream of a minimal TK promoter, in 10 μL Lipofectin (Invitrogen). Where indicated, cells were additionally co-transfected with both a luciferase construct as well as cDNA plasmids encoding 1) an empty vector for Jak2 2) wild type Jak2 cDNA 3) a Jak2 Y1007F mutant or 4) a Jak2 K882E mutant. All four Jak2 expression plasmids were kind gifts from Dr. James Ihle (St. Jude's Children Hospital). Following transfection, the cells were seeded in 12-well plates at 2.5 x 10^5 cells per well, serum starved for 20 hrs, and then treated as indicated. Luciferase activity was measured from detergent extracts in the presence of ATP and luciferin using the Reporter Lysis Buffer System (Promega) and a luminometer (Monolight Model 3010). Luciferase values were recorded as relative light units (RLU)/μg protein. Each of the conditions were measured in replicates of six (n=6).

Results

Characterization of Jak2 Expression in the Stably Transfected γ2A Cells

The γ2A-derived stable cell lines were created as described in the Methods. In order to verify the relative expression of Jak2 in each cell line, 25 μg of whole cell

protein lysate from each cell line was separated by SDS-PAGE and subsequently Western blotted with anti-Jak2 polyclonal antibody (Fig. 2-1A, top). The results show that Jak2 protein expression is completely lacking in the γ2A cell line, but is readily detectable in the γ2A/Jak2 cell line. In order to demonstrate that both lanes were loaded equally, the same membrane was stripped and Western blotted with anti-STAT1 polyclonal antibody to detect endogenous STAT1 protein (Fig. 2-1A, bottom). The results show that both lanes had roughly equal levels of STAT1 protein.

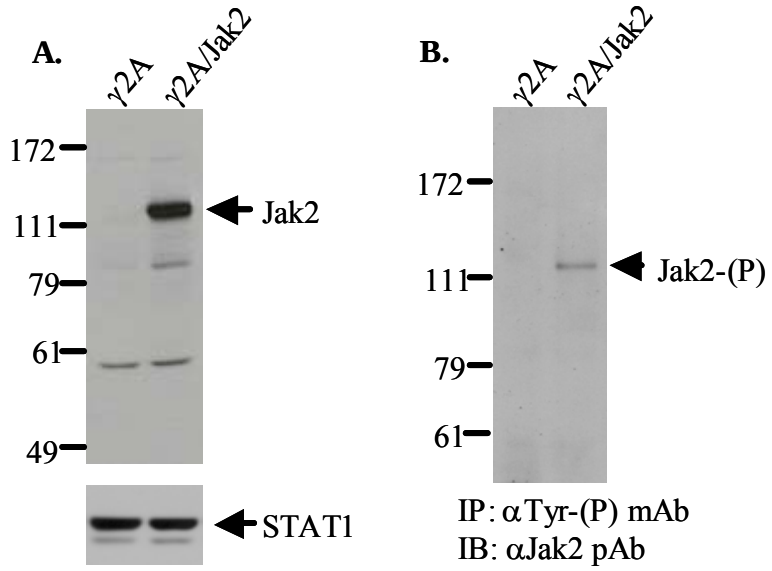


Figure 2-1. Characterization of Jak2 expression in γ2A-derived cells. A) Whole cell protein lysates from the γ2A and γ2A/Jak2 cell lines were Western blotted with anti-Jak2 antibody to detect expressed Jak2 protein (top). The blot was subsequently stripped and re-blotted with anti-STAT1 antibody to ensure equal loading (bottom). B) γ2A and γ2A/Jak2 whole cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and then Western blotted with anti-Jak2 antibody to measure Jak2 tyrosine phosphorylation levels. Shown is one of two (A) or three (B) representative results. Printed with permission of publisher

Jak2 has a basal level of tyrosine kinase activity that is significantly increased in response to ligand treatment (Duhé and Farrar, 1998). The relative kinase activity of Jak2 is directly proportional to its own tyrosine phosphorylation levels (Feng *et al.,* 1997;

VonDerLinden *et al.,* 2002). To determine whether the Jak2 protein expressed in the γ2A/Jak2 clone had proper basal level tyrosine phosphorylation, equal amounts of whole cell lysate from each clone were immunoprecipitated with anti-phosphotyrosine antibody and then Western blotted with anti-Jak2 antibody (Fig. 2-1B). The results show that the Jak2 protein expressed in the γ2A/Jak2 clone does have detectable levels of tyrosine phosphorylation, which is consistent with cells that endogenously express Jak2.

Collectively, the results in Fig. 2-1 demonstrate that while the γ2A cell line completely lacks Jak2 protein expression, the γ2A/Jak2 cell line has readily detectable levels of this protein. Furthermore, the expressed Jak2 protein shows normal, basal level tyrosine phosphorylation.

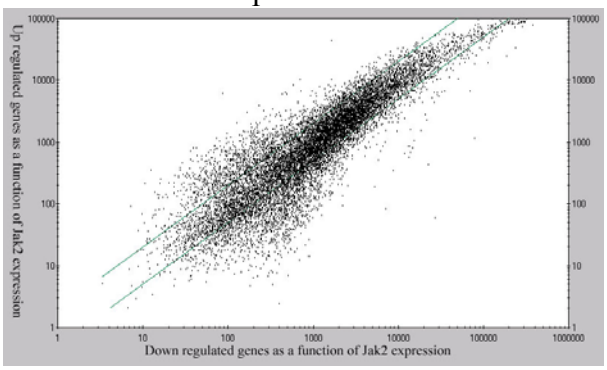
Microarray Analysis Demonstrates that Jak2 Mediates the Expression of Many Diverse Genes

We next wanted to determine whether the basal level expression of Jak2 in a cell, independent of exogenous ligand addition, has a measurable effect on gene expression. To do this, we compared gene expression profiles in γ2A versus γ2A/Jak2 cells. Total RNA was harvested from both cell lines and then prepared for Affymetrix microarray analysis as described in the Methods. The Affymetrix U95A GeneChip® was used as the differential expression platform. This chip contains the probe sequences representing ~12,000 fully sequenced human genes. The expression signals generated from the hybridization of probes from both cell lines were then compared and analyzed. Fig. 2-2 shows a graphical illustration of the mRNA expression levels from this experiment (Experiment #1). Each dot on the plot represents one of the 12,000 different genes on the chip. Genes falling outside the two parallel lines had a greater than 2.0-fold change in gene expression as a result of the presence of Jak2. Genes falling above the two parallel

lines had increased gene expression while those falling below the two parallel lines had decreased gene expression. The data indicated Jak2 expression in a cell, devoid of exogenously added ligand, altered the expression of 1,251 genes by at least 2-fold.

This entire procedure was then repeated a second independent time. The results of this experiment are shown (Fig. 2-2, Experiment #2). This time the analysis indicated that 1,042 genes had at least a 2-fold change in gene expression as a function of expressed Jak2.

Experiment #1



Experiment #2

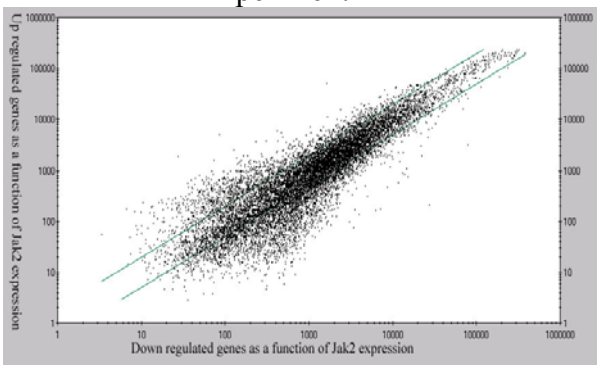


Figure 2-2. Global Analysis of Jak2-Dependent Gene Expression. Graphical illustration of the mRNA expression levels from two replicated experiments using Affymetrix MicroArray Suite, Version 4. The plots compare hybridization signal intensities from arrays probed with cRNA from the γ2A and γ2A/Jak2 cell lines. Each dot on the plot corresponds to a different gene. The two parallel dashed lines represent the level for a 2-fold change in expression. Printed with permission of publisher

The results gathered in Fig. 2-2 were further analyzed using Venn Diagram analysis. This analysis allows for the identification of genes that were present in both

experiments. The results demonstrated that 621 genes were consistently differentially expressed greater than 2-fold in both experiments. These 621 genes were further analyzed to distinguish up-regulated genes from down-regulated genes (Fig. 2-3A). The analysis showed that 474 genes were up regulated and 147 genes were down regulated. Notably, the range of fold changes of these genes was quite impressive, spanning from 2- to 78-fold. Not surprisingly, the majority of genes found to be present in only one of the two experiments had induction numbers falling close to the 2-fold cutoff. In this case, they were identified in one experiment with a value that was at 2-fold or higher, but not in the other experiment because the value was just under the 2-fold cutoff threshold.

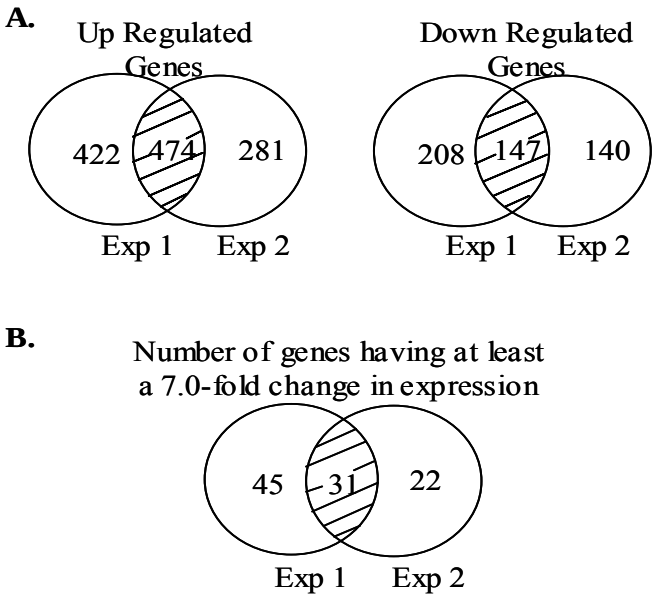


Figure 2-3. Venn Diagrams Illustrating the Number of Up and Down Regulated Genes Consistent Between the Two Replicated Experiments. A) The data for Exp. #1 and #2 were merged so genes common to both experiments could be identified as having at least a 2-fold change in gene expression. A total of 621 genes were differentially expressed in both experiments. These 621 genes were further analyzed to distinguish up regulated from down regulated genes. The hatched lines indicate the area of overlap between the two experiements. B) The data for experiments #1 and #2 were analyzed so that genes having at least a 7-fold change in expression could be identified. A total of 31 genes were identified as being common to both experiments and having at least a 7-fold change in expression. Printed with permission of publisher

The full list containing all 621 genes is found in Appendix A. Of the 621 genes on this list, 390 have a known ontological function. When these 390 genes were queried as to whether any were cytokine regulated, 56 genes were identified. Appendix B contains this list of 56 genes. Several examples include the interferon γ–inducible protein (Fan et al., 1989), the Type 1 and 3 IP_3 receptors (Rozovskaia et al., 2003) and the inhibitor of activated STAT protein (Liu et al., 1998). Collectively, the identification of genes that have previously been shown to be cytokine and/or Jak2- regulated suggest that the microarray experiments had in fact identified genes that are Jak2 targets and not genes that are differentially expressed due to clonal artifact.

For our initial analysis, we shortened the list of 621 genes to include only those genes that were differentially expressed by at least 7-fold. Again, Venn Diagram analysis was performed to identify those genes that had at least a 7-fold change in gene expression, in both experiments (Fig. 2-3B). The results show 76 genes in experiment #1 and 53 genes in experiment #2 had at least a 7-fold change in expression. Of these genes, 31 were common to both groups. Table 3-1 lists these 31 genes. As previously explained, for the genes found to be present in only one of the two experiments, the majority had induction numbers falling close to the 7-fold cutoff. As such, they were detected in one experiment with a value that was 7-fold or greater, but not detected in the second experiment because the value was just below the 7-fold cutoff threshold. Overall however, there was a strong concordance between the genes on both lists. Interestingly, when ontological functions of these genes were classified, they were found to encompass diverse categories of cellular function including transcription factors, cell cycle control

Accession Number	*Gene Name*	*Induction #1*	*Induction #2*	*Average Induction*	*Category*
W25845	13h9	-78.4	-78.1	-78.25	NF
W26787	15d8	-39.2	-25.3	-32.25	NF
W27474	31d8	-23.7	-21.3	-22.5	NF
W28170	43a12	-22.5	-17.3	-19.9	NF
W27997	43 e3	-7.5	-8.1	-7.8	NF
U24152	Pak1	7	7.6	7.3	Signaling
Y09616	putative intestinal carboxylesterase	7.2	7.6	7.4	Serine Esterase
U18271	Thymopoietins (TMPO)	7.5	7.4	7.45	Cell Cycle
AL080203	DKFZp434F222	8.6	7.6	8.1	NF
L47345	Elongin A	8.3	7.2	7.75	Transcription
U68485	Bridging integrator protein-1 (BIN1)	8.3	9.4	8.85	Tumor suppressor
AF016371	U4/U6 snRNP-associated cyclophilin	8.4	14.5	11.45	Cyclophilin
W28235	43h8	8.5	8.6	8.55	NF
S78187	CDC25 Hu2	8.6	10.6	9.6	Cell Cycle
AB017430	Kid-kinesin-like DNA binding protein	8.9	8.9	8.9	Cell Cycle
AD001530	XAP-5	9.2	9.1	9.15	NF
AF035292	23584 clone	10.8	8.9	9.85	NF
M68864	Human ORF mRNA	12.1	10.4	11.25	NF
X79865	Mrp17	12.2	8.3	10.25	Cell growth
X71345	Trypsinogen IV-b form	12.3	17.8	15.05	Proteolytic enzyme
AL096723	DKFZp564H2023	12.6	14.3	13.45	NF
X96484	DGCR6 gene	13.7	11.7	12.7	Development
AF026031	hTOM	15.1	15.8	15.45	Mitochondrial transport
L23959	E2F-releated transcription factor	15.9	11.2	13.55	Transcription
N53547	yv43b12.s1	15.9	18.2	17.05	NF
X03656	G-CSF	17.2	15.8	16.5	Cell Defense
D83492	EphB6	18.5	12.7	15.6	Angiogenesis
D64142	Histone HI subtype	19.5	8.1	13.8	Transcription
U66061	Trypsinogen-C	23	28.6	25.8	Proteolytic enzyme
AF026977	Microsomal glutathione S-transferase III	31	36.8	33.9	Peroxidase
L37127	RNA polymerase II subunit	43.9	53	48.45	Transcription

Table 2-1 Summary of Jak2-dependent genes. The 31 genes having at least a 7-fold change in gene expression in both experiments are represented. Shown are the accession number, gene name, relative fold changes and a brief description of cell function. (NF = Currently, no known function). Printed with permission of publisher

genes, cell surface receptors, and intermediate signaling molecules. As such, the data indicates that Jak2 strongly regulates an important, but diverse, set of genes.

Validation of Jak2-dependent Gene Expression in γ2A and γ2A/Jak2 Cells

We next wanted to validate the apparent changes in Jak2-dependent gene expression identified via the microarray experiments. In order to obtain a representative sample from the list, we selected genes that represented a diverse set of fold changes and ontological functions. Northern blot analysis was then performed on several of these genes. For the intermediate signaling molecule, Pak1, Affymetrix predicted that Jak2-expressing cells would have 7.3-fold more mRNA when compared to non-Jak2-expressing control cells. Northern blot analysis indicated that of the two splice variants of Pak1, the smaller transcript was ~4-fold higher in the Jak2-expressing cells (Fig. 2-4A). Similarly, for the 4-1BBL gene, Affymetrix analysis indicated that the Jak2-expressing cells would contain 9.6-fold more mRNA when compared to the cells lacking Jak2. Northern blot analysis actually found the level closer to ~5-fold (Fig. 2-4A). Similarly, for the RNA splicing enzyme, USA-CyP, Affymetrix analysis predicted the Jak2-expressing cells would have 11-fold more mRNA when compared to the cells lacking Jak2. Again, densitometric analysis of the Northern blot found it to be ~7-fold greater (Fig. 2-4B). Finally, for the angiogenic cell surface receptor, EphB6, the Affymetrix prediction and the Northern blot were in close agreement, as both analyses found Jak2-expressing cells contained ~15-fold more EphB6 mRNA than the cells lacking Jak2 (Fig. 2-4C).

Collectively, the data in Fig. 2-4 demonstrate a reasonable correlation between the differential expression pattern predicted by the Affymetrix microarray analysis and the validation of the mRNA levels by Northern blot analysis. For some genes, the magnitude

of the prediction made by the Affymetrix analysis was higher than the actual measurement determined by Northern blot analysis. However, without exception, the genes that Affymetrix predicted to be differentially expressed were in fact differentially expressed in the same direction. Table 2-2 shows a complete summary of the validations.

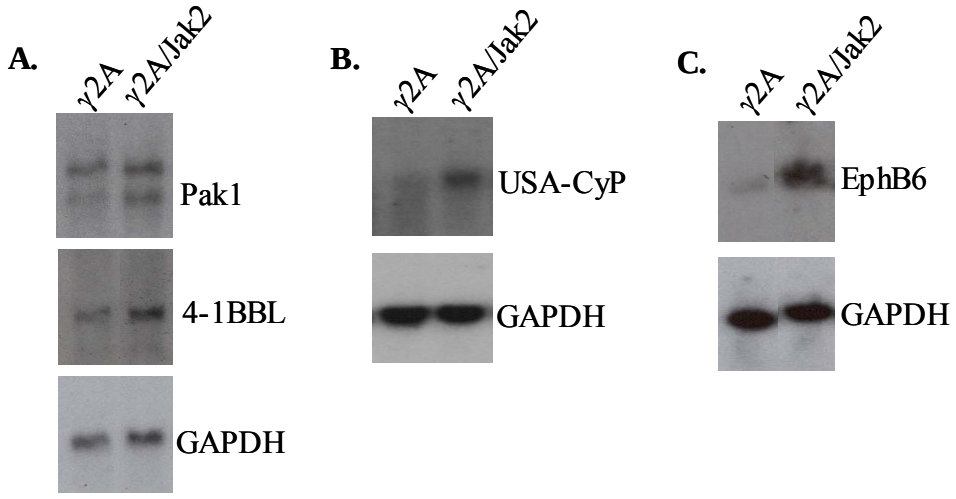


Figure 2-4. Confirmation of Jak2-dependent gene expression in the γ2A and γ2A/Jak2 cells via Northern blot analysis. Northern blot analysis of mRNA extracted from γ2A and γ2A/Jak2 cells. The blots were probed with cDNA's encoding either Pak1 and 4-1BBL (A), USA-CyP (B), or EphB6 (C). All blots were subsequently stripped and re-probed with GAPDH to control for loading. Printed with permission of publisher

To further validate the differential expression data generated by the microarray experiments, quantitative RT-PCR was also employed. Six separate genes were analyzed via quantitative RT-PCR. Graphs illustrating the derived fold changes between the γ2A and γ2A/Jak2 cell lines are shown in Fig. 2-5. For the EphB6 gene, quantitative RT-PCR found the level of differential expression to be ~12-fold greater in the Jak2-expressing cells (Fig. 2-5A). This was in close agreement with both the Affymetrix prediction and the Northern blot analysis shown in Fig.2-4C. For the protein tyrosine kinase gene

termed, FBK III16, Affymetrix predicted that the Jak2-expressing cells would have 12-fold less mRNA when compared to the non-Jak2 expressing controls. Quantitative RT-

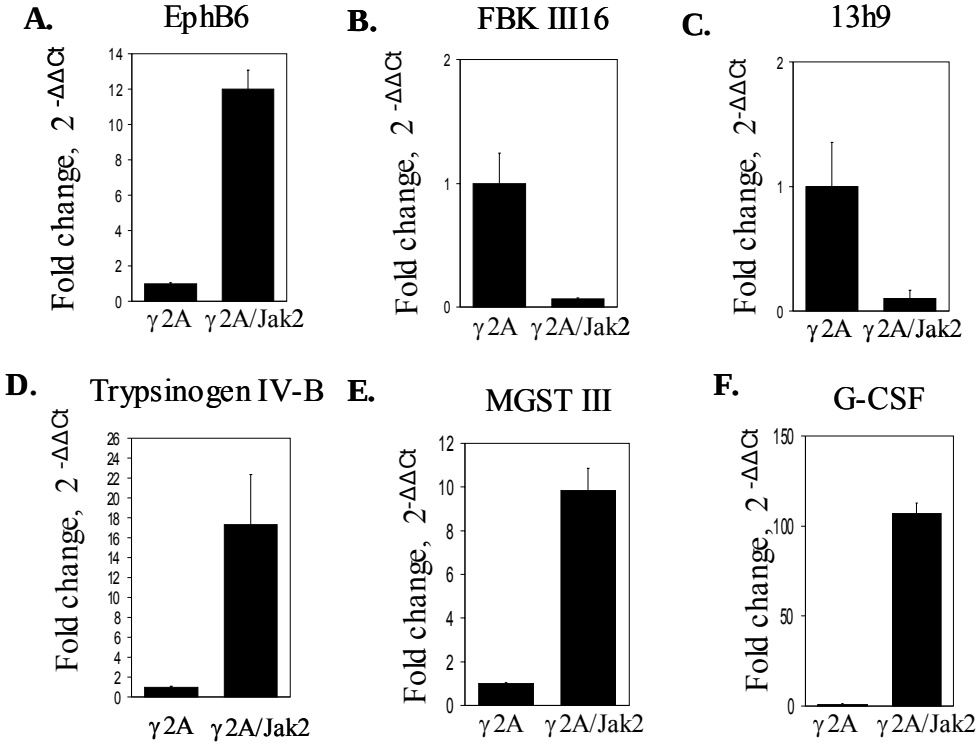


Figure 2-5. Confirmation of Jak2-dependent gene expression in the γ2A and γ2A/Jak2 cells via quantitative RT-PCR. Quantitative RT-PCR analysis of RNA extracted from γ2A and γ2A/Jak2 cells. Primers were designed for the genes encoding EphB6 (A), protein tyrosine kinase FBK III16 (B), 13h9 (C), trypsinogen IV-B (D), microsomal GST III (E) and G-CSF (F). Fold changes were derived from the $2^{-\clubsuit\clubsuit Ct}$ value and are indicated on each graph. Values are represented as the mean +/- SD. Printed with permission of publisher

PCR found the difference to be ~17-fold less (Fig. 2-5B). For the 13h9 gene, Affymetrix predicted a 78-fold decrease in mRNA levels in the Jak2-expressing cells. Quantitative RT-PCR actually found the level to be ~10-fold less in these cells (Fig. 2-5C). For the trypsinogen IV-B gene, Affymetrix predicted a 15-fold increase in mRNA levels in the Jak2-expressing cells. Quantitative RT-PCR found the level to be ~17-fold higher (Fig. 2-5D). For the microsomal GST III gene, the microarray studies predicted a 34-fold

increase in the mRNA levels in the Jak2-expressing cells. Quantitative RT-PCR found the level to be ~10-fold higher (Fig. 2-5E). Finally, for the G-CSF gene, Affymetrix predicted a 17-fold increase in mRNA levels in the Jak2-expressing cells when compared to the non-Jak2 expressing controls. Quantitative RT-PCR actually found the level to be ~107-fold higher in the Jak2 expressing cells (Fig. 2-5F).

Collectively, the quantitative RT-PCR data in Fig. 2-5 show similar trends in gene expression as was predicted by the microarray experiments. (Table 2-2)

Suppression of Endogenous Jak2 Kinase Activity via Over Expression of a Jak2 Dominant Negative Allele Similarly Inhibits Jak2-dependent Gene Expression

One interpretation of the data in Figs. 2-4 and 2-5 is that basal level Jak2 tyrosine kinase activity within a cell, independent of exogenous ligand addition, can significantly alter cellular gene expression. However, other interpretations might be that the results are due to artifact inherent to the γ2A-derived clones or that the effect might be unique only to γ2A-derived cells. To eliminate these alternate possibilities, we utilized rat aortic smooth muscle cells that stably express a Jak2 dominant negative cDNA (RASM DN). Expression of the dominant negative protein blocks function of wild type Jak2 normally found in these cells (Sayeski *et al.,* 1999a). In short, Jak2-dependent signaling in the dominant negative expressing cells is reduced by about 90% when compared to wild type controls. The control cells are rat aortic smooth muscle cells that express only a Neomycin resistant cassette (RASM WT). Thus, these cells allow for a determination of Jak2-dependent gene expression via a mechanism that is independent of the Jak2 null mutation.

Here, both sets of cells were serum starved for 48 hrs and then total RNA was harvested. Quantitative RT-PCR was subsequently performed on the several of the genes

shown in Figs. 2-4 and 2-5. Overall, the results were consistent with the Affymetrix-derived data as well as the Northern and quantitative RT-PCR experiments done in the γ2A cells (Table 2-2). Specifically, USA-CyP and 4-1BBL gene expression was ~10-fold higher in the RASM WT cells when compared to the RASM DN cells (Figs. 2-6A and 2-6B, respectively). 13h9 gene expression was ~8-fold less in the RASM WT cells when compared to the RASM DN cells (Figs. 2-6C). Finally, trypsinogen IV-B gene expression was ~7 fold greater in the RASM WT cells when compared to the RASM DN cells (Figs. 2-6D).

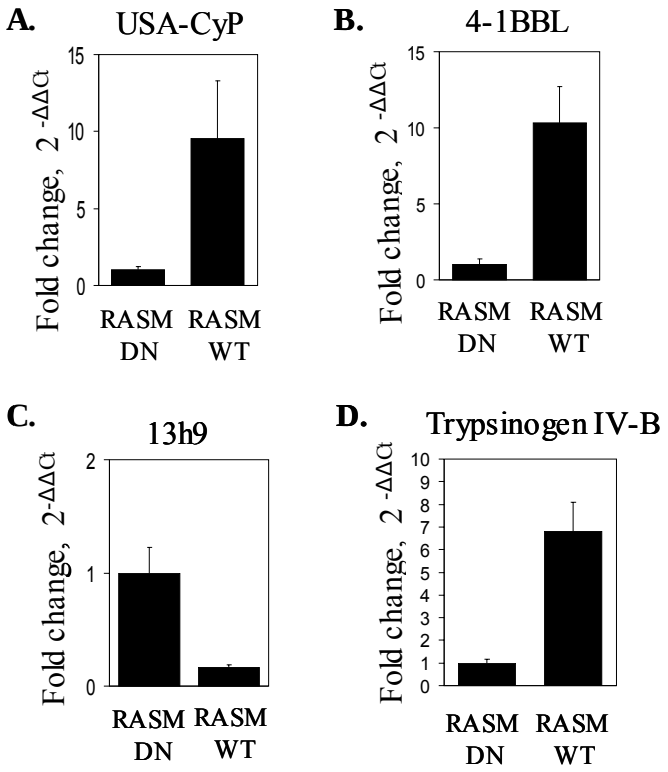


Figure 2-6. Confirmation of Jak2-dependent gene expression in the RASM DN and RASM WT cells via quantitative RT-PCR. Quantitative RT-PCR analysis of RNA extracted from RASM DN and RASM WT cells. Primers were designed for the genes encoding USA-CyP (A), 4-1BBL (B), 13h9 (C), and trypsinogen IV-B (D). Fold changes were derived from the $2^{-\spadesuit\spadesuit Ct}$ value and are indicated on each graph. Values are represented as the mean +/- SD. Printed with permission of publisher

Collectively, the data in Fig. 2-6 indicate that when endogenous Jak2 tyrosine kinase activity is reduced via expression of a Jak2 dominant negative allele, there is a corresponding change in gene expression that is similar to that seen in the γ2A-derived cells. As such, the data suggest that basal level Jak2 tyrosine kinase activity within a cell, independent of exogenous ligand addition, significantly alters cellular gene expression.

