Regulation of STAT Activation in the EGFRvIII Pathway

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1. 3 copies of

- **a. Progress report**
- b. **Submitted manuscript to MCB with acknowledgement to the Moran**
- c. **Abstract of presentation at international meeting**
- d. **Project activity statement**

2. 6 copies of

- **a. new application**
- b. **Budget justification**
- c. **Principle investigator's CV**

Progress Report:

Summary of the original objectives of the funded project

In 2003, we proposed to use fruit fly *Drosophila melanoganster* as the model system to test the molecular mechanism of the EGF receptor activation of the STAT. In order to establish a model system which is able to be applied to the examination of human homologies, we were first proposed to examine the Drosophila EGF receptor (DER) and its possible interactions with Drosophila endogenous STAT protein (STAT92E) by focusing on DER cytoplasmic region and STAT92E SH2 motif. All researches were originally planed to be tested in cell culture and further to be characterized with *in vivo* model.

The progress to date in achieving the research aims and in testing hypotheses

As part of the research project funded by the Moran Foundation and the NIH, the first few experiments were the verification of our previous discovery that EGFR distinctively activates STAT1 and STAT3 in mammalian cells. In order to test our hypothesis that distinct activation of STAT proteins by EGFR is determined by the EGFR cytoplasmic region and the specificity of SH2 domain of each individual STAT protein, we tested the DER and human STAT proteins and found there was no activation of STAT in response to the overexpression of DER. Due to the time limit, we are unable to overcome the technical difficulties to generate the Drosophila specific ligand for the receptor DER. However, in mammalian cell system, we further discovered that the distinct activation of STAT by EGFR is a result of the mediation of non-receptor tyrosine kinase c-Src. This recent discovery has been submitted to the journal of Molecular Cellular Biology (Mol. Cell. Biol.) and currently is under revision for resubmission (see appendix). In this report, we show that the activation of different members of the STAT protein family (STAT3 & STAT1) by the EGFR involves different mechanisms. Physiologically, STAT3 is the predominant STAT protein associated with the EGFR activation under the stimulation of ligand (TGFα or EGF). However, co-stimulation with arsenite and TGFα resulted in an activity shift from a STAT3 to a STAT1 dominant pattern. Although exposure to arsenite synergistically increases the ligand-induced EGFR activation, further experiments show that arsenite exposure down-regulates the activity of JAK1 and c-Src induced by EGFR activation. Our data revealed that these non-receptor tyrosine kinases are necessary for a maximal activation of STAT3 in the EGFR pathway. The activity shift from a STAT3 to a STAT1 dominant pattern is a combined result of arsenite-activation of EGFR and arsenite-inhibition of JAK1 and c-Src. Our study defines the molecular basis for the distinct activation of different members of STAT family (STAT1 and STAT3) by EGFR and demonstrates two independent effects of arsenite on EGFR-dependent signaling: (1) arsenite activation of the EGFR; (2) arsenite inhibition of the JAK and c-Src.

Have the time-lines for the research project changed? Has the direction of the research changed (from that specified in the original application)?

Our current progress of the research indicates that c-Src may play critical role in the mediation of EGFR activation of STAT proteins, particularly in the activation of STAT3. Although Dr. Perrimon at Havard Medical School provided us the cDNA constructs encoded Drosophila EGF receptor (DER) and Drosophila STAT protein (STAT92E). These constructs have been engineered into a *Drosophila* expressible vector (Gateway universal expression platform purchased from Invitrogen). We believe it is necessary to extend our research in mammalian cells and further examine the roles of c-Src may play in the regulation of STAT distinct activation by EGFR. Our data now convinced us that c-Src, in conjunction with other factors (SH2 domains of STAT proteins; tyrosine sites of EGFR), is necessary and sufficient for the distinct activation of STAT1 and STAT3. Since simultaneous activation of STAT1 and STAT3 is commonly observed in other growth factor and cytokine/interferon pathways, the molecular basis for the control of STAT distinct activation may be shared by them, in which c-Src play an indispensable role for the regulation. On the other hand, in various cancer cells, constitutively activated c-Src and STAT3 are easily detectable. Increased activity of c-Src and STAT3 are frequently associated with aberrantly expressed EGFR or mutation of EGFR. Our finding may suggest that "on/off " status of c-Src activity is determining the interaction of EGFR with STAT proteins via phosphor-tyrosine-SH2 binding and controls the directions of EGFR-dependent signaling. Our hypothesis is that c-Src affects the binding affinity of STAT protein to the EGFR cytoplasmic motifs, which becomes our current research focus. Therefore, we shifted our direction and are going to test this in the *Drosophila* S2 expression system we have established during 2003-2004 financial year (see new proposal for 2004-2005).

Distinct Activation of STAT3 and STAT1 by the Epidermal Growth Factor Receptor

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Running title: STAT activation by EGFR

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Abstract:

Ligand binding to the epidermal growth factor receptor (EGFR) triggers various signal transduction pathways including activation of signal transducer and activator of transcription (STAT) factors as well as non-receptor tyrosine kinases JAK1 and c-Src. In this report, we show that the activation of different members of the STAT protein family (STAT3 & STAT1) by the EGFR involves different mechanisms. Physiologically, STAT3 is the predominant STAT protein associated with the EGFR activation under the stimulation of ligand (TGFα or EGF). However, co-stimulation with arsenite and TGFα resulted in an activity shift from a STAT3 to a STAT1 dominant pattern. Although exposure to arsenite synergistically increases the ligand-induced EGFR activation, further experiments show that arsenite exposure down-regulates the activity of JAK1 and c-Src induced by EGFR activation. Our data revealed that these non-receptor tyrosine kinases are necessary for a maximal activation of STAT3 in the EGFR pathway. The activity shift from a STAT3 to a STAT1 dominant pattern is a combined result of arsenite-activation of EGFR and arsenite-inhibition of JAK1 and c-Src. Our study defines the molecular basis for the distinct activation of different members of STAT family (STAT1 and STAT3) by EGFR and demonstrates two independent effects of arsenite on EGFR-dependent signaling: (1) arsenite activation of the