Table 2-2 Summary of microarray validations

	γ2A cells			RASM cells
Gene Name	**Affymetrix**	**Northern**	**RT-PCR**	**RT-PCR**
Pak1	↑ 7.3	↑ ~4		
4-1BBL	↑ 9.6	↑ ~5		↑ 10
USA-CyP	↑ 11	↑ ~7		↑ 10
EphB6	↑ 15.6	↑ ~15	↑ 12	
FBK III16	↓ 12		↓ 17	
13h9	↓ 78		↓ 10	↓ 8
Trypsinogen IV-B	↑ 15		↑ 17	↑ 7
M-GST III	↑ 34		↑ 10	
G-CSF	↑ 17		↑ 107	

Shown are the gene expression fold changes predicted for each gene via Affymetix, Northern blot, and RT-PCR analysis in both γ2A and RASM cells.

Jak2 is a Critical Mediator of Both Basal Level and Ligand-induced Gene Transcription

The data in the preceding figures suggest that Jak2 is capable of significantly mediating gene transcription independent of exogenous ligand addition. This is a novel concept in that Jak2 has classically been viewed as a mediator of ligand-induced gene expression. We therefore hypothesized that Jak2 can act as a critical mediator of both basal level and ligand-induced gene transcription. To test this, we investigated the ability of angiotensin II (AngII) to further mediate mRNA gene expression. Numerous independent laboratories, including our own, have shown that AngII is a potent activator of Jak2, both *in vitro* and *in vivo* (Frank *et al.,* 2002; Marrero *et al.,* 1995; Sandberg *et*

al., 2004; Sayeski *et al.,* 1999; Seki *et al.,* 2000). Both γ2A-derived cell lines utilized in this study stably express the AngII type 1 (AT_1) receptor via the stable integration of cDNA expression plasmids (Sandberg *et al.,* 2004). In short, the γ2A cell line expresses the AT_1 receptor on a background that is devoid of Jak2. However, the γ2A/Jak2 cell line expresses the AT_1 receptor with similar affinity and abundance as the γ2A cell line, but also expresses wild type Jak2 protein. Thus, these cells allow for a determination of the role of Jak2 in gene expression, under both the basal- and ligand-activated states.

We first investigated the ability of Jak2 to become phosphorylated in response to AngII treatment. To characterize both the basal and ligand-induced tyrosine phosphorylation levels of Jak2, both sets of cells were either left untreated (-) or treated for 5 min with 100 nM AngII (+). Equal amounts of whole cell lysate from each condition were then immunoprecipitated with anti-phosphotyrosine antibody and subsequently Western blotted with anti-Jak2 antibody (Fig. 2-7A). Since the γ2A cells lack Jak2, AngII treatment failed to increase the tyrosine phosphorylation levels of the protein (lanes 2 vs 1). However, in the γ2A/Jak2 cells, Jak2 was found to be tyrosine phosphorylated prior to AngII treatment (lane 3), and ligand treatment further increased its tyrosine phosphorylation levels (lane 4). Thus, the data in Fig. 2-7A suggest that these cells appear to be suitable vehicles for studying gene expression that is both Jak2- and ligand-dependent.

One gene that showed remarkable consistency in its Jak2-dependent regulation in the microarray studies was EphB6. Specifically, Affymetrix, Northern blot, and quantitative RT-PCR analyses all indicated that the levels of EphB6 mRNA were about 15-fold higher in the Jak2-expressing cells (Figs. 2-4 & 2-5 and Table 2-1). To

determine the role of basal- and ligand-activated Jak2 on EphB6 gene expression, both sets of cells were either left untreated (-) or treated for 4 hrs with 100 nM AngII (+). RNA was then extracted and Northern blot analysis was performed (Fig. 2-7B, top). The results show that in the cells lacking Jak2, there is little to no EphB6 message, either with or without ligand treatment (lanes 1 and 2). However, in the Jak2 expressing cells, there was a marked increase in EphB6 mRNA levels that was completely independent of ligand treatment (lane 3). This result recapitulates the observation seen in Figs. 2-4C and 2-5A as it once again demonstrates that basal level Jak2 tyrosine kinase activity in a cell is sufficient to significantly increase expression of this gene. Finally, when the Jak2 expressing cells were treated with AngII, there was a further increase in EphB6 mRNA levels (lane 4). The nylon membrane was subsequently stripped and re-probed with the cDNA encoding GAPDH, in order to demonstrate similar loading across all lanes (Fig. 2-7B, bottom). Interestingly, the most striking increase in EphB6 gene expression does not occur in response to AngII treatment (i.e. ligand-activated Jak2), but rather occurs when Jak2 is simply expressed in the cell (i.e. basal activation state of Jak2).

To determine whether this effect could be conferred onto a heterologous Jak2-responsive promoter, we transfected these same γ2A and γ2A/Jak2 cells with a luciferase reporter construct containing four tandem repeats of the Jak2-responsive, GAS element, upstream of a minimal TK promoter. The cells were subsequently serum starved for 20 hrs, treated with 100 nM AngII for 0 or 24 hours and then luciferase activity was measured (Fig. 2-7C). In the cells lacking Jak2, there was minimal basal level luciferase activity that increased modestly with the addition of ligand (lane 2 vs. 1). However, in the Jak2-expressing cells, there was substantial luciferase activity measured at basal

levels (lane 3 vs. 1) that was significantly increased following AngII treatments (lane 4 vs. 3). Clearly however, of the four conditions, the largest increase in luciferase activity was seen in lane 3, where Jak2 expression significantly increased luciferase activity, independent of exogenous ligand addition.

To demonstrate that this observation is not an artifact unique to the γ2A/AT_1 receptor expressing cell lines, we transfected the same luciferase reporter construct into γ2A cells stably expressing either the growth hormone receptor alone (γ2A/GHR) or the GHR along with Jak2 (γ2A/GHR/Jak2). The creation and characterization of these cells has been previously described (He *et al.,* 2003). In short, both cell lines express the GHR at similar affinity and abundance, but only the second cell line expresses Jak2. In the absence of growth hormone, Jak2 displays low level, basal tyrosine phosphorylation. Upon treatment with growth hormone however, there is a marked increase in Jak2 tyrosine phosphorylation levels.

The luciferase activity in the γ2A cells expressing the GHR were similar to that seen in the γ2A cells expressing the AT_1 receptor. Specifically, the γ2A cells lacking Jak2 again demonstrated little luciferase activity, which did not increase upon treatment with GH (lane 1 and 2). Conversely however, in untreated Jak2-expressing cells, there was a dramatic increase in luciferase activity, roughly 2.5-fold higher than found in equivalent cells lacking Jak2 (lane 3 vs 1). Furthermore, as with AngII treatment, GH further increased luciferase activity in cells expressing Jak2. In this case, addition of GH increased luciferase activity ~3-fold above the untreated cells (lane 4 vs. 3). Thus, the data demonstrate that the magnitude by which Jak2 increases ligand-dependent gene transcription (~3-fold) is nearly equivalent to the magnitude by which Jak2 increases

ligand-independent gene transcription (~2.5-fold). As such, these data help strengthen the argument that Jak2 may act as a mediator of both ligand-independent and ligand-dependent gene transcription.

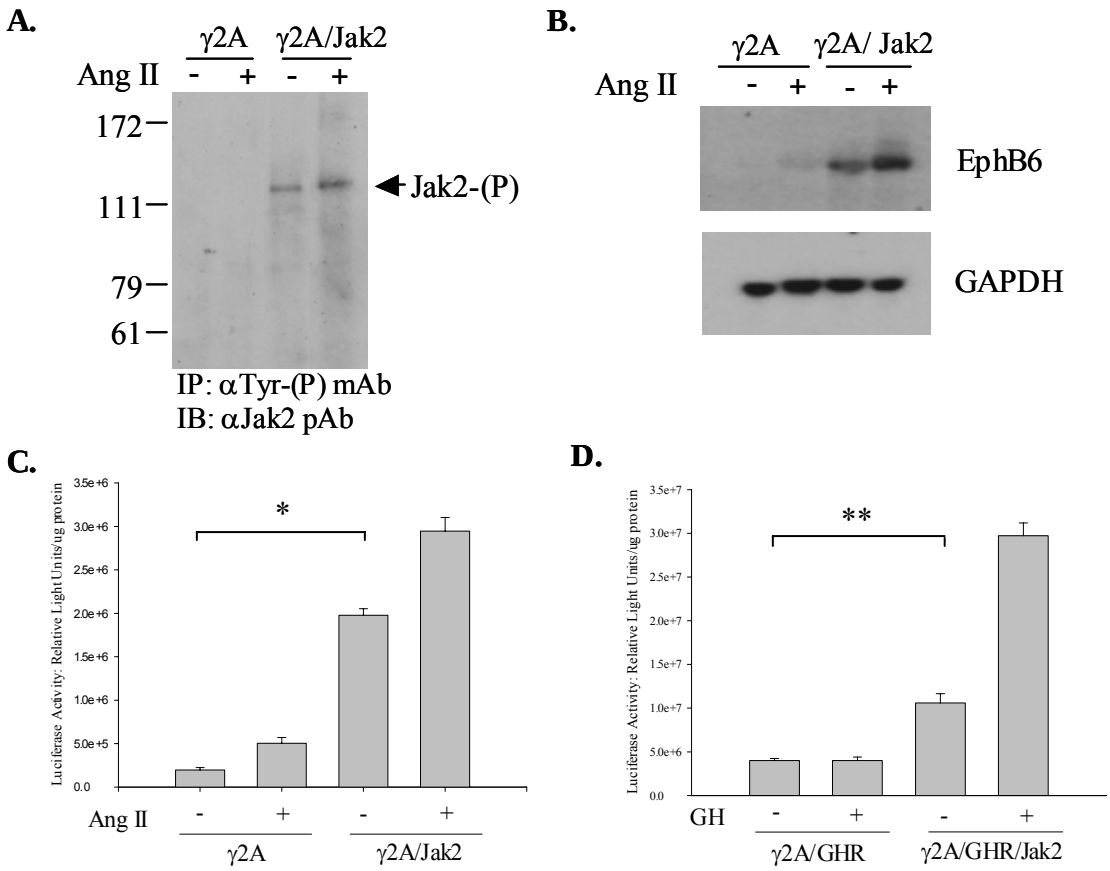


Figure 2-7. Jak2 plays a key role in basal, as well as ligand activated, gene transcription A) Quiescent γ2A and γ2A/Jak2 cells were either left untreated (-) or treated for 5 min with 100nM AngII (+). Lysates were immunoprecipitated with anti-phosphotyrosine antibody and subsequently Western blotted with anti-Jak2 antibody to measure Jak2 tyrosine phosphorylation levels. Shown is one of 3 representative results. B) Quiescent γ2A and γ2A/Jak2 cells were either left untreated (-) or treated for 4 hrs with 100nM AngII (+). Poly $(A)^+$ mRNA was then isolated from the cells and subsequently Northern blotted with the cDNA encoding for either EphB6 (top) or GAPDH (bottom). C) γ2A and γ2A/Jak2 cells were transfected with 0.5 μg of a luciferase reporter construct containing four tandem repeats of a GAS element. Cells were treated for 24 hrs with either vehicle control (-) or 100nM AngII (+) and then luciferase activity was measured. Values are plotted as the mean +/- SD. The difference in luciferase values between lanes 1 and 3 was statistically significant as determined by Student's *t*-test. *, $p = 1.23 \times 10^{-13}$. Shown is one of three

independent results. D) γ2A/GHR and γ2A/GHR/Jak2 cells were transfected with 5.0 μg of the same luciferase reporter construct described above. The cells were subsequently treated for 24 hrs with either vehicle control (-) or 600ng/ml GH (+) and then luciferase activity was measured. Values are plotted as the mean +/- SD. The difference in luciferase values between lanes 1 and 3 was statistically significant as determined by Student's *t*-test. **, $p = 2.94 \times 10^{-7}$. Shown is one of three independent results. Printed with permission of publisher

To further investigate the precise mechanism of Jak2 in mediating ligand-independent transcription, we utilized various Jak2 mutants. The mutants selected for investigation were chosen based upon a recent paper by Chatti and colleagues, in which they demonstrated that kinetically, the tyrosine kinase function of Jak2 exists in at least two independent states; namely, a basal state and a ligand-activated state (Chatti *et al.,* 2004). Specifically, the authors generated an activation loop mutant of Jak2 by changing the conserved tyrosine at position 1007 to phenylalanine. While this Jak2 mutant was unable to propagate cytokine-dependent signaling, it was nonetheless able to bind ATP and autophosphorylate, albeit less efficiently than wild type protein. As such, they concluded that Jak2 exists in at least two kinetically distinct states of activity; a high-activity catalytic state and a low-efficiency basal catalytic state. However, what remained uncertain was whether this low-efficiency basal state had any biological consequence.

In an attempt to investigate if perhaps the "low activation state" described by Chatti was mediating the ligand-independent activation of Jak2, we utilized a number of Jak2 mutant expression plasmids. Along with the STAT-responsive luciferase reporter construct previously described, γ2A/GHR cells lacking Jak2 expression were co-transfected with cDNA plasmids encoding either 1) an empty vector for Jak2 2) a plasmid containing wild type Jak2 3) a Jak2-Y1007F mutant which has low level ATP utilization,

but cannot activate in response to ligand treatment or 4) a Jak2-K882E mutant which has absolutely no kinase activity as it is unable to bind ATP. After transfection, the cells were treated with GH to activate Jak2 and luciferase activity was subsequently measured (Figure 2-8A). For the cells transfected with empty vector control, there was a minimal level of luciferase activity that did not change with ligand addition. The Jak2-K882E mutant, which has absolutely no kinase activity, had virtually the same luciferase expression pattern as the empty vector control. However, for the Jak2-Y1007F mutant, there was an 8-fold increase in luciferase activity at the 0 hr time point over both the empty vector control and the Jak2-K882E transfected cells. These data indicate that a Jak2 protein that possesses basal level kinase activity, but cannot activate in response to exogenous ligand addition due to mutation of tyrosine 1007, can greatly increase gene transcription in the basal catalytic state. Not surprisingly, cells expressing the Jak2-Y1007F mutant do not exhibit increased luciferase activity in response to GH treatment. Finally, for cells expressing Jak2-WT, prior to ligand addition, there was luciferase activity that was similar to the Jak2-Y1007F mutant. However, 6 hrs of growth hormone treatment resulted in a 2.5-fold increase in luciferase activity presumably due to phosphorylation of tyrosine 1007. The data show that simply expressing a Jak2 protein which possesses only basal level kinase activity (i.e. the Jak2-Y1007F mutant) results in an 8-fold increase in gene expression whereas ligand-dependent activation of Jak2-WT only results in a further 2.5-fold increase in gene transcription. Thus, the degree by which Jak2 influences basal level gene transcription is much greater than the degree by which it influences ligand-dependent gene transcription.

The relative levels of expressed Jak2 protein for each condition were determined via anti-Jak2 Western blot analysis (Figure 2-8B). In summary, the data indicate that the transcriptional effect of Jak2 in the basal catalytic state (i.e. ligand-independent) is greater than that seen in the ligand-activated state.

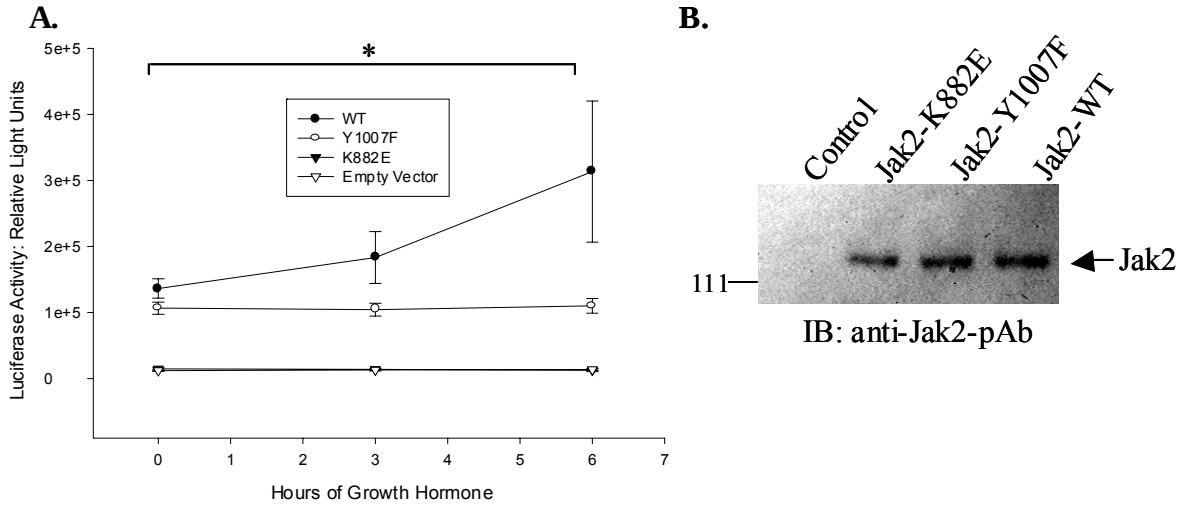


Figure 2-8. A Jak2 mutant that possesses only basal level kinase activity, significantly influences gene transcription. A) γ2A/GHR cells were co-transfected with 5.0 μg of a luciferase reporter construct containing four tandem repeats of a Jak2-responsive GAS element and either empty vector for Jak2 (Control), the Jak2-K882E mutant, the Jak2-Y1007F mutant, or Jak2-WT. The cells were serum starved and subsequently treated for either 0, 3, or 6 hours with 250 ng/ml GH and then luciferase activity was measured. Each condition was measured in replicates of six (n=6). Values are expressed as the mean $\pm$ SD. The difference in luciferase values between the Jak2-WT transfected cells at time 0 hr versus 6 hrs was significantly different as determined by Student's *t*-test. *, $p < 0.05$. B) Lysates from each of the four transfected conditions were Western blotted with anti-Jak2 antibody to assess Jak2 expression levels. Printed with permission of publisher

Discussion

Jak2 is a key mediator of cellular gene expression. A variety of ligands that bind cytokine, tyrosine kinase growth factor and G protein-coupled receptors, are all known to signal through Jak2. This study was therefore designed to help elucidate the critical role that Jak2 has in regulating cellular gene transcription. Here, we found that when Jak2

was expressed in a cell, 621 genes had a greater than 2-fold change in gene expression when compared to non-Jak2 expressing control cells.

This work is significant for several reasons. First, in the realm of cellular transcription, genes can be expressed at either basal levels or under activated conditions such as when a ligand binds its receptor. Jak2 has long been regarded as a key mediator of this ligand-activated state of transcription and has never thought to be important in basal transcriptional regulation. This dissertation shows for the first time that, when Jak2 is expressed in a cell at basal level conditions, it appears to play a central role in cellular transcriptional regulation that is independent of exogenous ligand addition.

Second, a classification of these differentially regulated genes was done in an attempt to discover prominent classes of Jak2 signaling targets. Uncovering functional classes of genes could potentially lead to predictions about genomic targets of Jak2. Interestingly however, no prominent class of genes appeared evident. The classification revealed a large assortment of genes encoding many diverse proteins such as transcription factors, intermediate signaling molecules and cell surface receptors. The data suggest that Jak2 shows no single prominent function at the basal level, but rather maintains a global influence within the cell.

Third, the Jak2 knockout mouse dies during development therefore indicating that this tyrosine kinase is required for survival (Neubauer *et al.,* 1998; Parganas *et al.,* 1998). These same studies showed that Jak2 is required for proper signaling through a variety of cytokine receptors. Subsequent studies further demonstrated that Jak2 is a critical mediator of growth factor and G protein-coupled receptor signaling. However, the downstream target genes of Jak2 tyrosine kinase remain largely unknown. Here, we

identified 621 genes that have at least a 2-fold change in gene expression as a function of expressed Jak2. As such, additional downstream target genes of Jak2 may now be known.

As mentioned previously, the major focus of this study did not include the genes falling within the differential signal expression range of 2- to 7-fold. This does not suggest these genes are not biologically important. To the contrary, genes having a 2-fold change in gene expression have previously been shown to have important biological consequences (Cook *et al.,* 2002; Rome *et al.,* 2003). However, given the vast number of genes that were identified in this study, we narrowed our focus and chose to study genes having larger fold changes.

Interestingly, Jak2 has been regarded as an activator of ligand-dependent gene transcription. However, this study revealed that nearly one-quarter of all Jak2-dependent genes were down regulated. One possible explanation for this is that Jak2 is having an indirect effect on these gene promoters via the activation of transcriptional repressor genes. Once expressed, the repressors would subsequently bind other promoters and, in turn, reduce gene transcription. Alternatively, recent studies have shown that the Jak/STAT pathway itself is capable of directly inhibiting expression of specific gene promoters. Specifically, recent work demonstrated the γ-globin gene promoter is inhibited by STAT3ß (Foley *et al.*, 2002). Currently, further experiments are required in order to determine which of these scenarios might be happening in the γ2A-derived cells.

As indicated above, a major finding of this work is that Jak2 may function as a critical mediator of ligand-independent gene transcription. An important concern however, is whether Jak2 is already in a "ligand-activated" state prior to exogenous

ligand addition. For several reasons, we believe the answer is no. First, the level of Jak2 protein that is expressed in the γ2A-derived cells used in these studies is at a level that is similar to cells that endogenously express Jak2, such as Jurkat cells. As such, this would tend to minimize Jak2 autophosphorylation in the absence of exogenously added ligand. Second, the cells were washed extensively with phosphate-buffered saline and serum starved prior to use. This made the cells quiescent and in turn minimized the tyrosine kinase activity of proteins such as Jak2, prior to any ligand treatment. Third, the addition of exogenous ligand subsequently activated Jak2 suggesting that Jak2 was not fully activated prior to ligand addition. Fourth, the phenomena of Jak2 mediating ligand-independent gene transcription was observed in multiple independent cell lines (γ2A/AT_1, γ2A/GHR, and RASM) therefore suggesting that the effect is not due to clonal artifact. Fifth, in the case of the RASM-derived cells, when endogenous Jak2 tyrosine kinase activity was reduced via the expression of the dominant negative Jak2 allele, there was a subsequent alteration in gene expression that correlated with the microarray predictions. This demonstrates that when the tyrosine kinase function of endogenously expressed Jak2 (i.e. non-transfected) is reduced from its basal state, there is a significant corresponding change in Jak2-dependent gene transcription. And sixth, recent work by Chatti and colleagues identified that the tyrosine kinase function of Jak2 exists in at least two independent states; namely, a basal state and a ligand-activated state (Chatti *et al.,* 2004). Our data here suggest that the basal state of Jak2, previously characterized biochemically as being capable of binding ATP and tyrosine autophosphorylating, is in fact an important mediator of gene transcription.

In conclusion, this study showed that expression of Jak2 can alter the transcriptional regulation of 621 genes in γ2A-derived cells. These numbers are indicative of the critical role that Jak2 tyrosine kinase has within a cell and suggest that Jak2 plays a key role in basal, as well as ligand-activated, cellular gene transcription. Therefore we believe these studies suggest that Jak2 can significantly regulate gene expression outside of the classical, ligand-activated signaling paradigm.

CHAPTER 3
IDENTIFICATION OF JAK2 TARGETS IN RESPONSE TO ANGIOTENSIN II SIGNALING

Introduction

Angiotensin II (AngII) is a major regulator of cardiovascular and renal homeostasis. In addition to its role as a vasoconstrictor, AngII also acts as a potent growth factor by activating several non-receptor tyrosine kinases through the AT_1 receptor (Leduc *et al*., 1995; Schieffer *et al*., 1996). Jak2 is one example of a non-receptor tyrosine kinase that is activated by AngII (Marrero *et al*., 1995). Activated Jak2 is recruited to the AT_1 receptor upon treatment with AngII where it subsequently initiates signaling cascades that result in the regulation of gene transcription (Marrero *et al*., 1995; Ali *et al*., 1997).

While Jak2 is traditionally known to be an important mediator of cytokine signaling, recent studies have suggested it also contributes to various cardiovascular pathologies, such as neointimal formation and cardiac hypertrophy (Seki *et al.*, 2000; Mascareno *et al.*, 2001; Kodama *et al*., 1997). Interestingly, increased circulating levels of AngII correlate to similar cardiovascular pathologies as recently shown to be associated with Jak2. Given the link between these two signaling molecules, we hypothesize that Jak2 has a significant role in mediating AngII-induced gene transcription.

To date, the downstream targets of Jak2 activation via the AT_1 receptor remain largely unknown. By identifying these targets, we will be better equipped to determine the specific contributions of Jak2 in various cardiovascular pathologies.

Here, similar to the study detailed in Chapter 2, we utilized microarray technology to compare the gene expression of Jak2-deficient cells with the gene expression of Jak2-expressing cells. In this study however, we sought to compare the expression profiles of both cell lines in response to AngII treatment. We hypothesize that since Jak2 is recruited to and activated by the AT_1 receptor, it has a large role in mediating AngII-dependent gene transcription. Furthermore, we believe the identification of AngII-inducible genes that require Jak2 for their expression may provide meaningful insight on the specific roles of Jak2 within the cardiovascular system.

Microarray experiments determined that a large number of genes were differentially expressed greater than 2-fold in response to 1 and 4 hours of AngII treatment, when comparing the human fibroblast γ2A and γ2A/Jak2 cells. Amongst the many genes identified, some had been previously associated with Jak2 and/or AngII signaling. Conversely however, many of the genes identified by the microarray experiments were novel targets of both AngII and/or Jak2. These genes therefore offered novel insight into the effects of AngII-mediated cellular transcription.

In conclusion, using gene-profiling technology, we identified a large number of AngII-inducible genes that require Jak2 for regulation. By identifying the downstream targets of Jak2 activation via the AT_1 receptor, we may now be able to better elucidate of the role of Jak2 in the progression of cardiovascular diseases through AngII-dependent signaling.

Materials and Methods

Cell Culture

The γ2A and γ2A/Jak2 cell lines were described previously in Chapter 2. Cells were grown in DMEM +10% FBS at 37ºC in 5% CO_2 humidified atmosphere. All cells were made quiescent by washing them extensively with phosphate-buffered saline and then placing them in serum-free media for 20 hours prior to use.

Preparation of Total RNA

γ2A and γ2A/Jak2 cells were serum starved for 20 hrs and then treated for either 0, 1, or 4 hours with 100nM AngII. Total RNA was subsequently isolated using the acid guanidine thiocyanate/phenol/chloroform method of extraction (Chomczynski and Sacchi, 1987) exactly as described in Chapter 2. For each of the conditions, three confluent 100-mm culture dishes of cells were lysed and extracted RNA was then pooled together in order to avoid artifact that was unique to any one individual plate.

Microarray Expression Profiling

For the 0- and 4-hour conditions, cRNA probes were prepared for hybridization to Affymetrix microarray chips following the manufacturer's instructions (Affymetrix GeneChip® Expresssion Analysis Manual). Briefly, double stranded DNA was prepared from 10 μg of total RNA isolated from both cell lines using the Superscript® Double Stranded cDNA Synthesis kit (Invitrogen). Newly synthesized double stranded DNA was subsequently cleaned using Phase Lock Gels (PLG)-Phenol/Chloroform Extraction. 5 μl of double stranded DNA was then Biotin-labeled following the Enzo Bioarray High Yield RNA Transcript Labeling Kit protocol (Affymetrix). Biotinylated cRNA was subsequently cleaned using a Qiagen RNeasy column and quantitated. 20μg of unadjusted cRNA was then fragmented and hybridized to Affymetrix Test3 chips in order

to verify the quality of each preparation. Samples having similar metrics values were then hybridized to U95A GeneChips® at the University of Florida ICBR MicroArray Core Laboratory.

For the 0- and 1-hour conditions, total RNA was isolated as described. The resulting total RNA was shipped on dry ice to GenUs Biosystems, Inc (Chicago, IL) where the microarray hybridization was performed. Briefly, total RNA samples were quantitated by UV spectrophotometry at OD260/280 and the quality was assessed using an Agilent Bioanalyzer (Agilent Technologies). Once the quality and concentration was confirmed, double stranded DNA was prepared. Biotinylated cRNA targets were subsequently prepared from the DNA template and again verified on the Bioanalyzer. The appropriate amounts of cRNA were next fragmented to uniform size. The fragmented cRNA samples were hybridized to CodeLink™ Human Whole Genome Bioarrays (GE Healthcare, Amersham Biosciences) and stained with Cy5-streptavidin. Slides were scanned on an Axon GenePIX 4000B scanner (Molecular Devices, Axon Instuments).

Microarray Data Analysis

Affymetrix data was analyzed using the Affymetrix Software Package, Microarray Suite Version 5.0. Probe intensities for both cellular conditions were compared and reported in both tabular and graphical formats. GenUs data was analyzed with CodeLink and GeneSpring software packages. To compare individual expression values across arrays, raw intensity data from each probe was normalized to the median intensity of the array. Only genes with normalized expression values greater than background intensity in at least one condition were used for further analysis.

Results

Microarray Analysis of Jak2-dependent Gene Transcription Following 4 hours of AngII Treatment

The γ2A and γ2A/Jak2 cells used in this study have been stably transfected to establish expression of the AT_1 receptor on the plasma membrane. Since we are examining Jak2 signaling in response to AngII, it was necessary to ensure that these cells have the proper machinery to propagate AT_1 receptor-induced Jak/STAT signaling cascades. Previous studies from our lab investigated the ability of γ2A and γ2A/Jak2 cells to function normally in response to AngII treatment (Sandberg *et al.,* 2004). Experiments were conducted in these cells that established the following three parameters: 1) Jak2 is able to become tyrosine phosphorylated in response to AngII, 2) Jak2 forms a physical co-association with the AT_1 receptor following AngII treatment, and 3) STAT1 and STAT3 (downstream targets of Jak2) are able to become tyrosine phosphorylated in response to AngII treatment (Sandberg *et al.,* 2004). As expected, these parameters were only identified in the γ2A/Jak2 cells and not the control cells, which lack Jak2 expression. Furthermore, both cell lines were shown to tyrosine phosphorylate paxillin in response to AngII, which is a Jak2-indepndent target of the AT_1 receptor. Paxillin phosphorylation thereby confirms that the loss of Jak/STAT signaling in the γ2A cells is due to the specific loss of Jak2 function and not due to a clonal artifact inherent in these cells. Thus, these studies determined that the γ2A/Jak2 cell line is a good model for elucidating AngII signaling effects through Jak2 (Sandberg *et al.,* 2004).

We next sought to identify AngII-inducible genes that require Jak2 for their regulation. To do this, four different cellular conditions were created. First, two control conditions were prepared from the γ2A and the γ2A/Jak2 cell lines. These conditions

received no ligand, and therefore served as reference conditions. Next, both cell lines were treated for 4 hours with 100nM AngII. Previous work has determined that AngII is able to induce gene transcription in as little as 15 minutes and for as long as 24 hours after treatment (Taubman *et al.,* 1989; Sadoshima *et al.,* 1997). Given this wide range of transcriptional activation, we decided to examine 4 hours of AngII treatment in an attempt to identify the majority of AngII-responsive genes that would be differentially expressed.

Total RNA was harvested from the four experimental conditions, pooling three plates from each condition to minimize any artifacts. The extracted total RNA was reverse transcribed and biotin-labeled in preparation for hybridization to the Affymetrix U95A microarray chip. The Affymetrix U95A GeneChip® contains probe sequences for ~12,000 fully sequenced human genes. After the RNA probes were hybridized to the Affymetrix microarray chips, pair-wise analyses identified genes having a greater than two-fold change in expression between each condition. The summary of this data is shown as Fig 3-1. For the cells lacking Jak2 (γ2A), 68 genes showed a greater than two-fold change in expression after 4 hours of AngII treatment. However, for the Jak2-expressing cells (γ2A/Jak2), 482 genes had a greater than two-fold change in expression after the same 4-hour AngII treatment. These numbers suggest that the majority of the 482 genes that are differentially expressed in response to AngII are dependent upon Jak2 for regulation. Similar to as was expected, when the two different AngII-treated cell lines where compared to each other, the microarray experiments identified 364 genes to be differentially expressed. Ideally, these 364 genes were identified as AngII-inducible genes that require Jak2 for their transcriptional regulation.

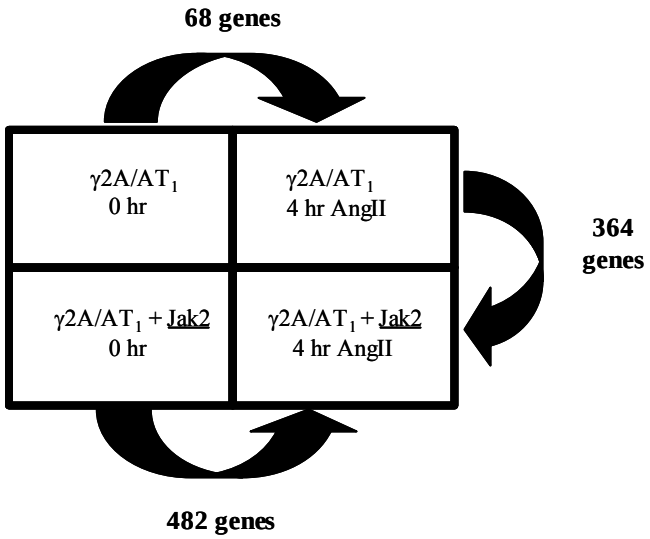


Figure 3-1 Summary of the number of differentially expressed genes identified by the microarray experiments following 4 hours of AngII treatment.

Since there was a possibility that some of the 364 AngII-inducible genes were not dependent upon Jak2, we conducted further analyses. To ensure the genes we had identified where in fact AngII-inducible genes that required Jak2 for their regulation, we combined the lists that represented genes that were regulated by AngII, irrespective of Jak2 (i.e. the lists of 64 and 482 genes). We next compared the 364 genes to this new combined list of AngII-inducible genes and subtracted any gene that was duplicated. By doing this, we ensured that the genes we identified in the microarray experiments were in fact regulated by *both* AngII treatment and Jak2 expression. We assumed that any gene that was duplicated was not dependent upon Jak2, but simply regulated by AngII. The final list of AngII-inducible genes found to be regulated through Jak2 was 254 genes.

Fig 3-2 shows a graphical illustration of mRNA expression levels from the AngII-treated conditions. Each dot on the graph represents one of the 12,000 different genes on the U95A chip. Genes falling outside the two parallel lines demonstrate a greater than 2.0 fold change in gene expression between the two conditions; genes falling above the

two parallel lines had increased gene expression, while genes falling below the two parallel lines had decreased gene expression. The further away from the 2-fold cut-off line that a gene lies, the greater the differential expression that gene displayed between the two conditions. In addition, the farther up the slope a gene lays, the greater the significance of differential gene expression. For example, Trypsinogen IV-B and Trypsinogen C both demonstrate large induction folds (67- fold and 308-fold, respectively). These genes both fall noticeably far from the 2.0 fold cut-off line and relatively high up the slope, away from the origin of the graph.

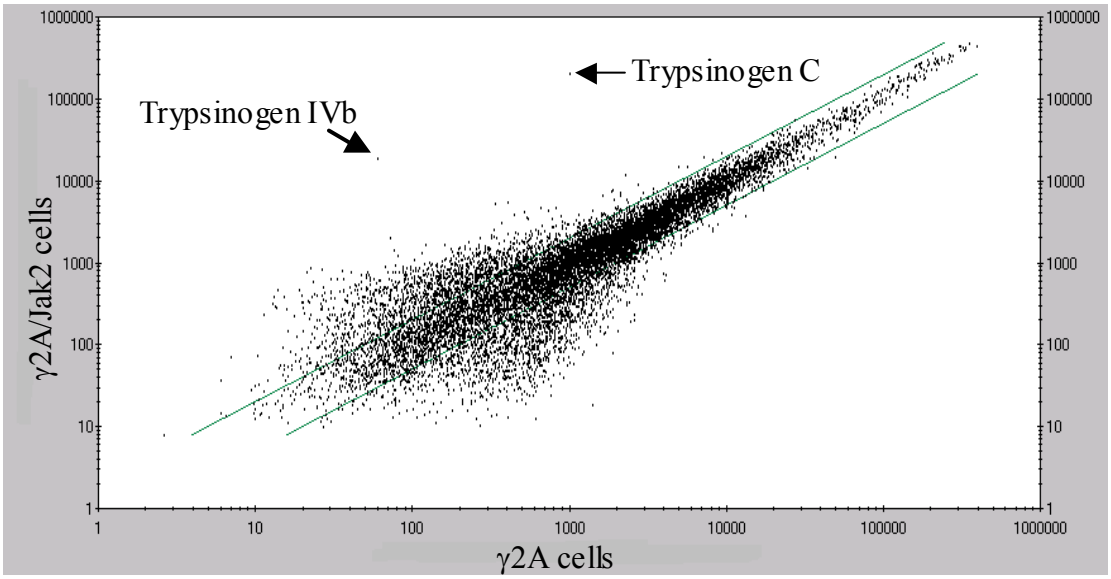


Figure 3-2. Scatter plot analysis of all genes identified during microarray expression profiling of γ2A cells verse γ2A/Jak2 cells treated for 4 hours with AngII. Each dot is the mean value for an individual gene from two arrays. The parallel lines indicate the two-fold differential expression levels.

In summary, these data suggest that Jak2 is responsible for regulating 254 genes by at least 2-fold when activated by AngII. More importantly, this entire procedure was repeated a second, independent time, and very similar results were obtained.

Statistical Analysis of the Affymetrix Microarray Replicated Experiments

The data obtained from both replicates were then further analyzed using various statistical comparisons. Amongst these statistical methods performed were *t*-test comparisons and identification of genes consistent between replicates. Statistical calculations of the microarray data were performed using both Affymetrix MAS5 software as well as Cormibia software. The Cormibia program is a software package that uses more stringent parameters in determining statistical significance of hybridization intensities. Table 3-1 provides a list of five representative genes all demonstrating statistical significance between the 2 independent microarray replicates. These genes represent an important set of cellular functions including angiogenesis, hematopoesis, and Ca^{2+} mobilization.

Table 3-1 Jak2-dependent genes following 4 hours of AngII treatment

Accession #	Fold Change	Gene Name	Category
U23850	↓ 5.2	Type 1 IP_3 receptor	Calcium Signaling
D11151	↓ 3.5	Endothelin-A Receptor	Vasoconstriction
X03656	↑ 13.0	G-CSF	Cell Defense
D83492	↑ 5.5	EphB6	Angiogenesis
U66061	↑ 308.1	Trypsinogen C	Proteolytic Enzyme

Shown are the gene accession numbers, the direction and magnitude of the fold change, the gene name, and a brief category summarizing the gene's function.

Microarray Analysis of Jak2-dependent Gene Transcription Following 1 hour of AngII Treatment

The previous microarray experiments examined genes regulated after 4 hours of AngII treatment. However, AngII can modulate the expression of some genes, such as *c-fos*, in as little as 15 minutes (Naftilan *et al.,* 1990; Viard *et al.,* 1992). The preceding microarray experiments failed to identify *c-fos* as being differentially expressed, either with or without Jak2. Clearly, we know that this gene is AngII-responsive. Therefore,

we hypothesized that there may be a significant number of genes that are expressed prior to the 4 hour time point we analyzed. Thus, we repeated the above experiments as before, this time shortening the AngII treatment to 1 hour.

γ2A and γ2A/Jak2 cells were treated exactly as described above, only this time cells were treated for one hour with AngII. Total RNA was extracted and prepared for hybridization to microarray gene chips. The cRNA probes, representing each of the four conditions, were hybridized to the CodeLink™ Human Whole Genome Bioarray (GenUs Biosystems). This particular expression platform targets ~57,000 transcripts and ESTs, including ~45,000 well characterized human genes and transcript targets.

The data from the four treatment groups was analyzed using CodeLink™ and GeneSpring software packages. Genes having a greater than 2-fold change in expression between the AngII-treated γ2A and γ2A/Jak2 cells were tabulated. The original statistical parameters predicted the expression of over 400 genes as being different between the AngII-treated cell lines. These 400 genes were further filtered down using an array of statistical measures, including the Cross-Gene Error Model algorithm offered by GeneSring software. This Cross-Gene Error Model generates *t*-test *p*-values for each gene as well as standard deviation and standard error. As before, the entire procedure was repeated a second independent time, and in total 65 genes were identified as statistically regulated in both replicates.

Overall, 65 AngII-inducible genes were found to be dependent upon Jak2. Table 3-2 provides a list of three representative genes all demonstrating statistical significance between the 2 independent microarray replicates. Again, a diverse set of genes was identified as being regulated by Jak2.

Table 3-2 Jak2-dependent genes following 1 hour of AngII treatment

Accession #	Fold Change	Gene Name	Category
NM_000581.1	↓ 2.3	Glutathione peroxidase 1	Oxidant Defense
NM_005627.2	↑ 3.3	SGK1	Na^+ Reabsorption
NM_002192.1	↑ 5.89	Erythroid differentiation protein	Cell Differentiation

Shown are the gene accession numbers, the direction and magnitude of the fold change, the gene name, and a brief category summarizing the gene's function.

Discussion

Using gene-profiling technology, this study provides new evidence to support the hypothesis that Jak2 tyrosine kinase is a key mediator of AT_1 receptor signal transduction. While previous studies have implicated Jak2 activation in a number of cardiovascular pathologies, such as neointimal formation, no clear functional consequence of this activation has been defined (Mascareno *et al.,* 2001).

Here, we demonstrate that the recruitment of Jak2 to the AT_1 receptor facilitates AngII-mediated signal transduction that results in the activation of many diverse genes. Some of these genes have previously been identified as targets of AngII signaling. One such example is the IP_3 receptor (Alexander *et al*., 1985). Through the activation of heterotrimeric G-proteins, AngII causes an increase in the production of the intermediate signaling molecule, inositol 1,4,5 trisphosphate (IP_3). Following its production, IP_3 binds to the IP_3 receptor and thereby causes the activation and regulation of the receptor. Furthermore, other genes identified by the microarray experiments have been previously associated with Jak2 signaling, such as the EphB6 gene (Chapter 2). Identifying genes that have been previously established as targets of AngII or Jak2 thereby strengthen the quality of the microarray predictions. The majority of genes that were identified however were novel targets of both AngII and Jak2. These genes offer new insights into the possible mechanisms of Jak2 when activated via the AT_1 receptor.

The number of genes identified as being differentially expressed after 1 hour of AngII treatment differed dramatically when compared to the number of genes regulated after 4 hours AngII treatment. Specifically, the microarray experiments identified 65 AngII-inducible genes as being Jak2-dependent after 1 hour of treatment. Alternatively, after 4 hours of AngII-treatment, the microarray experiments identified over 400% more Jak2-dependent genes (254 genes). One possible explanation for the dramatic difference in the number of genes identified could be the alternate time points. After 4 hours of AngII treatment, the potential for activation of secondary and tertiary genes increases greatly. While these genes may have important biological merit, they may not be directly mediated via Jak2. When the γ2A and γ2A/Jak2 cells were treated for only 1 hour with AngII, the potential for secondary and tertiary genes is dramatically reduced and results in a lower number of gene targets overall.

Another plausible explanation for the variation in the number of differentially expressed genes is the type of expression platforms utilized in each experiment. Microarray technology has undergone increasing popularity over the past decade. Concurrent with the increase in the number of studies using microarray technology, there has been an increase in the development of commercially available analytical software programs. These programs use varying statistical parameters to reduce the tremendous amount of raw data produced. Here, we used both Affymetrix and CodeLink™ expression platforms. Furthermore, the statistical software used for analysis also varied between experiments. Gene-profiling software is continually changing in stringency and methodology to better determine appropriate changes in gene expression. As such, the

difference in the amount of genes identified by the microarray experiments could be a result of the different parameters used in the software analysis.

In summary, using gene-profiling technology, we identified a large number of AngII-inducible genes that are downstream targets of Jak2. The large number of genes identified in this study indicates the critical role Jak2 plays in AngII-mediated transcription. Furthermore, the genes identified in this study can possibly elucidate our current understanding of the role Jak2 plays in the progression of various cardiovascular diseases.

CHAPTER 4
ANGIOTENSIN II INDUCES SGK1 GENE EXPRESSION VIA A JAK2-DEPENDENT MECHANISM

Introduction

The studies presented thus far have focused on the global role of Jak2 in mediating cellular gene transcription. In order to draw meaningful conclusions as to the function of Jak2 in a cell, specific genes must be analyzed. Previous gene profiling experiments identified numerous AngII-inducible genes that require Jak2 for their regulation. One such gene that was found to be dependent upon Jak2 for regulation was the serum and glucocorticoid regulated kinase 1 (*sgk1)*. Here, we sought to determine the precise mechanisms that control the expression and function of *sgk1* in response to AngII treatment.

sgk1 was originally identified as a "serum and glucocorticoid-regulated kinase" in rat mammary tumor cells (Webster *et al.,* 1993a). In the kidney, *sgk1* is an early-induced aldosterone target gene whose product, a serine-threonine kinase, appears to primarily regulate expression and function of the Na^+ epithelial channel (ENaC), as well as possibly other ion transporters. In addition to corticosteroids, a variety of other agonists increase *sgk1* gene transcription in a cell-type specific manner (Alliston *et al.,* 1997; Cowling *et al.,* 2000b; Kumar *et al.,* 1999; Lang *et al.,* 2000; Webster *et al.,* 1993b; Waldegger *et al.,* 1998). However, the specific signaling pathways that mediate the activation of *sgk1* gene transcription by the different agonists have not been well defined.

Currently, the functions of *sgk1* are best characterized in response to its induction via aldosterone. Aldosterone treatment increases *sgk1* gene expression within 15 minutes. Maximum *sgk1* induction peaks at 60 minutes and then returns back toward basal levels over the ensuing 24 hours (Chen *et al.,* 1999). This activation has been found in multiple cells types including; A6 cells, mpkCCD cells, and in the rat collecting duct *in vivo* (Chen *et al.,* 1999; Neray-Fejes-Toth *et al.,* 1999; Shigaev *et al.,* 2000).

Following induction via aldosterone, SGK1 is translated and subsequently phosphorylates its substrate, Nedd4-2. In its unphosphorylated form, Nedd4-2 binds proline-rich motifs (PY) located in the carboxy terminus of ENaC (Kamynina and Staub, 2002). The association of Nedd4-2 with ENaC targets the channel for endocytosis. SGK1 mediated phosphorylation of Nedd4-2 results in its disassociation from ENaC and thereby causes an increase in ENaC abundance and activity at the plasma membrane of epithelial cells. The importance of SGK1 on ENaC regulation has been corroborated in an SGK1 knockout mouse (Wulff *et al.,* 2002; Huang *et al.,* 2004). The *sgk1*-/- mice exhibit normal kidney structure and function under physiological salt intake. However, when dietary salt is restricted, a defect in sodium retention by the kidney leads to a significant decrease in blood pressure (Wulff *et al.,* 2002). Given its roles in regulating ENaC function and expression, SGK1 is regarded as an important signaling molecule in blood pressure regulation.

Here, we explore the regulation of *sgk1* in response to AngII in human fibroblast cells. To date, no direct link has been established between *sgk1* and AngII signaling. Furthermore, we explore the specific Jak2-dependent mechanisms responsible for AngII-induced *sgk1* transcription.

Materials and Methods

Cell Culture

The γ2A and γ2A/Jak2 cell lines were described previously in Chapter 2. The γ2A cells stably expressing either the growth hormone receptor alone (γ2A/GHR) or the growth hormone receptor along with wild type Jak2 (γ2A/GHR/Jak2) have also been described (He *et al.,* 2003). Cells were grown in DMEM +10% FBS at 37ºC in a 5% CO_2 humidified atmosphere. All cells were made quiescent prior to experimentation by washing them extensively with phosphate-buffered saline and then placing them in serum-free media for 20 hours prior to use. Cell culture reagents were obtained from Life Technologies, Inc.

Quantitative RT-PCR

A two-step quantitative RT-PCR method was used to quantify changes in *sgk1* mRNA levels. Specifically, γ2A and γ2A/Jak2 cells were serum starved then treated for 0 or 1 hour with 100nM AngII. Following treatment, total RNA was isolated using the acid guanidine thiocyanate/phenol/chloroform method of extraction (Chomczynski and Sacchi, 1987), exactly as described in Chapter 2. The total RNA was subsequently reversed transcribed using the SuperScript II RNase H^- Transcriptase Kit (Invitrogen). Primers were designed against the *sgk1* gene using PrimerBank, a public resource for PCR primers (http://pga.mgh.harvard.edu/primerbank/) (Wang and Seed, 2003). The PrimerBank ID number for the primer pair used in the experiments was 25168263a1. PCR reactions were prepared using the SYBR Green PCR Core Kit (Applied Biosystems) and performed on the GeneAmp 5700 Sequence Detector machine (Applied Biosystems). 18s primers were used as a standard internal reference and analyses were accomplished

by calculating the $2^{-\Delta\Delta Ct}$ values for each condition (Giulietti *et al*., 2001;Livak and Schmittgen, 2001).

Northern Analysis

Northern Blot analysis was performed as previously described in Chapter 2. Briefly, γ2A and γ2A/Jak2 cells were serum starved and treated for 0 or 1 hour with 100nM AngII. Following treatments, total RNA was isolated and quantitated. 25 μg of total RNA was separated on a 1% agarose-6% formaldehyde-containing gel. RNA samples were transferred onto a charged nylon membrane (Millipore Corporation) and then hybridized to ^{32}P-labeled cDNA probes. Probes were labeled using the Random Primers DNA Labeling System Kit (Invitrogen). The cDNA encoding for *sgk1* was a kind gift from Dr. Florian Lang (University of Tubingen, Germany). Densitometrical analysis was performed using the automated digitizing software, Un-Scan-It, Version 5.1 (Silk Scientific).

Western Blot Analysis

Western blot analysis was performed exactly as was previously described in Chapter 2. Briefly, γ2A and γ2A/Jak2 cells were treated for 0, 30, or 60 minutes with 100nM AngII and whole cell lysates were collected. Lysates were subsequently separated on an 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were Western blotted with an anti-SGK1 polyclonal antibody (Cell Signaling Technology) for 2 hours in 5% milk/TBST. Membranes were subsequently stripped and re-probed with an anti-STAT1 polyclonal antibody (Santa Cruz Biotechnology) to confirm equal loading of all samples.

Luciferase Assay

γ2A and γ2A/Jak2 cells were transfected with 5 μg of a luciferase reporter construct that contains a ~3,000 bp segment (–3142 to +117) of the *sgk1* promoter upstream of the luciferase cDNA (Itani *et al.*, 2002). This construct was a generous gift from Dr. Christie Thomas (University of Iowa). Transfections were performed using Lipofectin (Invitrogen). Following the transfection, the cells were seeded into 12-well plates at 2.5 x 10^5 cells per well, grown for 36 hours, serum starved for 20 hours, and then treated for 0, 4, or 24 hours with 100nM AngII. Luciferase activity was measured from detergent extracts in the presence of ATP and luciferin using the Reporter Lysis Buffer System (Promega) and a luminometer (Monolight Model 3010). Experiments were repeated exactly as described using γ2A/GHR and γ2A/GHR/Jak2 cells. These cells were treated with 600ng/ml GH. Each of the conditions were measured in replicates of six (n=6).

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed using the EZ ChIP™ Kit according to the manufacturer's protocol (Upstate). Briefly, 2 x 10^6 γ2A and γ2A/Jak2 cells were treated for 0 or 20 minutes with 100nM AngII and then cross-linked with 1% formaldehyde at room temperature for 10 minutes. Cells were washed with 2 volumes of ice-cold PBS and then lysed with 1 mL nuclei swelling buffer (5mM PIPES pH 8.0, 8.5mM KCl, 0.5% NP-40). Following a brief centrifugation at 5000 rpm, cells were further lysed in SDS lysis buffer and sonicated using the 60 Sonic Dismembrator (Fisher Scientific) at Output 4.5. Cells were sonicated on ice for 4 cycles in 10-second intervals and allowed to cool for one minute between cycles. Chromatin fractions were spun at 12,000 rpm and the supernatants were then diluted ten-fold in a ChIP dilution buffer. Samples were

subsequently "pre-cleared" by adding 60 μL of Protein G Agarose beads (50% slurry) and shook at 4ºC for 1 hour. Immunoprecipitations were carried out overnight at 4ºC using 2 μg of STAT1, STAT3, or STAT6 antibodies (Santa Cruz) or adding no antibody as a negative control. Following immune complex capture, beads were washed and the complexes were eluted. Cross-links were subsequently reversed by adding 5M NaCl and incubating for 5 hours at 65ºC. DNA was purified and subjected to PCR amplification using the following primers which recognize the STAT-recognition sequence in the *sgk*1 promoter region: forward 5'- GTTTGAAAACAAACATGCAAAAGT-3' and reverse 5'-TTTAGGCAATTTCAAATCACAGTAAC-3'. The PCR products were analyzed by electrophoresis on a 2.5% agarose gel stained with ethidium bromide.

Results

AngII Induces *sgk1* Gene Expression in a Jak2-dependent Manner

Previous microarray experiments identified *sgk1* as a potential downstream target of Jak2 following 1 hour of AngII treatment (Chapter 3). These experiment compared gene expression profiling between a human fibroblast cell line that is devoid of Jak2 protein (γ2A) and the same cell line with the Jak2 protein expression restored via stable transfection (γ2A/Jak2). Briefly, the microarray experiments predicted *sgk1* gene expression to be up regulated by over 3-fold in the γ2A/Jak2 cells when treated with AngII for 1 hour. In order to confirm the validity of the microarray experiments, *sgk1* mRNA levels were analyzed after AngII treatment in γ2A and γ2A/Jak2 cells. Specifically, total RNA was isolated from both cell lines following 0 and 1 hour of treatment with 100nM AngII. The samples were probed with a 1,300 bp human *sgk1* cDNA and analyzed via Northern blot analysis as shown in Fig. 4-1A. Similar to the

microarray experiments, cells expressing Jak2 protein showed an increase in *sgk1* mRNA levels following treatment with AngII. Conversely, Jak2-deficent cells showed no increase in *sgk1* gene expression. Fig. 4-1B shows a quantitation of *sgk1* mRNA levels using densitometrical analysis. Specifically the data revealed that in γ2A/Jak2 cells there was a 3.5 fold increase in *sgk1* mRNA expression over the untreated controls, when corrected for loading and transfer efficiency with GAPDH. Thus, it appears that AngII increases *sgk1* mRNA levels in a Jak2-dependent manner in human fibroblast cells.

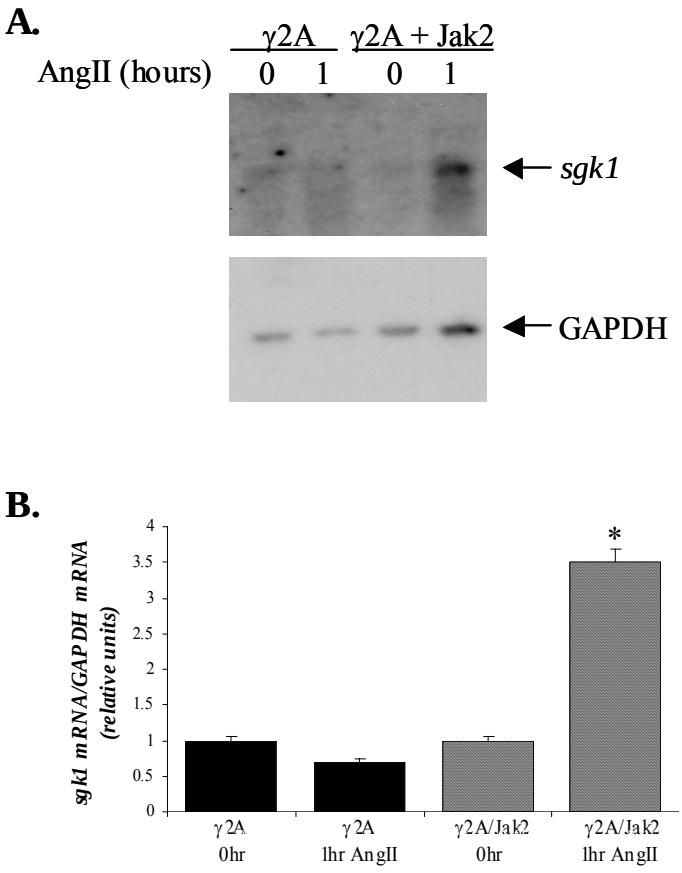


Figure 4-1 Activation of *sgk1* transcription by AngII requires Jak2. A) Northern blot analysis was performed using total RNA isolated from γ2A and γ2A/Jak2 cells. Membranes were probed with cDNA encoding for *sgk1*. Blots were subsequently stripped and re-probed with GAPDH to control for loading. Shown is one of three representative results. B) Densitometrical analysis of three Northern blots quantitating changes in *sgk1* gene expression. Significance was determined using Student's *t*-test.

sgk1 gene expression was further confirmed via quantitative RT-PCR analysis. γ2A and γ2A/Jak2 cells were treated for 1 hour with 100nM AngII. Total RNA was then extracted and samples were reverse transcribed. Quantitative RT-PCR analysis was performed using primers designed for *sgk1* (Fig. 4-2). The data confirms that AngII treatment causes an increase in *sgk1* gene expression in Jak2-expressing cells. This induction of mRNA was not seen in cells lacking Jak2 protein.

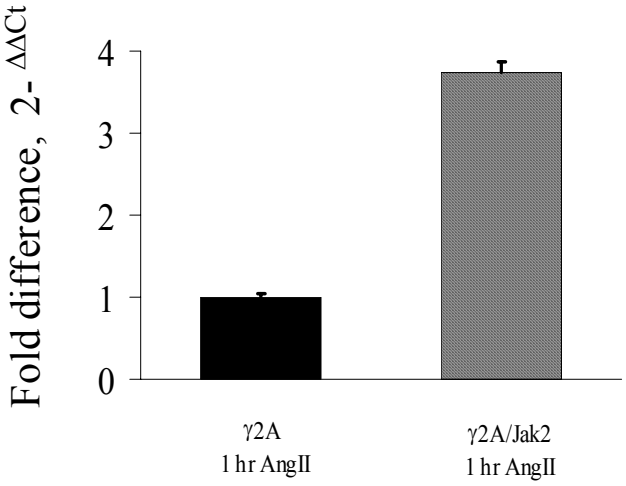


Figure 4-2 Jak2-expressing cells have a greater increase in *sgk1* gene expression than Jak2-deficient cells. Quantitative RT-PCR analysis of total RNA was performed using γ2A and γ2A/Jak2 cells treated for 1 hour with 100nM AngII. Primers were designed for *sgk1*. Fold changes were derived from the $2^{-\spadesuit\spadesuit Ct}$ value and are indicated on the graph. Values are represented as the mean +/- SD. Shown is one of three representative results.

Collectively, Fig. 4-1 and 4-2 strengthen the argument that AngII causes induction of the *sgk1* gene, independent of aldosterone action. Furthermore, it appears that AngII regulates *sgk1* transcription through a Jak2-dependent mechanism.

Jak2 is Critical for AngII-mediated Increases in SGK1 Protein Levels

To determine whether the induction of *sgk1* gene expression by AngII results in a corresponding increase in cellular SGK1 protein levels, whole cell lysates from γ2A and

γ2A/Jak2 cells were analyzed via Western blot analysis. Cells were treated for 0, 30, and 60 minutes with 100nM AngII and then protein content was determined by Western blotting with a polyclonal SGK1 antibody (Fig 4-3). Membranes were subsequently stripped and re-blotted with a STAT1 polyclonal antibody to ensure equal loading. Similar to the gene expression analysis, the γ2A/Jak2 cells showed an increase in SGK1 protein expression after treatment with AngII. This increase was not seen in the γ2A cells, which lack Jak2 protein.

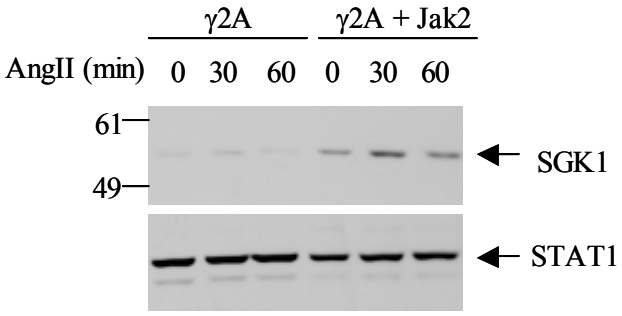


Figure 4-3 Western blot analysis of SGK1 protein expression in γ2A cells compared to γ2A/Jak2 cells following treatment with AngII. Cells were treated with 100nM AngII for 0, 30, and 60 min. Lysates were collected and blotted with an anti-SGK1 polyclonal antibody. The membrane was subsequently stripped and re-blotted with an anti-STAT1 polyclonal antibody to establish equal loading.

AngII, but not Growth Hormone, Causes Activation of the *sgk1* Promoter in Jak2-expressing Cells

We next sought to determine if AngII was causing *sgk1* induction through activation of the *sgk1* promoter. To do this, we transfected a luciferase reporter construct that contains ~3,000 bp of the *sgk1* promoter upstream of a luciferase-coding region into γ2A and γ2A/Jak2 cells. The cells were subsequently serum starved for 20 hrs, and then treated for 0, 4, or 24 hours with 100 nM AngII. Following cell lysis, luciferase activity was measured (Fig. 4-4A). In cells lacking Jak2 protein, there was no significant

increase in luciferase activity in response to AngII treatment. However, the γ2A/Jak2 cells showed a nearly 2.5-fold increase in luciferase activity following a 24-hour treatment with AngII. These data suggest that AngII causes a signaling cascade that results in the activation of the *sgk1* promoter. Furthermore, this transcriptional activation is only seen in cells expressing Jak2.

To determine whether the transcriptional activation of the *sgk1* promoter was specific for AngII treatment, we used γ2A cells that were stably transfected with the growth hormone receptor (GHR). γ2A/GHR and γ2A/GHR/Jak2 cells were transfected with the same luciferase construct as above. Cells were serum starved for 20 hours, treated for 0, 4, or 24 hours with 600ng/mL growth hormone (GH) and then luciferase activity was measured (Fig. 4-4B). This time, both cell types showed no increase in luciferase activity, irrespective of the presence of Jak2. These data indicate that contrary to AngII treatment, activation of Jak2 via GH has no effect on *sgk1* induction.

AngII Causes STAT1 Association with the *sgk1* Promoter Region in γ2A/Jak2 cells

The preceding data suggests that AngII induces *sgk1* transcription via the activation of Jak2. Traditionally, upon activation Jak2 propagates signaling cascades that activate the cytosolic transcription factors, termed STATs. Upon activation by Jak2, STATs will dimerize and translocate into the nucleus where they bind to STAT-recognition sites within the promoter region of a target gene. Most commonly, these STAT-recognition sequences are known as GAS motifs (gamma interferon activated sequences). GAS elements are palindromic response elements that share the general sequence motif $TTCN_mGAA$ (Lew *et al.,* 1991). In this study we questioned if AngII induction of *sgk1* was occurring through a Jak/STAT signaling cascade. Analysis of the ~3kb *sgk1*

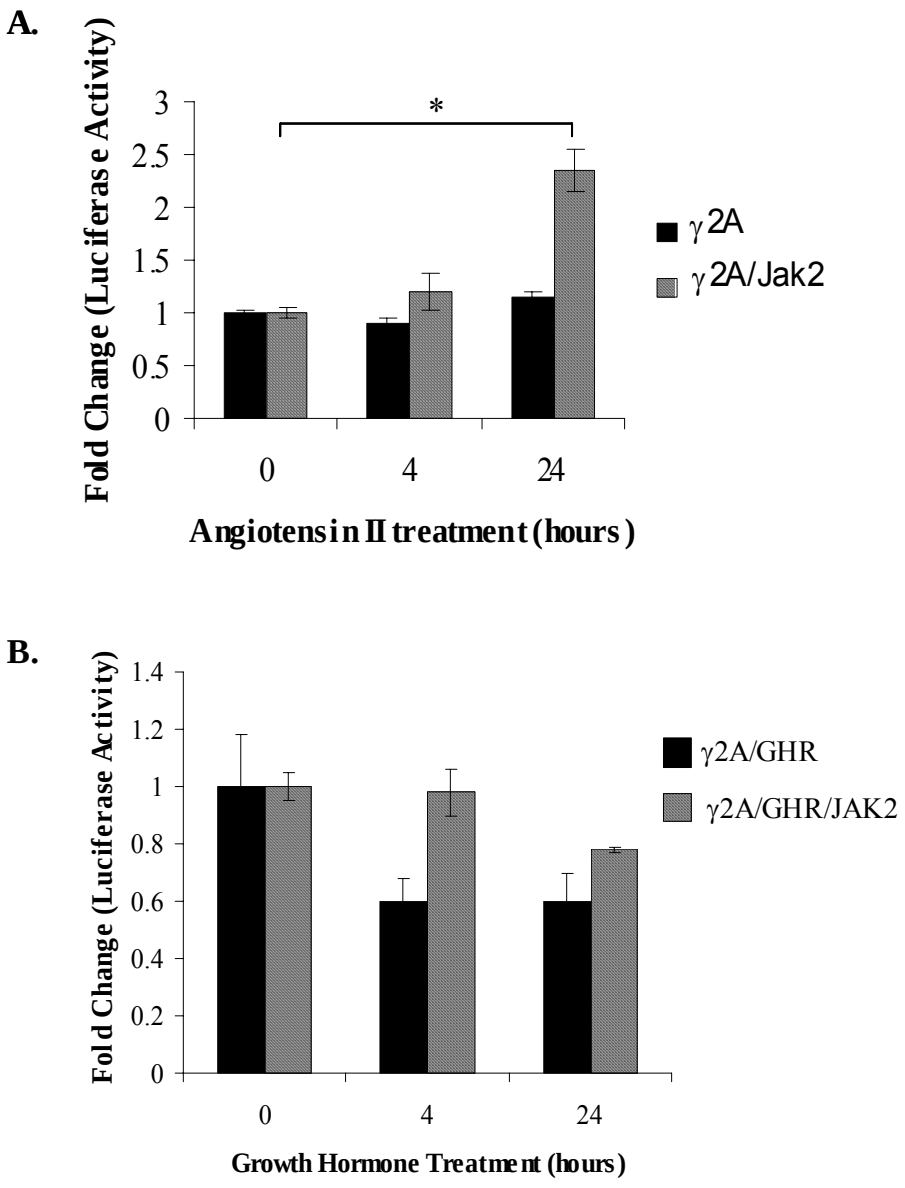


Figure 4-4 AngII activates the *sgk1* promoter in a ligand specific manner. A.) γ2A and γ2A/Jak2 cells were transfected with 5 μg of a luciferase reporter construct containing ~3,000 bp of the *sgk1* promoter upstream of a luciferase gene. Cells were treated with 100nM AngII and then luciferase activity was measured. The difference in luciferase activity between the 0 and 24 hour time points was statistically significant as determined by Student's *t*-test. *, $p = 9.48 \times 10^{-6}$. Shown is one of three independent results. B) γ2A/GHR and γ2A/GHR/Jak2 cells were transfected as above and treated with 600 ng/ml GH. Luciferase activity was then measured. Shown is one of three independent results.

promoter sequence revealed multiple GAS elements (Fig 4-5A). To examine whether STAT proteins associate with the *sgk1* promoter, γ2A and γ2A/Jak2 cells were analyzed by ChIP assays. A specific primer set was designed to amplify a 209 bp DNA fragment of the *sgk1* promoter that contained a GAS element identified at position –725 to -717. γ2A and γ2A/Jak2 cells were treated with 100nM AngII for 0 or 20 minutes, and subsequently analyzed by a ChIP assay (Fig 4-5B). PCR amplification revealed that STAT1 binds to the *sgk1* promoter in γ2A/Jak2 cells following treatment with AngII. As expected, this association was not found in the γ2A cells. Furthermore, when immunoprecipitations were performed using STAT1, STAT3, and STAT6 antibodies, PCR analysis suggested that STAT1 was the preferential STAT binding to the *sgk1* promoter in response to AngII (Fig. 4-5C). These data strengthen the argument that AngII is activating the Jak/STAT pathway to induce *sgk1* transcription.

Discussion

This study provides the first evidence that *sgk1* is induced by AngII via an aldosterone-independent mechanism. Specifically, we suggest that AngII is eliciting its effects on *sgk1* transcription through a Jak2-dependent mechanism.

To date, *sgk1* activation and function are best understood in response to aldosterone. The series of events leading to *sgk1* induction via aldosterone have been well studied. Traditionally, in response to a drop in blood volume, increased renin levels produce AngII. Amongst the many physiological effects of AngII, it acts directly on the adrenal glands to cause the secretion of aldosterone into the blood. Aldosterone subsequently binds to mineralocorticoid receptors within epithelial cells of the kidney

A.

Position	GAS element
(-2376 - -2347)	5' CAAAGTCACA **TTCTATGAA** GATTCCCTGC 3'
(-735 - -706)	5' CGGCGGCTCC **TTCAAGGAA** ACGTCAGTGC 3'

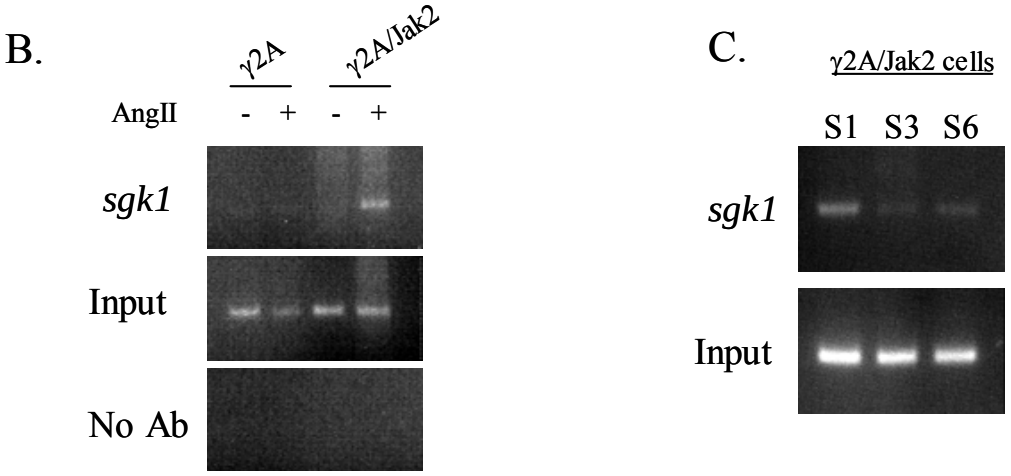


Figure 4-5 AngII causes STAT1 association with the *sgk1* promoter in γ2A/Jak2 cells. A.) Identification of GAS elements found within the ~3,000 bp region of the *sgk1* promoter B.) ChIP assay investigating STAT1 binding to the *sgk1* promoter in γ2A and γ2A/Jak2 cells. Cells were treated for 0 or 20 min with 100nM AngII and then subsequently subjected to formaldehyde cross-linking. Immunoprecipitation were performed using an anti-STAT1 antibody or no antibody (negative control). Purified DNA was analyzed by PCR with a primer set specific for the *sgk1* promoter region containing a GAS element. Input corresponds to 1/100 of the amount of DNA used in the assay. C.) ChIP assay analyzing which of the STATs binds preferentially to the *sgk1* promoter region. γ2A/Jak2 cells were treated for 20 min with 100nM AngII. Immunoprecipitations were performed using STAT1 antibody (S1), STAT3 antibody (S3), and STAT6 antibody (S6). Purified DNA was analyzed by PCR with a primer set specific for the *sgk1* promoter region containing a GAS element. Input corresponds to 1/100 of the amount of DNA used in the assay.

and directly causes an increase in *sgk1* transcription. Therefore, while previous studies have implicated an *indirect* role of AngII in mediating *sgk1* induction, there has been no evidence supporting a *direct* role. Here, we suggest that AngII is increasing *sgk1* transcription, but through an aldosterone-independent mechanism. As opposed to triggering aldosterone secretion, we propose that AngII is signaling through tyrosine

kinases to elicit its effects on *sgk1* transcription. Further investigations are required to determine the physiological consequences of this *sgk1* induction via AngII.

Our data show cells expressing Jak2 protein can increase *sgk1* expression following treatment with AngII. Alternatively, Jak2-deficient cells lack increases in *sgk1* expression. Therefore these studies suggest a critical role for Jak2 in regulating *sgk1* transcription. We hypothesized that AngII mediates *sgk1* transcription through the initiation of Jak/STAT signaling cascades. This hypothesis was supported by two main reasons.

First, Jak2 has been well established in the literature as being activated via the AT_1 receptor (Marrero et al., 1995). Specifically, AngII binding to the AT_1 receptor causes cytosolic Jak2 to become activated and subsequently form a physical association with the intracellular tail of the AT_1 receptor. After being recruited to the receptor, Jak2 initiates a tyrosine phosphorylation cascades that results in the activation and dimerization of the STATs. STAT dimers consequently translocate into the nucleus where they mediate gene transcription. Thereby, if *sgk1* transcription is being induced by a Jak2-dependent mechanism, it is probable that Jak2 is acting through the STATs.

Second, we identified multiple STAT-recognition sequences within the promoter region of *sgk1*. Previous work has elucidated the preferential binding parameters for the specific STATs. STAT6 dimers prefer $TTC(N)_4GAA$ whereas the remaining STAT dimers will traditionally recognize $TTC(N)_3GAA$ motifs (Schindler *et al.,* 1995; Seidel *et al.,* 1995; Horvath *et al.,* 1995). The specific motifs that were identified in this study suggest that a member of the STAT family, other than STAT6, may potentially be binding to the promoter of *sgk1* and initiating transcription. These observations further

strengthen the argument that AngII induces *sgk1* transcription via the activation of a Jak/STAT pathway

When the hypothesis was specifically investigated using the ChIP assay, we found that STATs were indeed involved in the AngII-induced increases in *sgk1* transcription. The data in Figure 4-5 shows that STAT1 is physically binding to the *sgk1* promoter region in Jak2-containing cells following treatment with AngII. While there was evidence that STAT3 and STAT6 may be having a minor contribution to *sgk1* transcription, we hypothesize that STAT1 is the preferential STAT involved in *sgk1* induction at position –725 to -717.

Interestingly, when cells are stimulated with GH, a well-known activator of Jak2, no significant increase in *sgk1* transcription is observed. While Jak2 is activated by a diverse set of ligands, it is unclear whether its downstream targets are ligand-specific. This work suggests that the transcriptional effects of Jak2 on *sgk1* induction are specific for treatment with AngII. This is in agreement with previous studies that suggest GH activates different STATs through specific mechanisms (Carter-Su *et al*, 1997). Namely, studies have shown that GH causes robust activation of STAT3 and STAT5b in certain cell types (Yi *et al*, 1996). Given the diversity of GH signaling, we believe activation of Jak2 via GH causes recruitment of different STATs as when AngII activates Jak2. Therefore, we conclude that Jak2 demonstrates ligand specificity, as evidenced by the lack of *sgk1* induction when GH activates Jak2.

In conclusion, these studies suggest that AngII mediates *sgk1* expression through a Jak2-dependent mechanism. Furthermore, AngII may now be regarded as a mediator of *sgk1* induction, independent of its actions through aldosterone.

CHAPTER 5
JAK2 PREVENTS ANGIOTENSIN II-MEDIATED INOSITOL 1,4,5 TRISPHOSPHATE RECEPTOR DEGRADATION

Introduction

The Type 1 inositol 1,4,5 trisphosphate (IP_3) receptor was amongst the genes identified in Chapter 3 as being a potential target of Jak2, when activated via the AT_1 receptor. While previous studies have established a relationship between AngII and the IP_3 receptor (Alexander *et al*., 1985), no such correlation has been made linking Jak2 to the IP_3 receptor. Therefore, we sought to elucidate the complex regulation of the IP_3 receptor in response to AngII. Specifically, we investigated the regulatory effects of signaling cascades initiated by Jak2.

As previously described in Chapter 1, the IP_3 receptors are intracellular calcium channels expressed on the membrane of the endoplasmic reticulum (ER). IP_3 is a second messenger produced through the stimulation of PLC-coupled receptors, such as the AT_1 receptor. IP_3 binding to its obligatory receptor results in a rapid release of calcium from internal stores via a non-selective cation pore in the C-terminal portion of the channel (Boehning *et al*., 2001). Three structurally distinct IP_3 receptors have been identified (Nakagawa *et al*., 1991). Of the three subtypes, Type 1 has the highest expression throughout all cell types studied (De Smedt *et al*., 1994; Wojcikiewicz, 1995).

Maintaining precise regulation of calcium signaling within a cell is critical for normal cellular functions. Regulation of calcium is maintained via a complex interplay between changes in cytosolic IP_3 concentration and IP_3 receptor expression on the

membrane of the ER. Regulation of IP_3 receptor expression and function can be mediated via its phosphorylation by multiple kinases such as cyclic-AMP-dependent protein kinase (PKA), protein kianse C (PKC) and Fyn tyrosine kinase (Ferris *et al*., 1991a, 1991b; Jayaraman *et al*., 1996). Specifically, Fyn has been shown to bind to and phosphorylate the IP_3 receptor at tyrosine 353 (Y^{353}) in activated T-cells (Jayaraman *et al*., 1996). Evidence suggests that the phosphorylation of Y^{353} via Fyn increases the binding affinity of IP_3 to its receptor when there are low concentrations of IP_3 within the cytosol (Cui *et al*., 2004). However, the effect of Y^{353} phosphorylation in response to ligand treatment (i.e., high IP_3 levels) has not yet been defined.

Here, we investigate the role of Jak2 in regulating the expression and function of the IP_3 receptor in response to AngII. Using rat aortic smooth muscle (RASM) cells over-expressing a dominant negative Jak2, we determined that the loss of a functional Jak2 contributes to AngII-mediated degradation of the IP_3 receptor. Since previous data show that Fyn, a downstream target of Jak2, is able to phosphorylate the IP_3 receptor at Y^{353}, we believe Jak2 prevents the AngII-mediated IP_3 receptor degradation via the activation of Fyn. In conclusion, these data suggest that Jak2 has a protective role in maintaining IP_3 receptor expression, potentially through activation of Fyn and subsequent phosphorylation of the IP_3 receptor.

Materials and Methods

Cell Culture

Creation of the γ2A and γ2A/Jak2 cells have previously been described in Chapter 2. Additionally, creation of the RASM-WT and RASM-DN cells have also been previously described (Sayeski *et al*., 1999a). All cells were maintained at $37^{B}C$ in a 5%

CO_2 humidified atmosphere. Prior to experimentation, all cells were made quiescent by washing them extensively with phosphate-buffered saline (PBS) and then placing them in serum-free media for either 20 hours (γ2A) or 48 hours (RASM). Cell culture reagents were obtained from Life Technologies, Inc. AG490, AG-9, PP-2, PP-3, and lactacystin were all purchased from Calbiochem. Losartan was from Merck.

Quantitative RT-PCR

A two-step quantitative RT-PCR method was used to quantify changes in IP_3 receptor gene expression. Specifically, the γ2A and the RASM-derived cell lines were serum starved and then treated for 0 or 4 hours with 100nM AngII. Following treatment, total RNA was isolated using the acid guanidine thiocyanate/phenol/chloroform method of extraction (Chomczynski and Sacchi, 1987). The total RNA was subsequently reversed transcribed using the SuperScript II RNase H^- Transcriptase Kit (Invitrogen). Primers were designed against the Type 1 IP_3 receptor gene using PrimerBank, a public resource for PCR primers (http://pga.mgh.harvard.edu/primerbank/) (Wang and Seed, 2003). The PrimerBank ID number for the primer pair used in the experiments was 598181a1. PCR reactions were prepared using the SYBR Green PCR Core Kit (Applied Biosystems) and performed on the GeneAmp 5700 Sequence Detector machine (Applied Biosystems). 18s primers were used as a standard internal reference and analyses were accomplished by calculating the $2^{-\Delta\Delta Ct}$ values for each condition (Giulietti *et al*., 2001;Livak and Schmittgen, 2001).

Western Blot Analysis

Western blot analysis was performed exactly as was previously described in Chapter 2. Briefly, whole cell lysates from RASM-WT and RASM-DN cells were

collected following the appropriate treatments described in each experiment. Lysates were subsequently separated on an 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were Western blotted with an anti-Type 1 IP_3 receptor polyclonal antibody (Upstate Biotechnology) for 2 hours in 5% milk/TBST. Membranes were subsequently stripped and re-probed with an anti-STAT1 polyclonal antibody (Santa Cruz Biotechnology) to confirm equal loading of all samples. Densitometrical analysis was performed using the automated digitizing software, Un-Scan-It, Version 5.1 (Silk Scientific).

Immunofluorescence

The Type 1 IP_3 receptor was visualized using immunofluorescence. Cells were grown on 2-chambered microscope slides composed of #1.0 German Borosilicate Coverglass (Lab-Tek). After treatment with AngII for 0 or 1 hour, cells were washed with K^+-free PBS and then fixed at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 minutes. Fixed cells were subsequently washed four times with K^+-free PBS, permeabilized for 10 minutes at room temperature with 0.2% Triton X-100 in K^+-free PBS, washed an additional four times, and then blocked with 5mg/ml bovine serum albumin in K^+-free PBS for 4 hours at room temperature. The cells were then incubated with a primary anti-IP_3 receptor antibody (1:200) overnight at 4^BC using 5mg/ml BSA in K^+-free PBS. The following day, cells were washed four times and incubated with a goat anti-rabbit secondary antibody conjugated to FITC (1:500) for 4 hours at room temperature. Cells were mounted with Vectashield mounting medium supplemented with Dapi (Vector Laboratories, Inc.). Images were collected using the

Zeiss Axioplan 2 Fluorescence Microscope. Cells were visualized using a magnification of 100x (oil emersion objective).

Calcium Studies

Fura-2/AM loading and intracellular calcium measurements were carried out as previously described (Xia *et al.,* 2004). In short, cells were loaded at room temperature for two hours in HEPES-buffered solution containing 5-10 μM Ca^{2+} indicator fura-2/AM (Calbiochem), then washed three times and incubated for an additional 20 minutes in dye-free solution to reduce the possibility of incomplete hydrolysis of the acetoxymethyl esters by intracellular esterases. Fura-2/AM was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the loading and experimental solution was below 0.5% (v/v).

The $[Ca^{2+}]_i$ measurements were made with an ratiometric imaging system (InCyt Im2, Intracellular Imaging, Inc., Cincinnati, OH), including a PC computer, a filter wheel of conventional design, a CCD camera, and a Nikon TE 300 microscope with 40× air objective (0.65 N.A.). Cells were continuously superfused with Ringer's solution (in mM, 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES; pH 7.4 with NaOH) or Ringer's solution plus AngII through a gravity-fed system at a rate of 3-4 ml•min^{-1}. Solutions were evacuated by suction. In each experiment, a number of either single cells or a group of cells were selected using the software setting. The fluorescent emissions as paired signals (F340 and F380) at wavelength of 510 nm from the region of interest were measured accordingly to excitation wavelengths of 340 nm and 380 nm, at a time interval of every three seconds. Background fluorescence was subtracted online from F340 and F380 signals.

Changes in $[Ca^{2+}]_i$ are reported as the mean fluorescence ratio of F340/F380 over time for a group of cells. The mean fluorescence ratio of F340/F380 was generated offline and expressed as R/R_0, with R being the fluorescence ratio change over time and R_0 the averaged fluorescence ratio of a period of 60-120 seconds before AngII addition.

The final results from each group of experiments (n) are reported as the mean peak response (means ± SE). Statistical significance was examined using Student' *t*-test. A value of $P < 0.05$ was considered significant.

Results

Jak2 Regulates IP_3 receptor Gene Expression Following Treatment With AngII

Chapter 3 describes gene-profiling experiments that identified AngII-inducible genes that require Jak2 for their regulation. Briefly, microarray experiments compared a Jak2-deficient cell line (γ2A) to a similar cell line expressing Jak2 (γ2A/Jak2) that had been treated with 100nM AngII for either 0 or 4 hours. Analysis revealed numerous AngII-inducible genes that had a greater than 2.0-fold change in expression as a function of Jak2. Amongst these genes was the Type 1 IP_3 receptor. Quantitative RT-PCR was used to validate the microarray data using the γ2A and γ2A/Jak2 cells. The IP_3 receptor expression pattern found by the quantitative RT-PCR analysis was similar to that observed in the microarray studies (Fig. 5-1A). To eliminate the possibility that the increase in IP_3 receptor expression was clonal artifact inherent to the γ2A-derived cells, we next investigated IP_3 receptor expression in rat cultured aortic smooth muscle cells over-expressing a dominant negative form of Jak2. Aortic smooth muscle cells were chosen because they have high expression of the Type 1 IP_3 receptor (Marks, 1992). In addition, these cells are a more physiologically relevant cell type for investigating AngII-

dependent changes since they are highly contractile cells. Specifically, the cells used in this study were rat aortic smooth muscle cells stably expressing either 1) a dominant negative Jak2 protein and the Neomycin selectable marker (RASM-DN) or 2) just the Neomycin selectable marker alone (RASM-WT). The RASM-DN cells suppress endogenous Jak2 function by roughly 90% when compared to the RASM-WT cells. The full characterization of these cells has been previously described (Sayeski *et al*., 1999a).

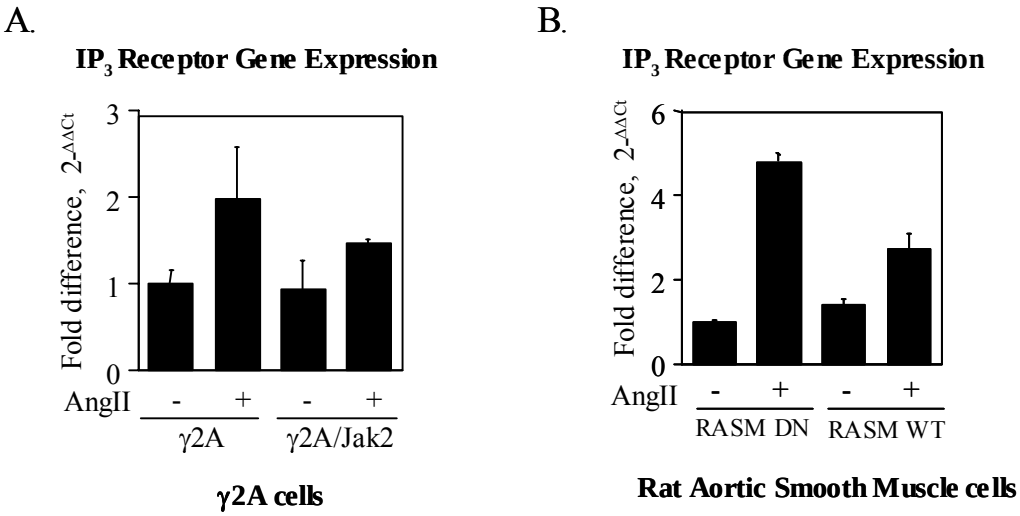


Figure 5-1. Cells having little to no functional Jak2 protein have a greater increase in IP_3 receptor gene expression than when compared to cells expressing Jak2. Quantitative RT-PCR analysis of total RNA was performed on either γ2A and γ2A/Jak2 cells (A) or RASM-WT and RASM-DN cells (B). Primers were designed for Type 1 IP_3 receptor. Fold changes were derived from the $2^{-\spadesuit\spadesuit Ct}$ value and are indicated on the graph. Values are represented as the mean +/- SD. Shown is one of three representative results. Printed with permission of publisher

Quantitative RT-PCR analysis found an expression profile of IP_3 receptor that was similar between the γ2A and RASM cells (Fig 5-1B). Specifically, in cells lacking a functional Jak2, there is a marked increase in IP_3 receptor gene expression following 4 hours of 100nM AngII (~2-fold in γ2A, ~4-fold in RASM-DN). Conversely, cells that do have functional Jak2 demonstrate a significantly lower increase of IP_3 receptor

expression in response to AngII (~0.5-fold in γ2A/Jak2, ~2-fold in RASM-WT). In summary, the quantitative RT-PCR experiments suggest that in cells *having little to no* Jak2, AngII-induced IP_3 receptor gene transcription is increased to a greater degree than in cells that *have* functional Jak2 protein.

Cells Lacking Functional Jak2 Undergo AngII-mediated Degradation of the IP_3 receptor

Western blot analysis was used to investigate the protein levels of IP_3 receptor in AngII-treated RASM-WT cells compared to RASM-DN cells. Cells were treated for 0, 1, and 4 hours with 100nM AngII. Whole cell protein lysates from each condition were then separated by SDS-PAGE and subsequently Western blotted with an anti-IP_3 receptor antibody (Fig 5-2A, top). Overall, RASM-DN cells have significantly less IP_3 receptor protein expression when compared to the RASM-WT cells. To ensure equal protein loading, the membrane was stripped and re-blotted with an anti-STAT1 antibody (Fig. 5-2A, bottom). Next, this experiment was repeated as before, but this time a 2-fold increase in RASM-DN whole cell lysate was loaded (relative to the RASM-WT lysate) as means to obtain detectable levels of the IP_3 receptor protein. Interestingly, when IP_3 receptor expression was visualized in the RASM-DN cells, we found that there was a marked decrease in IP_3 receptor protein following 1 and 4 hours of AngII treatment (Fig 5-2B, top). These data suggest the protein is being degraded in response to AngII. However, this did not occur in the RASM-WT cells. To confirm the levels of lysate loaded into each lane, the membrane was stripped and re-probed with anti-STAT1 antibody to confirm the roughly 2-fold increase in STAT1 protein in the RASM-DN cells (Fig 5-2B, bottom). These experiments indicate that while there is less IP_3 receptor

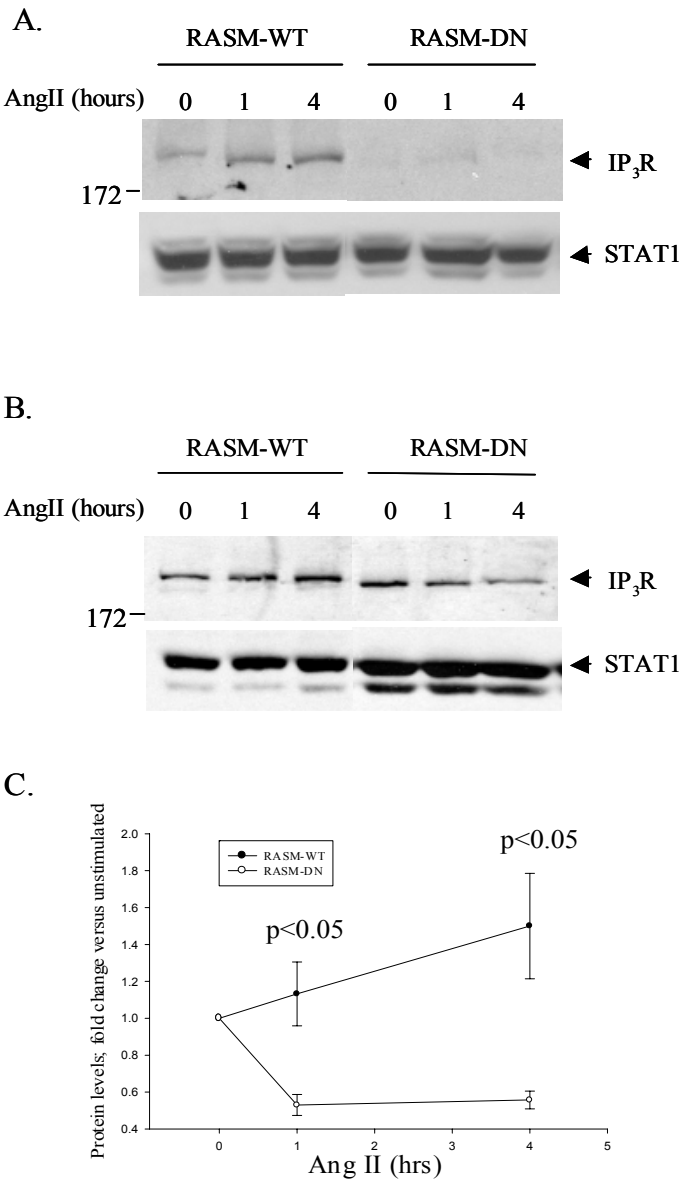


Figure 5-2. Cells lacking functional Jak2 undergo AngII-mediated degradation of the IP_3 receptor. A) Western blot analysis of IP_3 receptor expression in RASM-WT cells compared to RASM-DN cells following treatment with AngII. Cells were treated with 100nM AngII for 0, 1, and 4 hours. Lysates were collected and blotted with an anti-IP_3 receptor polyclonal antibody (specific for Type 1). The membrane was subsequently stripped and re-blotted with an anti-STAT1 polyclonal antibody to establish equal loading. B) Similar Western blot analysis was performed, but with twice as much RASM-DN whole cell lysate. C) Densitometry analysis of IP_3 receptor expression of eight representative Western blots. Significance was determined using Student's *t*-test. Printed with permission of publisher

protein expressed in Jak2-impared cells, the protein that *is* present, is degraded in response to AngII treatment.

Densitometry analysis of IP_3 receptor protein expression was done on eight representative Western Blots in order to quantitate the AngII-inducible degradation of the IP_3 receptor (Fig 5-2C). We found that there was a significant reduction in IP_3 receptor expression at 1 hour following AngII treatment in the RASM-DN cells when compared to untreated RASM-DN cells (47.0±5.7%, $P<0.05$). Conversely, although a mild increase in IP_3 receptor expression in RASM-WT cells was evident following 1 and 4 hours of AngII treatment, this was not significantly different when compared to untreated RASM-WT cells.

In conclusion, Western blot analysis of IP_3 receptor protein suggests that, in the absence of functional Jak2, there is AngII-mediated degradation of the receptor. Interestingly, this observation was the inverse of what we observed at the transcriptional level. In other words, in the RASM-DN cells, one hour after AngII treatment there is a marked degradation of IP_3 receptor protein when compared to the RASM-WT cells. However, 4 hours after AngII treatment in the RASM-DN cells, the mRNA levels are significantly greater than those in the RASM-WT cells, as seen in Fig 1B. Thus, we hypothesize that the increased mRNA levels observed in the RASM-DN cells 4 hours after AngII treatment is compensatory to the protein degradation seen after 1 hour of AngII treatment in these cells.

RASM-WT Cells Treated With AG490 Recapitulate AngII-mediated IP_3 receptor Degradation in RASM-DN Cells

In order to eliminate the possibility that the degradation of the IP_3 receptor was the result of clonal artifact within the RASM-DN cells, Jak2 was pharmacologically

inhibited using AG490 in RASM-WT cells. RASM-WT cells were pre-treated for 16 hours with 100μM of AG-9, an inactive analogue, or with the Jak2 pharmacological inhibitor, AG490. Following pretreatment with the inhibitor, the cells were treated for 0, 1, and 4 hours with 100nM AngII. Cells from each treatment condition were then lysed and subsequently Western blotted with an anti-IP_3 receptor polyclonal antibody (Fig 5-3, top). The data show that pharmacological inhibition of Jak2 function by AG490 induces AngII-mediated IP_3 receptor degradation similar to that shown in the RASM-DN cells. The membrane was then stripped and re-blotted using an anti-STAT1 polyclonal antibody to ensure equal protein loading (Fig 5-3, bottom).

Collectively, the data demonstrate that when Jak2 tyrosine kinase function is blocked with AG490, AngII treatment produces a similar IP_3 receptor degradation pattern as is seen when Jak2 function is blocked via the dominant negative protein.

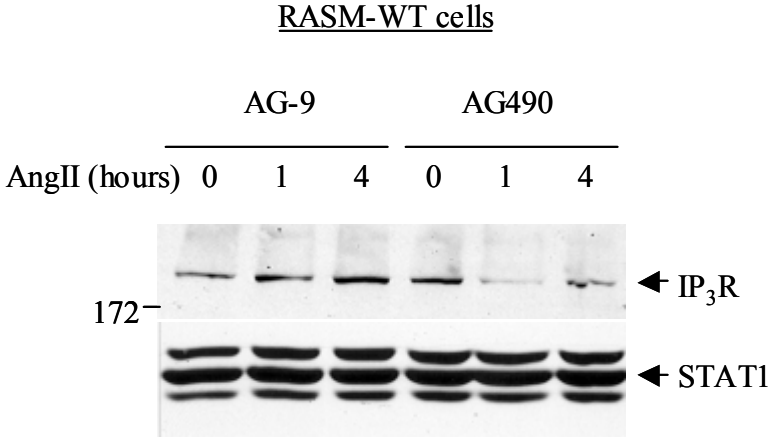


Figure 5-3. RASM-WT cells treated with AG490 recapitulates AngII-mediated IP_3 receptor degradation seen using RASM-DN cells. RASM-WT cells were pretreated with 100μM of either AG-9 or AG490 for 16 hours. Following pretreatment, cells were treated with 100nM AngII for 0, 1, or 4 hours and Western blotted with an anti-IP_3 receptor antibody. Membranes were re-blotted with an anti-STAT1 antibody to ensure equal protein loading. Shown is one of four representative results. Printed with permission of publisher

AngII-mediated IP_3 receptor Degradation is Reversible Following Recovery From AngII

To determine if AngII-mediated IP_3 receptor degradation in RASM-DN cells is reversible following recovery from AngII, RASM-DN cells were treated with 100nM AngII for 4 hours. The AngII-containing media was then removed and cells were allowed to recover for 2, 20, and 24 hours in media that lacked AngII. Whole cell lysates were prepared from each condition and Western blotted for IP_3 receptor as shown (Fig. 5-4, top). The results show that following 4 hours of AngII treatment, the IP_3 receptor undergoes degradation, as previously shown. However, within 2 hours of removal of AngII from the incubation medium, the IP_3 receptor expression began rapid recovery and demonstrated full restoration by 24 hours. The membrane was eventually re-blotted for STAT1 expression to establish equal loading across all lanes (Fig. 5-4, bottom).

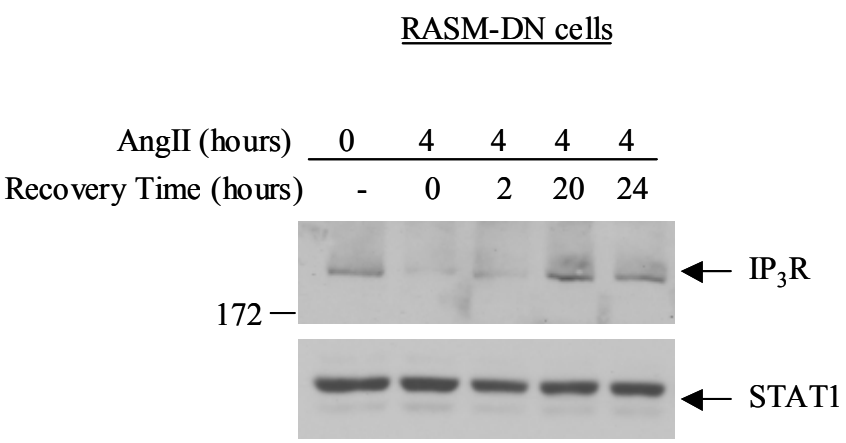


Figure 5-4. AngII-mediated IP_3 receptor degradation is reversible following recovery from AngII. RASM-DN cells were treated with 100nM AngII for 0 or 4 hours. The cells were allowed to recover for 0, 2, 20, or 24 hours in media that was free of AngII. IP_3 receptor expression was determined via Western blot analysis. STAT1 expression was analyzed to ensure equal protein loading. Shown is one of four representative Western blots. Printed with permission of publisher

In summary, these data show that the rapid IP_3 receptor degradation in cells lacking a functional Jak2 is reversible once the influence of AngII is removed from the culture media.

AngII-inducible Degradation of the IP_3 receptor Occurs via the AT_1 receptor and Through a Proteosome-dependent Mechanism

To elucidate the mechanism of AngII- inducible degradation of the IP_3 receptor, RASM-DN cells were treated with losartan, an AT_1 receptor blocker, or lactacystin, a specific proteasome inhibitor. Losartan was used to identify the specific

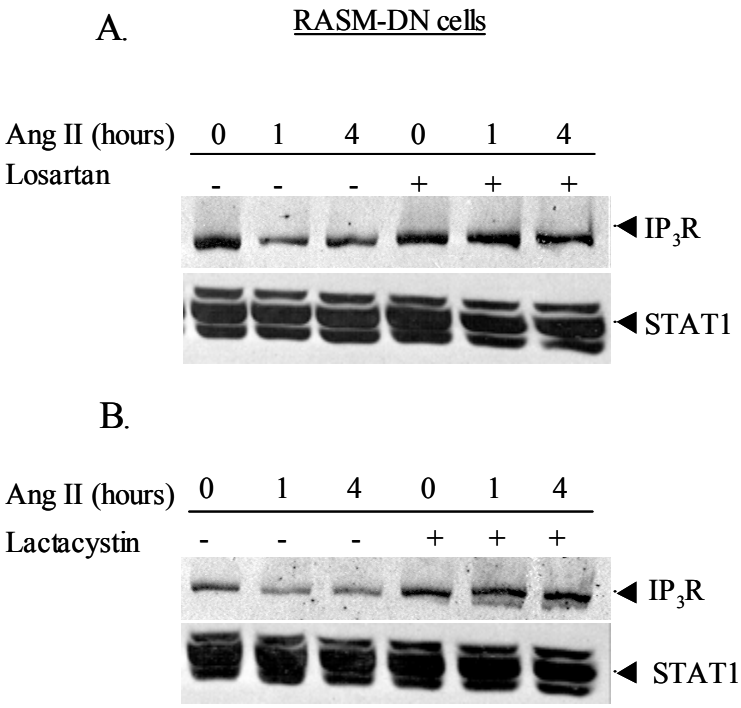


Figure 5-5. IP_3 receptor degradation is dependent upon AngII and occurs through a proteasome-dependent mechanism. A) RASM-DN cells were pretreated with 10μM losartan for 30 minutes and then treated with 100nM AngII for 0, 1, and 4 hours. Lysates were collected and Western blotted with an antibody against the IP_3 receptor. B) RASM- DN cells were pretreated with 8μM lactacystin for 13 hours and then treated with 100nM AngII for 0,1, and 4 hours. Lysates were collected and Western blotted with an antibody against the IP_3 receptor. The samples were also Western blotted with anti-STAT1 antibody to confirm equal loading. Shown is one of three representative results for each. Printed with permission of publisher

type of AngII receptor that is linked to IP_3 receptor degradation. Lactacystin was used to determine the specific mechanism responsible for the IP_3 receptor degradation. RASM-DN cells were either pretreated with 10μM of losartan for 30 minutes or 8μM lactacystin for 13 hours. Following pretreatment, cells were treated for 0, 1, and 4 hours with 100nM AngII. Whole cell protein lysates were then separated by SDS-PAGE and subsequently Western blotted with anti-IP_3 receptor antibody (Fig. 5-5A and 5-5B). Analysis shows that AngII-dependent IP_3 receptor degradation was lost when the cells were pretreated with either losartan or lactacystin, suggesting the degradation of IP_3 receptor is dependent upon the AT_1 receptor and occurs through the proteaosome-dependent pathway.

Thus, we conclude that the mechanism by which AngII promotes IP_3 receptor degradation requires both the AT_1 receptor and a functional proteaosome.

Immunofluorescence Experiments Demonstrate that IP_3 receptor in RASM-DN cells is Rapidly Degraded Following AngII Treatment

We next used immunofluorescence analysis to visualize the changes in IP_3 receptor expression in RASM-WT and RASM-DN cells following treatment with AngII. Specifically, RASM-WT and RASM-DN cells were either left untreated or were treated for 1 hour with 100nM AngII. Cells were subsequently fixed and stained using an antibody specific for the Type 1 IP_3 receptor. The cellular localization of the IP_3 receptor was then visualized using florescent microscopy (Fig. 5-6). In the RASM-WT cells, prior to AngII treatment there was strong staining throughout the cell and this pattern did not change with AngII treatment. In the RASM-DN cells however, prior to AngII treatment there was marked perinuclear staining and this was lost following AngII treatment.

Collectively, the data further suggest that the loss of Jak2 within a cell, results in the AngII-dependent degradation of the IP_3 receptor.

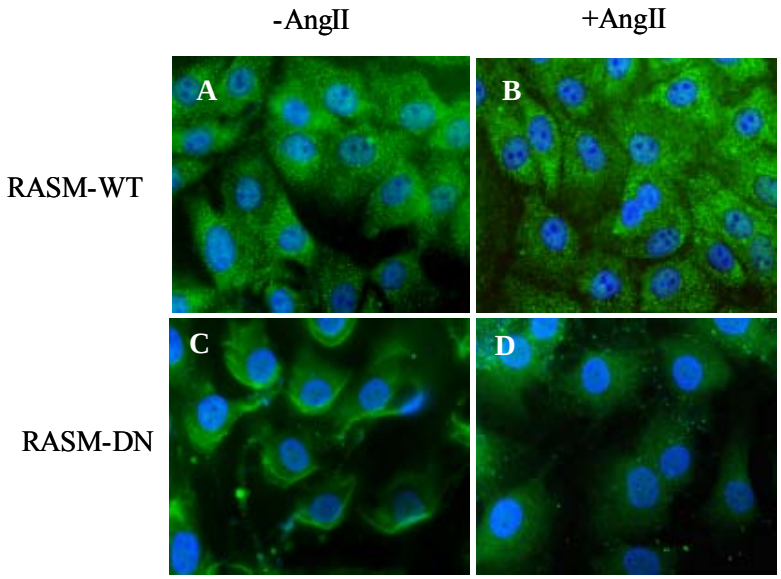


Figure 5-6. The IP_3 receptor is rapidly degraded following AngII treatment. The Type 1 IP_3 receptor was visualized using immunofluorescence. RASM-WT (A, B) and RASM-DN (C, D) cells were either left untreated or treated for 1 hour with AngII as indicated. Cells were visualized using an antibody specific for the Type 1 IP_3 receptor. A FITC-conjugated 2º antibody was used to detect IP_3 receptor (green) and the nuclei were counter stained with DAPI (blue). Images were collected using the Zeiss Axioplan 2 Fluorescence Microscope. Shown is one of three representative results. Printed with permission of publisher

RASM-DN Cells Have a Reduction in AngII-induced Calcium Mobilization When Compared to RASM-WT Cells

If the expression of IP_3 receptors were reduced in RASM-DN cells, it could affect the intracellular calcium release from IP_3-sensitive calcium stores. To test this hypothesis, we carried out intracellular calcium measurements using the calcium indicator fura-2. The ratio of emitted fura-2 fluorescence was used as an index of the increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Figure 5-7A shows that a short time (3 min) exposure of extracellular AngII could induce a transient increase in $[Ca^{2+}]_i$ in both RASM-WT and RASM-DN cells. However, the peak response of $[Ca^{2+}]_i$ in RASM-DN

cells was significantly reduced than that in RASM-WT cells. Figure 5-7B shows the comparisons of intracellular calcium response to 1nM AngII application (DN 27.4 ± 1.1 % vs. WT 42.2 ± 4.7 %; n = 5, respectively; $P = 0.002$) and to 10nM AngII application (DN 50.0 ± 8.1 % vs. WT 95.8 ± 20.9 %; n = 4, respectively; $P = 0.007$). These experiments indicate that the release of Ca^{2+} from IP_3-sensitive Ca^{2+} stores was affected in the RASM-DN cells.

To further verify the role of Jak2 signaling pathway in AngII-induced calcium response, we examined RASM-WT cells that were pre-treated with the Jak2 inhibitor AG490 (100 μM for 16 hours). Figure 5-7C shows that the mean peak calcium response was significantly reduced in the AG490-treated cells (control 88.3 ± 25.9 % vs. AG490 39.0 ± 6.9 %; n = 3, respectively; $P = 0.03$). This observation is consistent with the Western blot data in Figure 5-3 showing that AG490 causes a reduction in the IP_3 receptor levels.

Inhibition of Fyn tyrosine kinase Results in a Reduction of IP_3 receptor Expression

Fyn tyrosine kinase phosphorylates the IP_3 receptor at Y^{353} in activated T-cells (Cui *et al*., 2004). Furthermore, previous work from our group has shown that in response to AngII, Jak2 promotes Fyn activation (Sayeski *et al*., 1999a). The effect of Fyn on IP_3 receptor phosphorylation in response to AngII treatment in smooth muscle cells has not yet been determined. To investigate the role of Fyn in regulating IP_3 receptor expression, RASM-WT cells were pretreated for 16 hours with 8μM of PP2, a Src tyrosine kinase family inhibitor, or PP3, an inactive analogue. Following the pretreatment, cells were treated with 100nM AngII for 0 or 4 hours. Whole cell lysates were then collected and Western blot analysis was performed using an antibody against

the IP_3 receptor (Fig 5-8, top). RASM-WT cells pretreated with PP2 showed a dramatic decrease in IP_3 receptor expression when compared to control cells. The membranes were subsequently stripped and re-blotted with an anti-STAT1 antibody to confirm equal loading amongst all lanes (Fig 5-8, bottom).

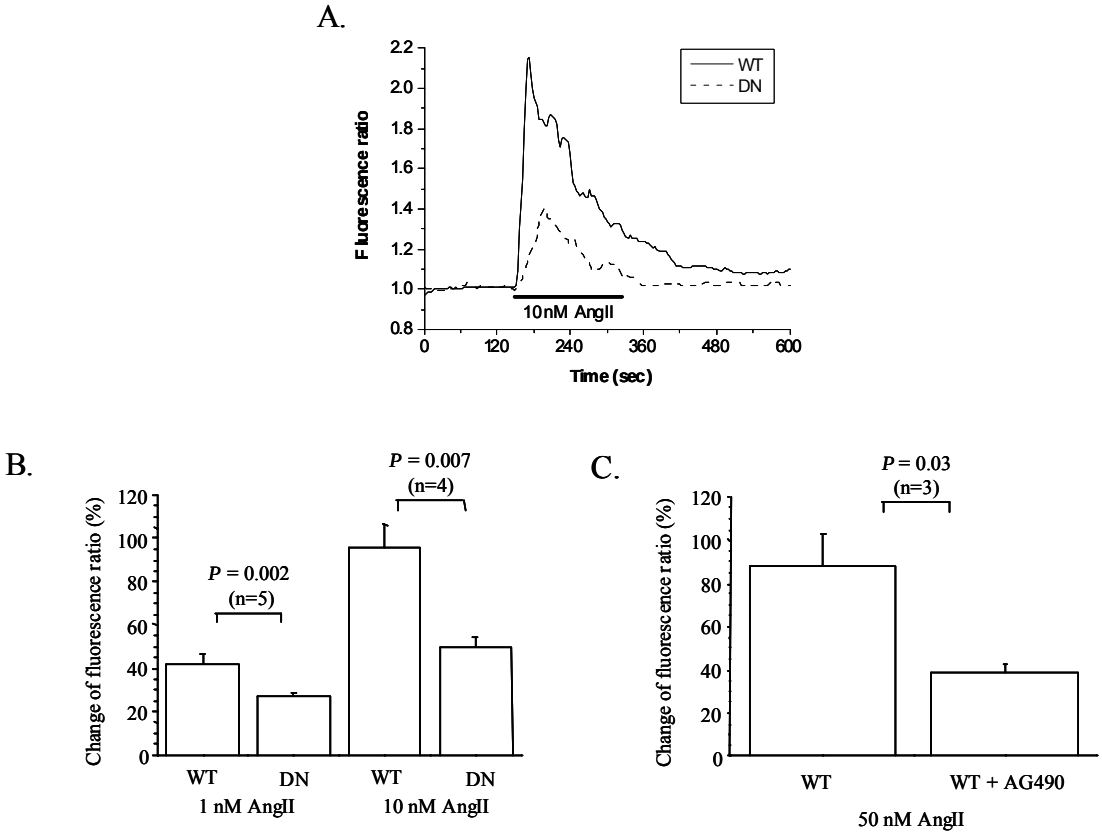


Figure 5-7. Functional difference of RASM-WT and RASM-DN cells in response to AngII. A) Representative traces from RASM-WT (solid line) and RASM-DN (dashed line) cells show the difference of intracellular calcium response to 10 nM AngII application. Each trace represents an averaged response of at least 5 cells. Bar indicates the duration (3 min) of AngII application in the bath. B) Peak calcium responses of RASM-WT cells to 1 nM and 10 nM AngII are compared to the peak calcium responses of RASM-DN cells, respectively. C) Mean peak calcium response to AngII (50 nM) in WT cells pre-treated with Jak2 inhibitor AG490 (100 M) was significantly reduced. The number in parentheses represents the number of experiments carried out to determine the mean peak increase in calcium signaling and each experiment represents the response from at least 50 cells. Statistical values are means ± SE from non-paired *t*-test. Printed with permission of publisher

These results suggest that Fyn contributes to IP_3 receptor protein expression in wild type RASM cells, potentially through the phosphorylation of Y^{353} in the IP_3 binding domain of the IP_3 receptor.

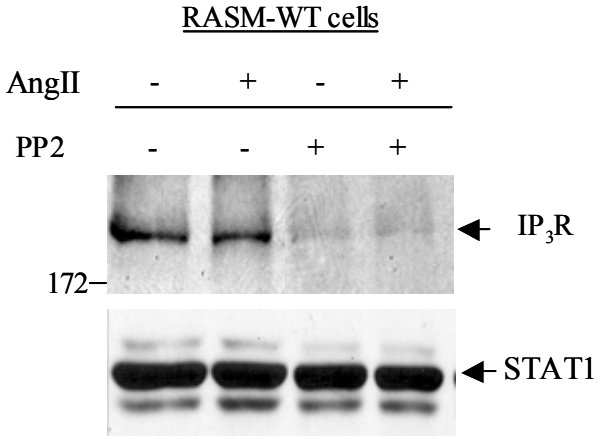


Figure 5-8. Inhibition of Fyn tyrosine kinase results in a reduction of IP_3 receptor expression. RASM-WT cells were pretreated for 16 hours with 8μM of PP2. Following the incubation with PP2, cells were treated with 100nM of AngII. Lysates were collected and IP_3 receptor expression was determined using an anti-IP_3 receptor antibody. The membrane was subsequently stripped and re-probed using an anti-STAT1 antibody to confirm protein loading. Shown is one of three independent results. Printed with permission of publisher

Discussion

In this study we investigated the role of Jak2 in regulating the expression and function of the Type 1 IP_3 receptor in response to treatment with AngII. Using RASM cells over-expressing a Jak2 dominant negative protein, we showed rapid AngII-mediated degradation of the IP_3 receptor in cells lacking a functional Jak2. Interestingly, this loss of IP_3 receptor correlated to a reduction in AngII-induced calcium mobilization. These data therefore suggest that when Jak2 is lacking from a cell, the IP_3 receptor is targeted for proteasome-dependent degradation.

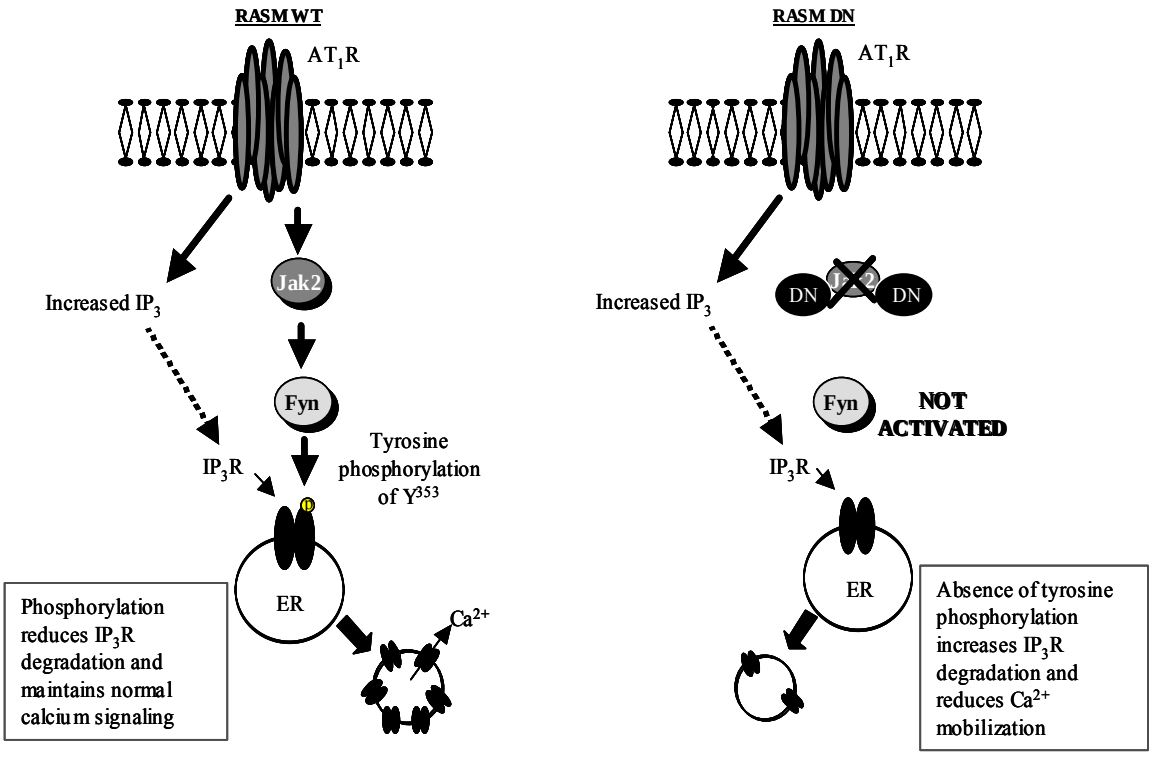


Figure 5-9. Proposed model for regulation of IP_3 receptor via Jak2. In this model, RASM-WT cells (left) generate high concentrations of IP_3 in response to AngII via heterotrimeric G-proteins that are coupled to the AT_1 receptor. In addition to IP_3 generation, the AT_1 receptor also activates Jak2. Jak2 in turn activates Fyn. Once activated, Fyn is able to phosphorylate the IP_3 receptor. Conversely, in the RASM-DN cells (right), the AT_1 receptor generates IP_3, but fails to activate Jak2, and as a result, Fyn is not activated. We propose that the lack of IP_3 receptor phosphorylation via Fyn make the receptor vulnerable to proteosome-dependent degradation. Printed with permission of publisher

Figure 5-9 illustrates our proposed model for AngII-mediated IP_3 receptor degradation in RASM cells. Briefly, prior data shows Jak2 is able to activate Fyn downstream of the AT_1 receptor (Sayeski *et al.*, 1999a). Fyn in turn has been shown to phosphorylate the IP_3 binding domain of the IP_3 receptor in activated T cells (Cui *et al.*, 2004). To date, there has been no functional consequence of this phosphorylation in response to AngII treatment. Here, we hypothesize that when the IP_3 receptor is phosphorylated by Fyn, possibly at Y^{353}, it is protected from AngII-induced degradation.

In the absence of Jak2, and therefore the absence of Fyn activation, the IP_3 receptor is unable to be phosphorylated and is therefore vulnerable to proteasome-dependent degradation events following AngII treatment.

Previous microarray studies within our lab identified the IP_3 receptor as a potential target of Jak2 downstream signaling (Chapter 3). Quantitative RT-PCR analysis found that, following chronic stimulation with AngII, the gene expression profile of the IP_3 receptor was different in cells that lack Jak2, versus cells that have functional Jak2. The increase in gene expression was significantly higher in cells lacking a functional Jak2. Interestingly, this data directly conflicts with the analysis of IP_3 receptor protein expression. Contrary to the increase in mRNA gene expression, our Western blot data of RASM-DN protein lysates demonstrate a dramatic decrease in IP_3 receptor protein in response to AngII. We believe that the increase in IP_3 receptor mRNA levels in the Jak2-DN cells is a compensatory effect of the rapid degradation of the IP_3 receptor protein. Once IP_3 receptor protein has recovered to normal levels from the effects of AngII, we find that the mRNA expression also return to normal levels. Therefore, we do not believe that Jak2 is having a direct transcriptional effect on the IP_3 receptor gene. Instead, we believe that Jak2 is mediating the IP_3 receptor at the protein level through signaling events within the cytosol, such as the activation of Fyn.

Reduced IP_3 receptor expression in a cell can correlate to decreases in intracellular calcium mobilization. When analyzed for their ability to mobilize calcium, the RASM-DN cells clearly demonstrated a significant reduction in calcium signaling when compared to RASM-WT control cells. Even WT cells treated with a Jak2 inhibitor demonstrated a significant decline in AngII-inducible calcium signaling, thereby

strengthening the critical role Jak2 has in the regulation of IP_3 receptor expression, and subsequently, calcium mobilization.

Our data summarized in Figure 2 through Figure 6 suggest that cells lacking a functional Jak2 undergo degradation of the IP_3 receptor following 1 or 4 hours of AngII treatment. Given our data, we hypothesized that the loss of the IP_3 receptor would contribute to a reduction in AngII-induced calcium signaling. Notably, the cells used in the calcium studies were not pre-treated with AngII for 1 or 4 hours prior to calcium measurements. However, it was not surprising to see a significant difference in calcium handling between the DN and WT cells at baseline (i.e., prior to AngII treatment). As shown in Figure 2A and Figure 6, there are significantly less amounts of IP_3 receptor expressed in the RASM-DN cells prior to any treatments with AngII. In addition, Figure 8 shows that when RASM-WT cells are pre-treated with PP2, there is a dramatic decrease in IP_3 receptor expression, and again this decrease is prior to AngII treatment. These figures collectively support that there is a reduction of IP_3 receptor expression, and thereby calcium signaling, in cells lacking a functional Jak2 (or Fyn) prior to AngII-mediated degradation of the receptor. Due to rapid desensitization of the AT_1 receptor, it was difficult to visualize calcium signaling following pre-treatment of AngII for 1 or 4 hours. However, we hypothesize that if we were able disregard the AT_1 receptor desensitization and perform calcium measurements following AngII treatment for 1 and 4 hours, there would be an even larger difference between RASM-WT and RASM-DN cells. Furthermore, we believe this increased difference in calcium handling could be attributed to the degradation of the IP_3 receptor in the DN cells.

While the involvement of AngII in cardiovascular disease has been well established, the role of Jak2 in cardiovascular pathology is an ongoing area of research. Numerous examples within the literature have found coinciding roles of the renin-angiotensin system and Jak2 activation. For example, Jak2 is activated in response to balloon injury of rat carotid arteries. This activation of the JAK/STAT signaling cascade leads to increased neointima formation as a result of vascular smooth muscle cell proliferation (Seki *et al*., 2000; Shibata *et al*., 2003). Additionally, Jak2 is activated in response to both mechanical stretch and acute pressure overload being partially mediated through an AngII-dependent mechanism (Pan *et al*., 1997, 1999). Given the numerous examples demonstrating the overlap of Jak2 and AngII in the progression of vascular disease states, we feel it is advantageous to identify the downstream targets of Jak2 via the AT_1 receptor. This study highlights one such downstream target of Jak2, the IP_3 receptor. Elucidating the precise role of Jak2 in regulating the IP_3 receptor in response to AngII may contribute to determining the role of Jak2 in the progression of some vascular pathologies.

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CHAPTER 6
CONCLUSIONS AND IMPLICATIONS

The data presented in this dissertation investigated the downstream signaling effects of Jak2 tyrosine kinase. This work is significant for a number of reasons. First, we examined the effects of Jak2 expression in cells, independent of its activation via an exogenous ligand. The data suggest that Jak2 has a large regulatory role in a cell that is outside of the traditional ligand-activated signaling paradigm (Wallace *et al.,* 2004). Second, we investigated the downstream targets of Jak2 activation via the AT_1 receptor. The data provide insight into the specific Jak2-dependent signaling cascades that are initiated in response to AngII treatment. Third, we investigated the specific regulation of *sgk1* in response to AngII. The data suggest that AngII induces *sgk1* expression through a Jak2-dependent mechanism. Lastly, we investigated the role of Jak2 in the regulation of IP_3 receptor expression and function. Specifically, the data suggest that Jak2 protects the IP_3 receptor from AngII-mediated degradation (Wallace *et al.,* 2005).

A more detailed and intricate discussion about the specific implications of the data presented within this dissertation is discussed below.

Ligand-Independent Activation of Jak2

Previously, Jak2 was thought to have a very one-dimensional role within the cell. It was believed to reside inertly within the cytoplasm and/or associated with its respective cell surface receptors (Frank *et al.,* 1994; Ali *et al.,* 1997; Sayeski *et al.,* 2001). Initial biochemical and cellular studies suggested that only when Jak2 was activated by ligand-binding, could it propagate the Jak/STAT signaling cascade to the nucleus, resulting in

transcriptional regulation (Witthuhn *et al.,* 1993; Argetsinger *et al.,* 1993; Rui *et al.,* 1994; Narazaki *et al.,* 1994; Marrero *et al.,* 1995). However, the one-dimensional view of Jak2 activation is beginning to be re-evaluated. Recent studies have suggested the existence of at least two distinct catalytic states of Jak2 (Chatti *et al.,* 2004). Currently, however, no functional consequence has been attributed to these different activation levels. Here, using gene-profiling technology we explored the hypothesis that Jak2 may in fact have varying degrees of catalytic activation and that these activation levels result in significant changes in gene transcription. The microarray experiments detailed in Chapter 2 identified a large number of genes that were differentially expressed by Jak2 in the absence of exogenous ligand treatment. These data thereby indicate the important cellular consequence Jak2 has in its basal level of activation.

In conclusion, the ability of Jak2 to regulate gene expression through basal level activation is a paradigm shift from previous schools of thought. The data presented in Chapter 2 suggest that the role of Jak2 deviates from the accepted presumption that it functions in a solely ligand-activated system, turned on and off as distinctly as one turns on and off a light switch. Instead the data presented here, as well as work from others, suggest that Jak2 functions more dynamically via its various catalytic activation states. The cartoon in Figure 6-1 illustrates this point graphically. The switch on the left illustrates the simple binary toggle between "on and off" while the switch on the right illustrates the potentially more dynamic range of Jak2 activation consistent with that seen using a dimmer switch. Overall, while the cartoon is simplistic in nature, it is used to illustrate the point that Jak2 appears to regulate gene transcription much more complexly than previously thought.

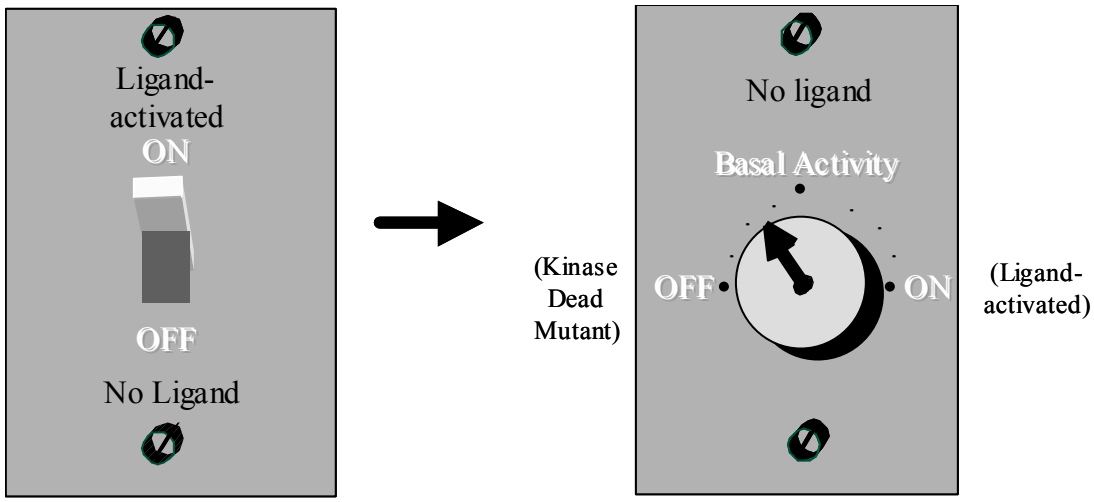

Figure 6-1. Comparison between previous and current signaling paradigms of Jak2. The switch on the left depicts the simple ON/OFF model of ligand-dependent gene transcription. In the absence of ligand, Jak2 is catalytically inactive and thereby is unable to mediate gene transcription. Conversely, in the presence of ligand, Jak2 becomes catalytically active and demonstrates a corresponding increase in gene transcription. The switch on the right depicts a model whereby the multiple catalytic states of Jak2 correspond to different levels of gene transcription. The OFF position is representative of a Jak2 that is kinase dead (i.e. Jak2-K882E mutant) and corresponds to a complete lack of gene transcription. The BASAL ACTIVITY position is representative of Jak2 being a low efficiency tyrosine kinase (i.e. unphosphorylated Y1007). In this state, there is a basal level of gene transcription occurring. The ON position is representative of fully activated Jak2 as that seen with ligand treatment (i.e. phosphorylated Y1007). In this state, gene expression levels are maximally turned ON.

Transcriptional Roles of Jak2 in Response to Angiotensin II Signaling

The work presented in Chapter 3 used gene-profiling technology to investigate the effects of Jak2 activation via the AT_1 receptor. The data confirms that Jak2 is an important mediator of AngII-induced gene transcription. This is evidenced by the large number of Jak2-dependent genes that were identified by the microarray experiments. A number of implications can be concluded from these experiments.

First, this work did not merely identify genomic targets of Jak2, as was the original intent. Rather, this work also provided novel insight into the non-transcriptional effects

Jak2 has within a cell. As discussed previously, the microarray experiments identified Jak2-dependent genes following 1 and 4 hours of AngII treatment. The data revealed a dramatic difference in the number of genes that were identified between the two time points. Namely, the 1-hour time point identified much fewer genes. We hypothesize that a majority of the genes identified after the 4-hours of AngII treatment may be secondary and tertiary response genes. This hypothesis is strengthened by the analysis of specific genes identified by the microarray experiments. For our studies, we chose one gene from the 1-hour time point and one gene from the 4-hour time point for further analysis. (*sgk1* and IP_3 receptor, respectively). Our data suggests that Jak2 is *directly* regulating *sgk1* transcription after 1 hour of AngII treatment and is *indirectly* effecting IP_3 receptor transcription after 4 hours of AngII treatment. Therefore, we conclude that after 1- hour of AngII treatment the microarray data provide a reliable source of direct transcriptional targets of Jak2. Conversely, the experiments identifying Jak2-dependent genes after 4 hours of AngII treatment are a compilation of both direct and indirect gene targets. These experiments therefore provide insight on the functional consequences Jak2 has that are independent of regulating gene expression.

Next, identification of novel targets of Jak2 may contribute in understanding the role Jak2 has in cardiovascular diseases. For example, glutathione peroxidase-1 (GPx-1) was identified as being down regulated by 3.5-fold in cells that express Jak2 following 1-hour of AngII treatment. GPx-1 is an antioxidant that is important for protecting cells against oxidative damage. Recently, studies have identified GPx-1 as a biomarker for cardiovascular disease risk. These studies showed that patients with reduced GPx-1 activity demonstrate a significant increase in cardiovascular risk (Schnabel *et al.,* 2005).

Further studies have implicated a role for GPx-1 in ischemia-reperfusion injury (Toufektsian *et al.,* 2000; Venardos *et al.,* 2004; Venardos *et al.,* 2005). Specifically, when GPx-1 is down regulated, recovery from ischemia-reperfusion injury is impaired. Interestingly, Jak2 activation has also been associated with cardiac injury during ischemia-reperfusion (Mascareno *et al.,* 2001). *In vivo* studies show that treatment with AG490, an inhibitor for Jak2, leads to a reduction in cardiac infarct size and a corresponding reduction in apoptosis of cardiomyocytes following ischemia-reperfusion injury. Nonetheless, despite the evidence supporting Jak2's activation in response to ischemia/reperfusion, there remains no elucidation of the functional significance of the activation. Additional studies are necessary to determine the specific relationship between Jak2 and GPx-1, but we hypothesize that Jak2 activation via the AT_1 receptor results in the transcriptional down-regulation of GPx-1. This down-regulation may play an important role in the detrimental effects associated with ischemia reperfusion injury. In conclusion, we believe that further studies investigating the target genes of Jak2 may elucidate the role Jak2 has in AngII-induced pathologies.

AngII Induces *sgk1* Transcription

In addition to suggesting a novel role for Jak2 in regulating *sgk1* transcription, these data suggest, for the first time, that AngII is directly inducing *sgk1* transcription independent of aldosterone production. Our data specifically suggests that AngII is activating a Jak/STAT pathway to regulate *sgk1* transcription in human fibroblast cells.

While these studies investigated the signaling cascades that cause AngII-mediated *sgk1* induction, the functional consequences of *sgk1* induction remain to be investigated. We hypothesize that the consequences of AngII-mediated *sgk1* induction in fibroblast cells are separate from the well-defined roles of aldosterone-mediated *sgk1*

induction in the kidney. This hypothesis is based upon the dramatic phenotype of the mineralcorticoid receptor (MR) knockout mouse (Berger *et al.,* 1998). As expected, MR knockout mice demonstrate severe pseudohypoaldosteronism. This is a condition characterized by severe loss of Na^+ despite evidence of hyponatremia, hyperkalemia, hyperreninemia, and elevated aldosterone levels (Rosler *et al.,* 1973). The aldosterone resistance in these animals is so severe the mice die within 2 weeks of birth from the effects of sodium wasting (Berger *et al.,* 1998). There is no evidence detailing any significant functions of aldosterone outside of its transcriptional actions and, to date, there are few other genomic targets of aldosterone in addition to *sgk1*. Therefore, it is probable that the sodium wasting in the MR knockout mice is so severe because there are no additional activators of *sgk1* transcription that are sufficient to compensate for the loss of aldosterone action. As such, we believe that the effects of AngII on *sgk1* induction are distinct from the effects of aldosterone.

Recent studies have investigated a number of potential physiological roles for SGK1, including its effects on regulating apoptosis and its contribution in the progression of fibrosing diseases. In addition to Nedd4-2, SGK1 phosphorylates several other proteins. The identification of SGK1 substrates was facilitated by its similarity to the anti-apoptotic protein, protein kinase B (PKB). The catalytic domain of SGK1 is 54% identical to that of PKB and studies have identified both protein kinases can phosphorylate similar substrates (Kobayashi and Cohen, 1999; Park *et al.,* 1999). For example, similar to PKB, SGK1 phosphorylates members of the Forkhead Family of transcription factors, termed FKHR, FKHRL1, and AFX (Brunet *et al.,* 2001). Phosphorylation of these transcription factors inhibits the transcription of apoptotic

signals, and thereby promotes cell survival. The role of SGK1 in apoptosis was further confirmed by studies that showed a significant decrease in apoptosis when SGK1 is over-expressed in cells. Furthermore, overexpression of a catalytically inactive mutant of SGK1 caused a significant increase in apoptosis (Mikosz *et al.,* 2001). Thus, we believe that since AngII can promote cell survival through activation of PKB signaling, it may be having similar effects on cell survival through its activation of SGK1.

In addition to its modulation of apoptosis, we hypothesize that SGK1 may be contributing to the progression of various fibrosing diseases. While the precise roles of SGK1 remain to be determined, studies have identified significant increases in SGK1 transcription in various pathologies such as diabetic nephropathy (Hoffman *et al.,* 1998; Kumar *et al.,* 2000; Lang *et al.,* 2000; Waldegger *et al.,* 1999) and fibrosing pancreatitis (Waldegger *et al.,* 1997). While further investigations are required to confirm, we suggest that AngII-induced activation of SGK1 may be a contributor of these pathologies.

In conclusion, *sgk1* is an appealing candidate for blood pressure regulation and possibility plays a role in essential hypertension (Busjahn et al., 2002). Therefore, understanding the mechanisms for AngII-mediated *sgk1* induction may have therapeutic merit in the future.

Jak2 Regulates AngII-mediated IP_3 receptor Degradation

The studies presented in Chapter 5 investigated the role of Jak2 in regulating the expression and function of the IP_3 receptor in smooth muscle cells. Understanding the precise role of Jak2 in regulating intracellular Ca^{2+} levels may elucidate its contributions to the progression of various cardiovascular pathologies.

The precise regulation of intracellular Ca^{2+} is critical for normal vascular smooth muscle (VSM) function (Santella, 1998). VSM cells are a non-proliferating, contractile

cell type under normal conditions. However, following vascular insults such as balloon injury, VSM cells adopt a highly proliferative, non-contractile phenotype (Thyberg *et al.,* 1995; Shanahan *et al.,* 1998). This switch in phenotype often exacerbates preexisting pathologies. Currently, the mechanisms involved in regulating the phenotype of the VSM cells are unclear. However, it appears that the initiating signal for the phenotype switch is dependent upon passage through a number of cell cycle checkpoints. These cell cycle events require specific changes in cytosolic Ca^{2+} concentrations (Ghosh *et al.,* 1991; Short *et al.,* 1993; Waldron *et al.,* 1994). Recent studies hypothesized that an alteration in intracellular Ca^{2+} signaling is the initiator for VSM cells to enter the cell cycle and begin proliferation (Wilkerson *et al.,* 2005). Specifically, the data suggests that an increase in IP_3 receptor-mediated-Ca^{2+}release is a requirement for VSM proliferation. Thus, increased IP_3 receptor expression and function may be critical regulators for pathologies associated with VSM cell proliferation, such as neointimal formation.

The data presented in Chapter 5 support previous studies that suggest a role for IP_3 receptor in contributing to cardiovascular pathologies that result from abnormal VSM cell function. Our data suggest that upon activation by AngII, Jak2 prevents IP_3 receptor degradation and leads to increases in cytosolic Ca^{2+} signaling. These increases in intracellular Ca^{2+} concentration may be a contributing factor to the proliferation of VSM cells. Therefore, we believe that Jak2 is regulating cytosolic Ca^{2+} levels and thereby contributing to the proliferation of VSM cells. These data may contribute to the elucidation of Jak2's role in neointimal formation.

In conclusion, the summation of the data presented within this dissertation elucidates the downstream signaling mechanisms of Jak2 within a cell. Specifically, the

data provides insight into the roles of Jak2 in contributing to AngII-induced pathologies both inside and outside the classical Jak/STAT signaling paradigm.

APPENDIX A
JAK2-DEPENDENT GENES

Accession Number	1st Exp.	2nd Exp.	Average Induction	Gene Name
W25845	-78.4	-78.1	**-78.25**	13h9
W26787	-39.2	-25.3	**-32.25**	15d8
W27474	-23.7	-21.3	**-22.5**	31d8
W28170	-22.5	-17.3	**-19.9**	43a12
AI701156	-16.7	-13.7	**-15.2**	we10f09.x1 Homo sapiens cDNA, 3
M57763	-9.9	-4.2	**-7.05**	ADP-ribosylation factor (hARF6)
W27997	-7.5	-8.1	**-7.8**	43 e3
L07648	-7.5	-6.3	**-6.9**	MXI1
AF035119	-7.2	-4.7	**-5.95**	Deleted in liver cancer-1 (DLC-1)
AA890010	-6.8	-4.2	**-5.5**	aj89h08.s1
W27761	-5.7	-6	**-5.85**	37c5
AJ000644	-5.7	-3.4	**-4.55**	SPOP
W29031	-5.6	-5.7	**-5.65**	55c6
AW043812	-5.6	-4.9	**-5.25**	wy81b07.x1 Homo sapiens cDNA, 3
AL009266	-5.5	-3.3	**-4.4**	Similar to C. elegans RNA binding protein
AF065389	-5.1	-5.3	**-5.2**	Tetraspan NET-4
L07648	-5.1	-5	**-5.05**	MXI1
AA630312	-4.9	-3.8	**-4.35**	ac08f05.s1
W28483	-4.9	-2.5	**-3.7**	47e 11
AF079221	-4.8	-4	**-4.4**	BCL2/adenovirus E1B 19kDa-interacting protein 3a
U59305	-4.8	-2.5	**-3.65**	Ser-thr protein kinase PK428
D11151	-4.7	-3.8	**-4.25**	Endothelin-A receptor, exon 8 and 3 flanking region
AB004848	-4.6	-6.1	**-5.35**	Placenta mRNA
AF117829	-4.6	-4.5	**-4.55**	8q21.3- RICK gene
D50840	-4.6	-2.6	**-3.6**	ceramide glucosyltransferase
W28740	-4.4	-9.1	**-6.75**	51a5
L06797	-4.4	-4.3	**-4.35**	Orphan G protein-coupled receptor mRNA
U54804	-4.4	-3.8	**-4.1**	Has2
AB023137	-4.3	-2.9	**-3.6**	KIAA0920 protein
U00943	-4.2	-4	**-4.1**	A9A2BRB2 (CAC)n/(GTG)n repeat-containing mRNA
Y08262	-4.1	-4.3	**-4.2**	SCA2 protein
W27998	-4.1	-4.2	**-4.15**	43e 5
D50310	-4.1	-3.7	**-3.9**	Cyclin I
L41827	-4.1	-3.6	**-3.85**	Sensory and motor neuron derived factor (SMDF)
U92715	-4.1	-3.1	**-3.6**	Breast cancer antiestrogen resistance 3 protein (BCAR3)
AF052143	-3.9	-3.6	**-3.75**	Clone 24466
D26070	-3.9	-3.3	**-3.6**	Type 1 inositol 1,4,5-trisphosphate receptor

AB002297	-3.8	-4.2	**-4**	KIAA0299 gene
	-3.8	-3.9	**-3.85**	Glial Growth Factor 2
X87949	-3.8	-2	**-2.9**	BiP protein
AF070670	-3.7	-4	**-3.85**	Protein phosphatase 2C alpha 2
M64349	-3.6	-4.8	**-4.2**	Cyclin D (cyclin D1)
M23263	-3.5	-3.7	**-3.6**	Androgen receptor
AL096752	-3.5	-2.4	**-2.95**	DKFZp434A012 (from clone DKFZp434A012)
AF047438	-3.5	-2.2	**-2.85**	GOS28/P28 protein
W28479	-3.4	-5.4	**-4.4**	47d8
U48807	-3.4	-3.5	**-3.45**	MAP kinase phosphatase (MKP-2)
M14333	-3.4	-3.3	**-3.35**	c-syn protooncogene
D28118	-3.4	-2.3	**-2.85**	DB1
D49489	-3.4	-2.3	**-2.85**	Protein disulfide isomerase-related protein P5
X92098	-3.3	-2.3	**-2.8**	Transmembrane protein rnp24
U50648	-3.3	-2.2	**-2.75**	Interferon-inducible RNA-dependent protein kinase (Pkr)
M97252	-3.2	-4.2	**-3.7**	Kallmann syndrome (KAL)
AB016789	-3.2	-3.2	**-3.2**	Glutamine-fructose-6-phosphate amidotransferase
U37407	-3.2	-3.1	**-3.15**	Phosphoprotein CtBP
D28118	-3.2	-3	**-3.1**	DB1
U09510	-3.2	-2.6	**-2.9**	Glycyl-tRNA synthetase
M31166	-3.2	-2.5	**-2.85**	Tumor necrosis factor-inducible (TSG-14)
U43286	-3.2	-2.1	**-2.65**	Selenophosphate synthetase 2 (SPS2)
X78947	-3.1	-3.2	**-3.15**	connective tissue growth factor
AC004380	-3.1	-3.2	**-3.15**	Chromosome 16 BAC clone
L20859	-3.1	-2.5	**-2.8**	Leukemia virus receptor 1 (GLVR1)
AF016266	-3.1	-2.4	**-2.75**	TRAIL receptor 2
U43142	-3.1	-2.2	**-2.65**	Vascular endothelial growth factor protein VRP
U09510	-3.1	-2.1	**-2.6**	Glycyl-tRNA synthetase
U83246	-3	-3.3	**-3.15**	Copine I
D87953	-3	-2.9	**-2.95**	RTP
U01062	-3	-2.2	**-2.6**	Type 3 inositol 1,4,5-trisphosphate receptor
Z50022	-3	-2	**-2.5**	Surface glycoprotein
AI097085	-2.9	-3.4	**-3.15**	oz22c10.x1
D26362	-2.9	-3.2	**-3.05**	KIAA0043 gene
AB012130	-2.9	-2.9	**-2.9**	Sodium bicarbonate cotransporter2
M81601	-2.9	-2.4	**-2.65**	Transcription elongation factor (SII)
M55630	-2.9	-2.3	**-2.6**	Topoisomerase I pseudogene 2
D87077	-2.8	-3.6	**-3.2**	KIAA0240 gene
L37043	-2.8	-3.1	**-2.95**	Casein kinase I epsilon
AB014888	-2.8	-3	**-2.9**	MRJ
M23379	-2.8	-2.8	**-2.8**	GTPase-activating protein ras p21 (RASA)
AA996066	-2.8	-2.3	**-2.55**	os33d01.s1
M13509	-2.8	-2.2	**-2.5**	Skin collagenase mRNA
AF032886	-2.8	-2.1	**-2.45**	Forkhead protein (FKHRL1)
AB018301	-2.7	-4.9	**-3.8**	KIAA0758 protein
AI742087	-2.7	-3	**-2.85**	wg38g10.x1
AL050164	-2.7	-2.8	**-2.75**	DKFZp586C1622 (from clone DKFZp586C1622)

AB018259	-2.7	-2.5	**-2.6**	KIAA0716 protein
AF014837	-2.7	-2.3	**-2.5**	m6A methyltransferase (MT-A70)
M24069	-2.7	-2	**-2.35**	DNA-binding protein A (dbpA)
AF038966	-2.6	-2.8	**-2.7**	Secretory carrier-associated membrane protein (SCAMP)
AL050006	-2.6	-2.6	**-2.6**	DKFZp564A033 (from clone DKFZp564A033)
AI052724	-2.6	-2.5	**-2.55**	oz27a12.x1
AL049954	-2.6	-2.2	**-2.4**	DKFZp564A1523 (from clone DKFZp564A1523)
X63679	-2.6	-2.2	**-2.4**	TRAMP protein
M60278	-2.6	-2.2	**-2.4**	Heparin-binding EGF-like growth factor
D50683	-2.6	-2.1	**-2.35**	TGF-betaIIR alpha
L10284	-2.6	-2.1	**-2.35**	Integral membrane protein, calnexin, (IP90)
Y00970	-2.6	-2	**-2.3**	Acrosin
L06845	-2.6	-2	**-2.3**	Cysteinyl-tRNA synthetase mRNA
AF002697	-2.5	-2.8	**-2.65**	E1B 19K/Bcl-2-binding protein Nip3 mRNA
AI028290	-2.5	-2.7	**-2.6**	ov84f11.x1
AA133246	-2.5	-2.4	**-2.45**	zl17h10.r1
AL049957	-2.5	-2.3	**-2.4**	DKFZp564J0323 (from clone DKFZp564J0323)
U60061	-2.5	-2.2	**-2.35**	FEZ2
W28616	-2.4	-5.1	**-3.75**	49b9
X55005	-2.4	-4.6	**-3.5**	Thyroid hormone receptor alpha 1 THRA1(c-erbA-1 gene)
AL031290	-2.4	-3.1	**-2.75**	DNA sequence from clone 774I24 chr. 1q24.1-24.3
X02747	-2.4	-2.8	**-2.6**	Aldolase B
AB011104	-2.4	-2.7	**-2.55**	KIAA0532 protein
D26070	-2.4	-2.5	**-2.45**	1 inositol 1,4,5-trisphosphate receptor
L07956	-2.4	-2.4	**-2.4**	1,4-alpha-glucan branching enzyme (HGBE)
AF070606	-2.4	-2.4	**-2.4**	Clone 24411
AB018294	-2.4	-2.3	**-2.35**	KIAA0751 protein
L13773	-2.4	-2.3	**-2.35**	AF-4
AF039656	-2.4	-2.2	**-2.3**	Neuronal tissue-enriched acidic protein
AF070616	-2.4	-2.1	**-2.25**	Clone 24772 BDP-1 protein
AL050021	-2.4	-2.1	**-2.25**	DKFZp564D016 (from clone DKFZp564D016)
U50523	-2.4	-2	**-2.2**	BRCA2
AB002803	-2.4	-2	**-2.2**	BACH1
Z29064	-2.4	-2	**-2.2**	AF-1p
AB011155	-2.4	-2	**-2.2**	KIAA0583 protein
AB005293	-2.3	-4.1	**-3.2**	Perilipin
AI743133	-2.3	-2.6	**-2.45**	wg87f07.x1
AI808712	-2.3	-2.4	**-2.35**	wf57c05.x1
AL050091	-2.3	-2.4	**-2.35**	DKFZp586F1918 (from clone DKFZp586F1918)
AF039945	-2.3	-2.2	**-2.25**	Synaptojanin 2B mRNA
AF038187	-2.3	-2.2	**-2.25**	clone 23714
L05424	-2.3	-2.1	**-2.2**	Cell surface glycoprotein CD44 (CD44)
AI743507	-2.3	-2.1	**-2.2**	wf72a06.x2
AB015051	-2.3	-2.1	**-2.2**	Daxx
D31887	-2.3	-2	**-2.15**	KIAA0062 gene
D86425	-2.3	-2	**-2.15**	Osteonidogen
L16895	-2.2	-2.6	**-2.4**	Lysyl oxidase (LOX) gene

AB007900	-2.2	-2.6	**-2.4**	KIAA0440
D50919	-2.2	-2.3	**-2.25**	KIAA0129 gene
AI732885	-2.2	-2.3	**-2.25**	oe64d04.x5
AI365215	-2.2	-2.2	**-2.2**	qz41a06.x1
U07806	-2.2	-2.1	**-2.15**	Camptothecin resistant CEM/C2 DNA topoisomerase I
AJ131245	-2.2	-2	**-2.1**	Sec24 protein (Sec24B isoform)
Z78388	-2.1	-2.1	**-2.1**	HSZ78388
	-2.1	-2	**-2.05**	Oncogene E6-Ap, Papillomavirus
S78771	-2.1	-2	**-2.05**	CpG island-associated gene
U17999	-2.1	-2	**-2.05**	HSU17999
W28760	-2	-3.7	**-2.85**	W28760:51c8
X78565	-2	-2.5	**-2.25**	Tenascin-C
N90755	-2	-2.4	**-2.2**	zb22c08.s1
M93284	-2	-2.1	**-2.05**	Pancreatic lipase related protein 2 (PLRP2)
M23114	-2	-2	**-2**	Calcium-ATPase (HK1)
D17517	2	2	**2**	Sky
X71973	2	2	**2**	Phospholipid hydroperoxide glutathione peroxidase
U49278	2	2	**2**	UEV-1 (UBE2V)
AF061034	2	2	**2**	FIP2 alternatively translated
M26683	2	2	**2**	Interferon gamma treatment inducible
	2	2.1	**2.05**	Rad2
AI547262	2	2.1	**2.05**	PN001_AH_H03.r
U72514	2	2.1	**2.05**	C2f
AI655015	2	2.1	**2.05**	wb66a10.x1
AJ245416	2	2.1	**2.05**	G7b protein
M19267	2	2.2	**2.1**	Tropomyosin
AB000449	2	2.2	**2.1**	VRK1
AL035398	2	2.3	**2.15**	DNA sequence from clone 796I17 on chr. 22q13.2.
M80261	2	2.4	**2.2**	Apurinic endonuclease (APE)
AA926959	2	2.4	**2.2**	om68h08.s1
L07493	2	2.5	**2.25**	Replication protein A 14kDa subunit (RPA)
U73379	2	2.6	**2.3**	Cyclin-selective ubiquitin carrier protein
AB006624	2	2.6	**2.3**	KIAA0286 gene
U50939	2	2.7	**2.35**	Amyloid precursor protein-binding protein 1
Y09008	2	2.7	**2.35**	Uracil-DNA glycosylase
AI032612	2	2.7	**2.35**	ow17e07.x1
AF023462	2	2.9	**2.45**	Peroxisomal phytanoyl-CoA alpha-hydroxylase (PAHX)
N98670	2	2.9	**2.45**	yy66d08.r1
AI541308	2	3.1	**2.55**	pec1.2-4.F11.r
AL050050	2	2.5	**2.25**	DKFZp566D133 (from clone DKFZp566D133)
J05448	2.1	2	**2.05**	RNA polymerase subunit hRPB 33
M69023	2.1	2	**2.05**	Globin gene
L08069	2.1	2	**2.05**	Heat shock protein, E. coli DnaJ homologue
AL120687	2.1	2	**2.05**	Follistatin-related protein (FRP)
D45906	2.1	2.1	**2.1**	LIMK-2
W28498	2.1	2.1	**2.1**	W28498:50e2
L07540	2.1	2.1	**2.1**	Replication factor C, 36-kDa subunit

AF011468	2.1	2.2	**2.15**	Serine/threonine kinase (BTAK)
AF067656	2.1	2.2	**2.15**	ZW10 interactor Zwint
U04209	2.1	2.2	**2.15**	Associated microfibrillar protein
AB011131	2.1	2.2	**2.15**	KIAA0559 protein
U19142	2.1	2.3	**2.2**	GAGE-1 protein
AF067139	2.1	2.3	**2.2**	NADH-ubiquinone oxidoreductase NDUFS3 subunit
D42073	2.1	2.3	**2.2**	Reticulocalbin
AA524058	2.1	2.4	**2.25**	ng33b12.s1
L03532	2.1	2.5	**2.3**	M4 protein
AI740522	2.1	2.5	**2.3**	wg16b07.x1
U52112	2.1	2.6	**2.35**	N-acetyl transferase related protein
AF035555	2.1	2.6	**2.35**	Short chain L-3-hydroxyacyl-CoA dehydrogenase
D38076	2.1	2.6	**2.35**	RanBP1 (Ran-binding protein 1)
X06956	2.1	2.9	**2.5**	HALPHA44 gene for alpha-tubulin
U51007	2.1	3.1	**2.6**	26S protease subunit S5a
U03911	2.1	3.2	**2.65**	Mutator gene (hMSH2)
D14697	2.1	3.3	**2.7**	KIAA0003 gene
AA121509	2.1	4.2	**3.15**	zk88c10.s1
Z69043	2.1	4.3	**3.2**	Translocon-associated protein delta subunit precursor
D50929	2.1	2.3	**2.2**	KIAA0139 gene
AL080088	2.1	2.4	**2.25**	DKFZp564K2062 (from clone DKFZp564K2062)
U20979	2.2	2.1	**2.15**	Chromatin assembly factor-I p150 subunit
AF046798	2.2	2.1	**2.15**	untitled
D80006:	2.2	2.1	**2.15**	KIAA0184
U92538	2.2	2.1	**2.15**	Origin recognition complex subunit 5 homolog (Orc5)
D26598	2.2	2.2	**2.2**	Proteasome subunit HsC10-II
U18934	2.2	2.2	**2.2**	Receptor tyrosine kinase (DTK)
D79987	2.2	2.2	**2.2**	KIAA0165 gene
L47276	2.2	2.2	**2.2**	Alpha topoisomerase truncated-form
D23662	2.2	2.3	**2.25**	Ubiquitin-like protein
U10860	2.2	2.3	**2.25**	Guanosine 5-monophosphate synthase
L08069	2.2	2.4	**2.3**	Heat shock protein, E. coli DnaJ homologue
Y07846	2.2	2.4	**2.3**	GAR22 protein
D10656	2.2	2.4	**2.3**	CRK-II
AL049365	2.2	2.5	**2.35**	DKFZp586A0618 (from clone DKFZp586A0618)
AC004770	2.2	2.5	**2.35**	Chromosome 11, BAC CIT-HSP-311e8 (BC269730)
D28364	2.2	2.5	**2.35**	Annexin II, 5 UTR (sequence from 5 cap to start codon)
R38263	2.2	2.6	**2.4**	yc92c11.s1
AL031228	2.2	2.7	**2.45**	WD40 protein BING4
D11094	2.2	2.7	**2.45**	MSS1
AA733050	2.2	2.7	**2.45**	zg79b05.s1
AI345944	2.2	2.8	**2.5**	Cqp47e09.x1
AA447559	2.2	3	**2.6**	zw81e11.s1
U61145	2.2	3	**2.6**	Enhancer of zeste homolog 2 (EZH2)
AA570193	2.2	3.3	**2.75**	nf38c11.s1
X59268	2.2	3.5	**2.85**	General transcription factor IIB
AB028069	2.2	3.6	**2.9**	Activator of S phase Kinase

AB007893	2.2	2.2	**2.2**	KIAA0433 mRNA
AF030186	2.2	2.4	**2.3**	Glypican-4 (GPC4)
AB007891	2.2	2.4	**2.3**	KIAA0431 mRNA
D89937	2.3	2	**2.15**	KIAA0116 gene
X97548	2.3	2	**2.15**	TIF1beta zinc finger protein
U09759	2.3	2	**2.15**	Protein kinase (JNK2)
H10201	2.3	2.1	**2.2**	ym02c07.s1
AB023421	2.3	2.1	**2.2**	Heat shock protein apg-1
D49738	2.3	2.2	**2.25**	Cytoskeleton associated protein (CG22)
AI827793	2.3	2.2	**2.25**	wf33b11.x1
D14812	2.3	2.2	**2.25**	KIAA0026 gene
U87459	2.3	2.3	**2.3**	Autoimmunogenic cancer/testis antigen NY-ESO-1
J03824	2.3	2.3	**2.3**	Uroporphyrinogen III synthase
AL050282	2.3	2.5	**2.4**	DKFZp586H2219 (from clone DKFZp586H2219)
U78027	2.3	2.6	**2.45**	Brutons tyrosine kinase (BTK), a
AL050353	2.3	2.7	**2.5**	DKFZp547C0410 (from clone DKFZp547C0410)
X81789	2.3	2.8	**2.55**	Splicing factor SF3a60
U61837	2.3	2.8	**2.55**	Putative cyclin G1 interacting protein
AF035940	2.3	2.8	**2.55**	MAGOH
M34079	2.3	2.8	**2.55**	Immunodeficiency virus tat transactivator binding protein-1
L13848	2.3	3.1	**2.7**	RNA helicase A
AA527880	2.3	3.8	**3.05**	nh86h10.s1
AF047470	2.3	4.9	**3.6**	Malate dehydrogenase precursor (MDH)
AF048731	2.3	2.6	**2.45**	Cyclin T2a
AF065485	2.3	2.9	**2.6**	Sorting nexin 4
W25866	2.4	2	**2.2**	14c12
U88871	2.4	2	**2.2**	HsPex7p (HsPEX7)
U33632:	2.4	2.1	**2.25**	Two P-domain K+ channel TWIK-1
AA628946	2.4	2.1	**2.25**	af28f05.s1
AF070559	2.4	2.1	**2.25**	Clone 24463
X90999	2.4	2.2	**2.3**	Glyoxalase II
X70944	2.4	2.2	**2.3**	PTB-associated splicing factor
S68271	2.4	2.3	**2.35**	Cyclic AMP-responsive element modulator
L24804	2.4	2.4	**2.4**	(p23)
AI525633	2.4	2.4	**2.4**	PT1.3_04_A08.r
AF070537	2.4	2.4	**2.4**	Clone 24606
U01923	2.4	2.5	**2.45**	BTK region clone ftp-3
D16581	2.4	2.7	**2.55**	8-oxo-dGTPase
U58087	2.4	2.7	**2.55**	Hs-cul-1 mRNA
D00760	2.4	3.1	**2.75**	Proteasome subunit HC3
Z84718	2.4	3.1	**2.75**	DNA sequence from clone 322B1 on chr. 22q11-12
AA768912	2.4	3.1	**2.75**	nz82h06.s1
AB002330	2.4	4	**3.2**	KIAA0332 gene
M60748	2.4	2.4	**2.4**	Histone H1 (H1F4)
AL042733	2.4	2.8	**2.6**	DKFZp434B2222
AF090988	2.5	2.1	**2.3**	U5 snRNP-specific 40 kDa protein
X98296	2.5	2.1	**2.3**	Ubiquitin hydrolase

U37012	2.5	2.2	**2.35**	Cleavage and polyadenylation specificity factor
AF077953	2.5	2.2	**2.35**	Protein inhibitor of activated STAT protein PIASx-alpha
AF035292	2.5	2.3	**2.4**	Clone 23584
L04270	2.5	2.4	**2.45**	Tumor necrosis factor receptor 2 related protein
X97544	2.5	2.4	**2.45**	TIM17 preprotein translocase
AF052134	2.5	2.4	**2.45**	Clone 23585
U90426	2.5	2.6	**2.55**	Nuclear RNA helicase
N23137	2.5	2.6	**2.55**	yx67h12.s1
N23137	2.5	2.6	**2.55**	C17orf1 protein
J03473	2.5	2.7	**2.6**	Poly(ADP-ribose) synthetase
N90862	2.5	2.7	**2.6**	zb11b06.s1
D88674	2.5	2.7	**2.6**	Antizyme inhibitor
D50922	2.5	3.1	**2.8**	KIAA0132 gene
AF005392	2.5	3.1	**2.8**	Alpha tubulin (TUBA2)
AF029890	2.5	3.4	**2.95**	Hepatitis B virus X interacting protein (XIP)
AB028639	2.5	2.6	**2.55**	CAPN7 mRNA for PalBH
AI033692	2.6	2	**2.3**	ow26f02.x1
D82061	2.6	2	**2.3**	Member of the short-chain alcohol dehydrogenase family
AC005329	2.6	2.1	**2.35**	Chromosome 19, cosmid R34382
AJ243937	2.6	2.1	**2.35**	G18.1a and G18.1b proteins
AF053305	2.6	2.1	**2.35**	Mitotic checkpoint kinase Bub1 (BUB1)
AI417075	2.6	2.3	**2.45**	tg78e09.x1
U11313	2.6	2.3	**2.45**	Sterol carrier protein-X/sterol carrier protein-2
M19267	2.6	2.3	**2.45**	Tropomyosin
AB002324	2.6	2.3	**2.45**	KIAA0326 gene
X79882	2.6	2.4	**2.5**	lrp
U79273	2.6	2.5	**2.55**	Clone 23933
U30872	2.6	2.6	**2.6**	Mitosin
AL037557	2.6	2.7	**2.65**	DKFZp564H2472_r1
U03911	2.6	2.7	**2.65**	Mutator gene (hMSH2)
AL035304	2.6	2.9	**2.75**	PAC 295C6,
U37426	2.6	3.1	**2.85**	Kinesin-like spindle protein HKSP (HKSP)
X84194	2.6	3.3	**2.95**	Acylphosphatase, erythrocyte (CT) isoenzyme
X92518	2.6	3.4	**3**	HMGI-C protein
AF068180	2.6	2.8	**2.7**	B cell linker protein BLNK
AF086904	2.6	3	**2.8**	Protein kinase Chk2 (CHK2)
AA128249	2.6	3.1	**2.85**	zl29d09.r1
U90841	2.7	2	**2.35**	SSX4 (SSX4)
AB011542:	2.7	2	**2.35**	MEGF9
M62762	2.7	2	**2.35**	Vacuolar H+ ATPase proton channel subunit
AA808961	2.7	2	**2.35**	nw16h03.s1
X04412	2.7	2.3	**2.5**	Plasma gelsolin
AL031681	2.7	2.3	**2.5**	Splicing factor, arginine/serine-rich 6 (SRP55-2) isoform 2
AB029001	2.7	2.4	**2.55**	KIAA1078 protein
U67058	2.7	2.4	**2.55**	Proteinase activated receptor-2
U12022	2.7	2.4	**2.55**	Calmodulin (CALM1)
U90840	2.7	2.6	**2.65**	SSX3 (SSX3)

AF074723	2.7	2.6	**2.65**	RNA polymerase transcriptional regulation mediator
AJ238096	2.7	2.7	**2.7**	Lsm4 protein
	2.7	2.7	**2.7**	Rna Polymerase II, 14.5 Kda Subunit
J04080	2.7	2.8	**2.75**	Complement component C1r
AI360249	2.7	2.9	**2.8**	qy84f07.x1
AB007455	2.7	3.1	**2.9**	P53TG1-A
X67155	2.7	3.1	**2.9**	Mitotic kinesin-like protein-1
L25876	2.7	3.2	**2.95**	Protein tyrosine phosphatase (CIP2)
D84109	2.7	3.9	**3.3**	RBP-MS/type 3
AF052288	2.7	2.3	**2.5**	untitled
AF091433	2.7	2.6	**2.65**	Cyclin E2
AF068868:	2.8	2	**2.4**	TNFR-related death receptor-6 (DR6)
M69023	2.8	2	**2.4**	DKFZp762F2110_r1
L34075	2.8	2.1	**2.45**	FKBP-rapamycin associated protein (FRAP)
AL021546	2.8	2.2	**2.5**	DNA sequence from BAC 15E1 on chromosome 12.
AL050089	2.8	2.2	**2.5**	DKFZp586E0518 (from clone DKFZp586E0518)
AL031681	2.8	2.3	**2.55**	splicing factor, arginine/serine-rich 6 (SRP55-2)(isoform 2)
D42040	2.8	2.3	**2.55**	KIAA9001 gene
D15050	2.8	2.2	**2.5**	transcription factor AREB6
L28821	2.8	2.3	**2.55**	Alpha mannosidase II isozyme
AJ000186	2.8	2.5	**2.65**	CMAD2 protein
AF026939	2.8	2.5	**2.65**	CIG49 (cig49)
W52024	2.8	2.6	**2.7**	zd13a03.s1
AL080101	2.8	2.6	**2.7**	DKFZp564L0472 (from clone DKFZp564L0472)
W25933	2.8	2.6	**2.7**	15b2
AA005018	2.8	2.7	**2.75**	zh96a09.r1
Y11307	2.8	2.7	**2.75**	CYR61
J03040	2.8	2.7	**2.75**	SPARC/osteonectin
AF059617	2.8	3.1	**2.95**	Serum-inducible kinase
AA203545	2.8	3.3	**3.05**	zx59a05.r1
AI683748	2.8	3.7	**3.25**	tw53e07.x1
AF073362	2.8	4.2	**3.5**	Endo/exonuclease Mre11 (MRE11A)
U78722	2.8	2.9	**2.85**	Zinc finger protein 165 (Zpf165)
AI810807	2.8	3.1	**2.95**	tu26a01.x1
AL120687	2.9	2	**2.45**	L-iditol-2 dehydrogenase
U24389	2.9	2.1	**2.5**	CLysyl oxidase-like protein
X76538	2.9	2.1	**2.5**	Mpv17
U44378	2.9	2.1	**2.5**	Homozygous deletion target in pancreatic carcinoma
M94250	2.9	2.1	**2.5**	Retinoic acid inducible factor (MK)
Z95118	2.9	2.6	**2.75**	DNA sequence from clone 354J5 on chr. 6q21-22.
	2.9	3.1	**3**	Calmodulin Type I
AB009282	2.9	3.2	**3.05**	Cytochrome b5
D28423	2.9	3.4	**3.15**	pre-mRNA splicing factor SRp20
M11058	2.9	3.6	**3.25**	3-hydroxy-3-methylglutaryl coenzyme A reductase
AI375913	2.9	3.6	**3.25**	tc14c08.x1
AI950382	2.9	3.7	**3.3**	wp10g06.x1
X98253	2.9	3.8	**3.35**	ZNF183 gene

M91670	3	2.4	**2.7**	Ubiquitin carrier protein (E2-EPF)
	3	2.7	**2.85**	Rad2
AF000982	3	2.8	**2.9**	Dead box, X isoform (DBX)
AF053306	3	2.9	**2.95**	Mitotic checkpoint kinase Mad3L (MAD3L)
X96752	3	2.9	**2.95**	L-3-hydroxyacyl-CoA dehydrogenase
D64154	3	3	**3**	Mr 110,000 antigen
L07541	3	3.2	**3.1**	Replication factor C, 38-kDa subunit
U41813	3	3.3	**3.15**	Class I homeoprotein (HOXA9)
AB009398	3	3.4	**3.2**	26S proteasome subunit p40.5
AW024285	3	3.4	**3.2**	wt69d06.x1
M87339	3	3.5	**3.25**	Replication factor C, 37-kDa subunit
AF015287	3	3.5	**3.25**	Serine protease
S70154	3	4.1	**3.55**	Cytosolic acetoacetyl-coenzyme A thiolase
X84002	3	4.2	**3.6**	TAFII20 mRNA for transcription factor TFIID
U03877	3	4.4	**3.7**	Extracellular protein (S1-5)
AF064606	3	2.4	**2.7**	KB07 protein
AL050166	3	2.7	**2.85**	DKFZp586D1122 (from clone DKFZp586D1122)
U73960:	3.1	2.1	**2.6**	ADP-ribosylation factor-like protein 4
AC002073	3.1	2.3	**2.7**	PAC clone DJ515N1 from 22q11.2-q22
AF060902	3.1	2.6	**2.85**	Vesicle soluble NSF attachment protein receptor VTI2
AB002360	3.1	3	**3.05**	KIAA0362 gene
AF010187	3.1	3.2	**3.15**	FGF-1 intracellular binding protein (FIBP)
J04088	3.1	3.3	**3.2**	DNA topoisomerase II (top2)
S62140	3.1	3.3	**3.2**	Translocated in liposarcoma
U64028	3.1	3.8	**3.45**	NADH-ubiquinone oxidoreductase subunit B13
AB018283	3.1	3.8	**3.45**	KIAA0740 protein
AB019987	3.1	4	**3.55**	Chromosome-associated polypeptide-C
AL031432	3.1	4.6	**3.85**	Clone 465N24 on chromosome 1p35.1-36.13.
U95006	3.1	4.6	**3.85**	D9 splice variant A mRNA
AB011161	3.1	5.1	**4.1**	KIAA0589 protein
L29218	3.1	2.5	**2.8**	clk2 mRNA
AJ238097	3.1	3.4	**3.25**	Lsm5 protein
U79528	3.2	2	**2.6**	SR31747 binding protein 1
AB021663	3.2	2.1	**2.65**	Leucine-zipper protein
L09235	3.2	2.5	**2.85**	Vacuolar ATPase (isoform VA68)
M91670	3.2	2.8	**3**	Ubiquitin carrier protein (E2-EPF)
U16954	3.2	3.1	**3.15**	(AF1q)
W27939	3.2	3.2	**3.2**	39g3
M21154	3.2	3.3	**3.25**	S-adenosylmethionine decarboxylase
AI525379	3.2	3.4	**3.3**	PT1.1_06_H01.r
AF035959	3.2	3.8	**3.5**	Type-2 phosphatidic acid phosphatase-gamma (PAP2-g)
U16261	3.2	4.2	**3.7**	MDA-7 (mda-7)
AF098162	3.2	5.6	**4.4**	Timeless homolog
AF049105	3.2	7.5	**5.35**	Centrosomal Nek2-associated protein 1 (C-NAP1)
U11037	3.2	2.5	**2.85**	Sel-1 like
AB014543	3.2	3.2	**3.2**	KIAA0643 protein
U03109	3.3	2.2	**2.75**	Aspartyl beta-hydroxylase

AB023205	3.3	2.7	**3**	KIAA0988 protein
X04327	3.3	3	**3.15**	Erythrocyte 2,3-bisphosphoglycerate mutase
X52142	3.3	3.1	**3.2**	CTP synthetase
AF025441	3.3	3.4	**3.35**	Opa-interacting protein OIP5
U62961	3.3	3.5	**3.4**	Succinyl CoA-3-oxoacid CoA transferase precursor
AL080146	3.3	4	**3.65**	DKFZp434B174 (from clone DKFZp434B174)
AA173896	3.3	4.1	**3.7**	zp03b02.s1
U59912	3.3	4.4	**3.85**	Chromosome 4 Mad homolog Smad1
U16261	3.3	4.6	**3.95**	MDA-7 (mda-7)
X66358	3.3	2.5	**2.9**	KKIALRE for serine/threonine protein kinase
Z26317	3.3	3.3	**3.3**	Desmoglein 2
X78992	3.3	3.5	**3.4**	ERF-2
D00591	3.4	2	**2.7**	RCC1 gene, exons 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13
D50930:	3.4	2	**2.7**	KIAA0140 gene
AF047469	3.4	2	**2.7**	Arsenite translocating ATPase (ASNA1)
U19796	3.4	2.1	**2.75**	Melanoma antigen p15
X66899	3.4	2.2	**2.8**	EWS
AB014604	3.4	2.5	**2.95**	KIAA0704 protein
AA845349	3.4	2.6	**3**	ak01g01.s1
AA631698	3.4	3.1	**3.25**	np79a08.s1
U74612	3.4	3.2	**3.3**	Hepatocyte nuclear factor-3/fork head homolog 11A
D78275	3.4	3.8	**3.6**	Proteasome subunit p42, complete cds
AF070578	3.4	4.2	**3.8**	Clone 24674
AF047436	3.4	4.5	**3.95**	F1Fo-ATPase synthase f subunit mRNA
AB014460	3.4	5.4	**4.4**	TSC2, NTHL1/NTH1 and SLC9A3R2/E3KARP genes
AW051579	3.4	3.2	**3.3**	wy87g03.x1
U79256	3.4	3.3	**3.35**	Clone 23719
AF006751	3.5	2	**2.75**	ES/130
X82260	3.5	2.2	**2.85**	RanGTPase activating protein 1
D87075	3.5	2.6	**3.05**	KIAA0238 gene
L16782	3.5	2.9	**3.2**	Putative M phase phosphoprotein 1 (MPP1)
AB014458	3.5	3.4	**3.45**	hUBP mRNA for ubiquitin specific protease
M13452	3.5	4.1	**3.8**	Lamin A mRNA, 3end
AF073362	3.5	4.4	**3.95**	Endo/exonuclease Mre11 (MRE11A)
X70683	3.5	5.8	**4.65**	SOX-4
X13482	3.5	8.5	**6**	U2 snRNP-specific A protein
U17163	3.5	2.8	**3.15**	Transcription factor ETV1
U58970	3.6	2.3	**2.95**	Putative outer mitochondria membrane 34kDa translocase
X65550	3.6	2.8	**3.2**	mki67a mRNA for antigen of monoclonal antibody Ki-67
M12125	3.6	3.4	**3.5**	Fibroblast muscle-type tropomyosin
AL050261	3.6	3.8	**3.7**	DKFZp547E2110 (from clone DKFZp547E2110)
AI023044	3.6	3.8	**3.7**	ow65c01.s1
L38928	3.6	5.2	**4.4**	5,10-methenyltetrahydrofolate synthetase
AF058918	3.6	2.8	**3.2**	unknown
N58115	3.6	3	**3.3**	yv65a01.s1
X81889	3.7	2.4	**3.05**	p0071 protein
AF063020	3.7	3.1	**3.4**	lens epithelium-derived growth factor

Z48054	3.7	3.7	**3.7**	Peroxisomal targeting signal 1 (SKL type) receptor
L38696	3.7	3.9	**3.8**	Autoantigen p542
X12458	3.7	3.9	**3.8**	P3 gene
AF015254	3.7	4.2	**3.95**	Serine/threonine kinase (STK-1)
K03460	3.7	4.4	**4.05**	Alpha-tubulin isotype H2-alpha gene
L07493	3.7	4.5	**4.1**	Replication protein A 14kDa subunit (RPA)
L08835	3.7	2.4	**3.05**	DMR-N9, partial cds; and myotonic dystrophy kinase
M30474	3.8	2.6	**3.2**	Kidney gamma-glutamyl transpeptidase type II
AC002115	3.8	3.4	**3.6**	R31396, F25451, and R31076 containing COX6B
AF038406	3.8	4	**3.9**	NADH dehydrogenase-ubiquinone Fe-S protein
U34804	3.8	4.4	**4.1**	Thermostable phenol sulfotransferase (STP2)
U01038	3.9	2.4	**3.15**	pLK
X51688	3.9	2.7	**3.3**	Cyclin A
AF047432	3.9	3.3	**3.6**	ADP-ribosylation factor mRNA
U82938	3.9	4.2	**4.05**	CD27BP (Siva)
U37139	3.9	4.2	**4.05**	Beta 3-endonexin
D87440	3.9	4.5	**4.2**	KIAA0252 gene
X83928	3.9	2.9	**3.4**	Transcription factor TFIID subunit TAFII28
AF019612	3.9	3.1	**3.5**	S2P
X66364	4	2.5	**3.25**	PSSALRE for serine/threonine protein kinase
U77949	4	2.5	**3.25**	Cdc6-related protein (HsCDC6)
X69550	4	2.5	**3.25**	Rho GDP-dissociation Inhibitor 1
AC002398	4	2.6	**3.3**	DNA from chromosome 19-specific cosmid F25965
J00277	4	2.7	**3.35**	Clones RS-[3,4, 6]) c-Ha-ras1 proto-oncogene
M94362	4	3.8	**3.9**	Lamin B2 (LAMB2)
Y13115	4	3.8	**3.9**	Serine/threonine protein kinase SAK
X54942	4	4.7	**4.35**	ckshs2 mRNA for Cks1 protein homologue
D88460	4.1	3.8	**3.95**	N-WASP
J04131	4.1	4.5	**4.3**	Gamma-glutamyl transpeptidase (GGT) protein
J05614	4.1	5.5	**4.8**	Proliferating cell nuclear antigen (PCNA)
AJ011981	4.1	2.6	**3.35**	clone 417820
AF002715	4.2	3.4	**3.8**	MAP kinase kinase kinase (MTK1)
AI862521	4.2	5.3	**4.75**	wj15a06.x1
U57646	4.2	6.8	**5.5**	Cysteine and glycine-rich protein 2 (CSRP2)
AF029669	4.3	4.2	**4.25**	Rad51C (RAD51C)
Z11584	4.3	4.3	**4.3**	NuMA protein
AL080071	4.3	6.5	**5.4**	DKFZp564M082 (from clone DKFZp564M082)
U79266	4.4	2.8	**3.6**	Clone 23627
AF064093	4.4	3	**3.7**	KE04p mRNA
U10868	4.4	3.2	**3.8**	Aldehyde dehydrogenase ALDH7
Y10043	4.4	3.5	**3.95**	High mobility group protein HMG2a
X74794	4.4	4.4	**4.4**	P1-Cdc21
AF098462	4.4	4.5	**4.45**	Stanniocalcin-related protein
AI341574	4.4	4.5	**4.45**	qq94h09.x1
U76638	4.4	5	**4.7**	BRCA1-associated RING domain protein (BARD1)
U34044	4.4	5.2	**4.8**	Selenium donor protein (selD)
AJ223349	4.5	6	**5.25**	HIRIP3 protein

D26155	4.5	2.3	**3.4**	Transcriptional activator hSNF2a
U36341	4.6	4.3	**4.45**	Xq28 cosmid, creatine transporter (SLC6A8)
X05360	4.6	4.4	**4.5**	CDC2 gene involved in cell cycle control
AF042083	4.7	4.2	**4.45**	BH3 interacting domain death agonist (BID)
X87176	4.7	4.6	**4.65**	17-beta-hydroxysteroid dehydrogenase
U66619	4.7	5.5	**5.1**	SWI/SNF complex 60 KDa subunit (BAF60c)
AI693193	4.7	9.1	**6.9**	wd68f02.x1
X57398	4.8	3.3	**4.05**	pM5 protein
W28892	4.8	3.9	**4.35**	53c11
AF001383	4.8	4.2	**4.5**	Amphiphysin II
AF040707	4.9	3.6	**4.25**	Candidate tumor suppressor gene 21 protein isoform I
J03060	5	3	**4**	Glucocerebrosidase (GCB)
Z36714	5	3.1	**4.05**	Cyclin F
U25975	5.1	2.3	**3.7**	Serine kinase (hPAK65)
U37408	5.1	2.5	**3.8**	Phosphoprotein CtBP
U09759	5.1	3.8	**4.45**	Protein kinase (JNK2)
U52100	5.1	6	**5.55**	XMP
Z97029	5.1	10.1	**7.6**	Ribonuclease H I large subunit
W27050	5.2	3.3	**4.25**	19f7
U65011	5.2	3.8	**4.5**	Preferentially expressed antigen of melanoma (PRAME)
AF041210	5.2	8.5	**6.85**	Midline 1 fetal kidney isoform 3 (MID1)
L35013	5.3	2.1	**3.7**	Spliceosomal protein (SAP 49)
U15655	5.3	2.4	**3.85**	ets domain protein ERF
AC004770	5.3	2.6	**3.95**	hFEN1 gene
U01923	5.3	3.5	**4.4**	BTK region clone ftp-3
X65873	5.3	3.9	**4.6**	Kinesin (heavy chain)
U25165	5.3	3.9	**4.6**	Fragile X mental retardation protein 1 homolog FXR1
Y11681	5.3	4.1	**4.7**	Mitochondrial ribosomal protein S12
AF040958	5.4	3.4	**4.4**	Lysosomal neuraminidase precursor
AF038250	5.4	5	**5.2**	untitled
AB002308	5.4	9.3	**7.35**	KIAA0310 gene
L37747	5.5	2.3	**3.9**	Lamin B1
AA552140	5.5	2.5	**4**	ng48e07.s1
AL050015	5.5	3.7	**4.6**	DKFZp564O243 (from clone DKFZp564O243)
W27541	5.5	4.9	**5.2**	32c12
AF052111	5.5	5	**5.25**	Clone 23953
M12125	5.5	6.3	**5.9**	Fibroblast muscle-type tropomyosin
J03824	5.6	3.7	**4.65**	Uroporphyrinogen III synthase
M87338	5.6	4.9	**5.25**	Replication factor C, 40-kDa subunit (A1)
W22520	5.7	5.1	**5.4**	68G3
W28214	5.7	5.7	**5.7**	45f7
AF038961	5.7	5.8	**5.75**	SL15 protein
AI698103	5.7	6.8	**6.25**	we20h11.x1
AL096739	5.7	3.1	**4.4**	DKFZp586H0623 (from clone DKFZp586H0623)
U75370	5.8	3.9	**4.85**	Mitochondrial RNA polymerase
AF080561	5.8	5	**5.4**	SYT interacting protein SIP
X71490	5.9	4.6	**5.25**	Vacuolar proton ATPase, subunit D

U47926	6	6.7	**6.35**	Unknown protein B
X51688	6.1	4.6	**5.35**	Cyclin A
D88357	6.1	5.3	**5.7**	CDC2 delta T
U90842	6.1	5.8	**5.95**	SSX5 (SSX5)
Z49105	6.1	5.8	**5.95**	HD21
AI961220	6.1	6.1	**6.1**	wt15b04.x1
X55110	6.2	4.3	**5.25**	Neurite outgrowth-promoting protein
X14850	6.2	6.1	**6.15**	H2A.X mRNA encoding histone H2A.X
Y00630	6.2	6.2	**6.2**	Arg-Serpin (plasminogen activator-inhibitor 2, PAI-2)
AF091080	6.2	7	**6.6**	Clone 614 unknown
AL008583	6.3	4.9	**5.6**	Ortholog of rat Neuronal Pentraxin Receptor
X79200	6.3	5.4	**5.85**	SYT-SSX protein
M13452	6.3	5.9	**6.1**	Lamin A
AJ133769	6.3	8.8	**7.55**	Nuclear transport receptor
AB002328	6.4	2.6	**4.5**	KIAA0330 gene
	6.4	5.4	**5.9**	Tubulin, Alpha 1, Isoform 44
AA165701	6.4	6.8	**6.6**	zo75g08.s1
D87435	6.5	2.2	**4.35**	KIAA0248 gene
X75342	6.5	4.8	**5.65**	SHB
U31384	6.6	6.7	**6.65**	G protein gamma-11 subunit
M63167	6.8	2.9	**4.85**	Rac protein kinase alpha
AD000092	6.8	4.3	**5.55**	EKLF, GCDH, CRTC, and RAD23A genes
L04658	6.8	5.8	**6.3**	untitled
L25444	7	3.2	**5.1**	TAFII70-alpha
U24152	7	7.6	**7.3**	p21-activated protein kinase (Pak1)
U66685	7.1	5.4	**6.25**	HSU66685
Y09616	7.2	7.6	**7.4**	Putative intestinal carboxylesterase (iCE)
AI936826	7.2	8.9	**8.05**	wp69h10.x1
U07695	7.3	4.8	**6.05**	Tyrosine kinase (HTK)
X56777	7.3	5.6	**6.45**	ZP3
L78833	7.3	6	**6.65**	BRCA1, Rho7 and vatI
Y18483	7.4	4.6	**6**	SLC7A8 protein
U18271	7.5	7.4	**7.45**	Thymopoietin (TMPO)
AF057297	7.8	5.3	**6.55**	Ornithine decarboxylase antizyme 2 (OAZ2)
AL050019	8.3	6.7	**7.5**	DKFZp564C186 (from clone DKFZp564C186)
L47345	8.3	7.2	**7.75**	Elongin A
U68485	8.3	9.4	**8.85**	Bridging integrator protein-1 (BIN1)
U02566	8.4	5.9	**7.15**	Receptor tyrosine kinase tif
AF016371	8.4	14.5	**11.45**	U4/U6snRNP-associated cyclophilin (USA-CyP)
W28235	8.5	8.6	**8.55**	43h8
AL080203	8.6	7.6	**8.1**	DKFZp434F222 (from clone DKFZp434F222)
S78187	8.6	10.6	**9.6**	CDC25Hu2=cdc25+ homolog
M64595	8.8	5	**6.9**	Small G protein (Gx)
AB017430	8.9	8.9	**8.9**	kid-Kinesin-like DNA binding protein
AD001530	9.2	9.1	**9.15**	XAP-5
S76638	9.9	4.5	**7.2**	p50-NF-kappa B homolog [human, peripheral blood Tcells
AA121509	9.9	17.3	**13.6**	k88c10.s1

S82470	10	4.1	**7.05**	Malignant cell expression-enhanced gene
Y07604	10.2	5.1	**7.65**	Nucleoside-diphosphate kinase
AB028974	10.4	7	**8.7**	KIAA1051 protein
AF035292	10.8	8.9	**9.85**	Clone 23584
M68864	12.1	10.4	**11.25**	ORF mRNA
X79865	12.2	8.3	**10.25**	Mrp17
X71345	12.3	17.8	**15.05**	Trypsinogen IV b-form
AL096723	12.6	14.3	**13.45**	DKFZp564H2023 (from clone DKFZp564H2023)
X96484	13.7	11.7	**12.7**	DGCR6 protein
U03398	14.5	4.7	**9.6**	Receptor 4-1BB ligand
AF026031	15.1	15.8	**15.45**	Putative mitochondrial membrane protein import receptor
L23959	15.9	11.2	**13.55**	E2F-related transcription factor (DP-1)
N53547	15.9	18.2	**17.05**	yv43b12.s1
X03656	17.2	15.8	**16.5**	Granulocyte colony-stimulating factor (G-CSF)
U15655	17.3	6.5	**11.9**	ets domain protein ERF
D83492	18.5	12.7	**15.6**	EphB6
D64142	19.5	8.1	**13.8**	Histone H1x
U66061	23	28.6	**25.8**	Trypsinogen C
AF026977	31	36.8	**33.9**	Microsomal glutathione S-transferase 3 (MGST3)
L37127	43.9	53	**48.45**	RNA polymerase II subunit

APPENDIX B
CYTOKINE-INDUCIBLE JAK2-DEPENDENT GENES

Accession #	Gene Name	Average. Induction #
W25845	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	-78.4
AF035119	Deleted in liver cancer 1	-5.95
AF117829	Receptor-interacting serine-threonine kinase 2	-4.55
AF117829	Receptor-interacting serine-threonine kinase 2	-4.55
L06797	Chemokine (C-X-C motif) receptor 4	-4.35
L06797	Chemokine (C-X-C motif) receptor 4	-4.35
M23263	Androgen receptor (dihydrotestosterone receptor	-3.6
D26070	Inositol 1,4,5-triphosphate receptor, type 1	-3.6
X55005	Thyroid hormone receptor, alpha	-3.5
X78947	Connective tissue growth factor	-3.15
M31166	Pentaxin-related gene, rapidly induced by IL-1 beta	-2.85
D28118	Zinc finger protein 161	-2.85
D28118	Zinc finger protein 161	-2.85
L20859	Solute carrier family 20 (phosphate transporter), member 1	-2.8
M23379	RAS p21 protein activator (GTPase activating protein) 1	-2.8
AF016266	Tumor necrosis factor receptor superfamily, member 10b	-2.75
U01062	Inositol 1,4,5-triphosphate receptor, type 3	-2.6
D26070	Inositol 1,4,5-triphosphate receptor, type 1	-2.45
M60278	Diphtheria toxin receptor	-2.4
X63679	Translocating chain-associating membrane protein	-2.4
X78565	Tenascin C (hexabrachion)	-2.25
AB015051	Death-associated protein 6	-2.2
U17999	Beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)	-2.05
M26683	Chemokine (C-C motif) ligand 2	2
D17517	TYRO3 protein tyrosine kinase	2
M26683	Chemokine (C-C motif) ligand 2	2
U09759	Mitogen-activated protein kinase 9	2.15
U88871	peroxisomal biogenesis factor 7	2.2
U18934	TYRO3 protein tyrosine kinase	2.2
U19142	G antigen 1	2.2
D10656	v-crk sarcoma virus CT10 oncogene homolog (avian)	2.3
L03532	Heterogeneous nuclear ribonucleoprotein M	2.3
AI033692	Breakpoint cluster region protein, uterine leiomyoma	2.3
D38076	RAN binding protein 1	2.35
L04270	Lymphotoxin beta receptor (TNFR superfamily, member 3)	2.45
U67058	Coagulation factor II (thrombin) receptor-like 1	2.55
D16581	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	2.55

AF068180	B-cell linker	2.7
AF068180	B-cell linker	2.7
AF006751	Ribosome binding protein 1 homolog 180kDa (dog)	2.75
X82260	Ran GTPase activating protein 1	2.85
AB007455	TP53 target gene 1	2.9
U58970	Translocase of outer mitochondrial membrane 34	2.95
X69550	Rho GDP dissociation inhibitor (GDI) alpha	3.25
U74612	Forkhead box M1	3.3
AF073362	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	3.5
Z48054	Peroxisome receptor 1	3.7
AF002715	Mitogen-activated protein kinase kinase kinase 4	3.8
D88460	Wiskott-Aldrich syndrome-like	3.95
U37139	Integrin beta 3 binding protein (beta3-endonexin)	4.05
U82938	CD27-binding (Siva) protein	4.05
U82938	CD27-binding (Siva) protein	4.05
U09759	Mitogen-activated protein kinase 9	4.45
AF042083	BH3 interacting domain death agonist	4.45
AF098462	Stanniocalcin 2	4.45
M63167	v-akt murine thymoma viral oncogene homolog 1	4.85
X55110	Midkine (neurite growth-promoting factor 2)	5.25
X75342	SHB (Src homology 2 domain containing) adaptor protein B	5.65
X56777	Zona pellucida glycoprotein 3A (sperm receptor)	6.45
U02566	TYRO3 protein tyrosine kinase	7.15
AJ133769	Transportin-SR	7.55
U03398	"Tumor necrosis factor (ligand) superfamily, member 9"	9.6
D83492	EphB6	15.6
AF026977	Microsomal glutathione S-transferase 3	33.9

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BIOGRAPHICAL SKETCH

Tiffany A. Wallace was born on November 16, 1979, in the small suburb of Vernon, New Jersey. As a child, Tiffany always maintained a love of science. Her initial career aspirations included becoming a veterinarian and making documentaries for *National Geographic*. Tiffany's interest in research specifically was sparked during her undergraduate studies at Monmouth University. While pursuing her degree in biology, she worked under Dr. Dennis Rhoads to investigate neurotransmitter release in sea anemones. In the spring of 2001, Tiffany graduated with honors from Monmouth University with a B.S. degree in biology and dual minors in chemistry and business. In pursuit of a Ph.D., she moved to Gainesville, Florida in the summer of 2001 and enrolled in the Interdisciplinary Program in Biomedical Sciences at the University of Florida. Following the completion of her first year, Tiffany joined the Department of Physiology and Functional Genomics and began her graduate thesis work under the guidance of Dr. Peter Sayeski. Her primary area of interest included elucidating angiotensin II signaling through the tyrosine kinase, Jak2. She will earn her doctorate in December 2005.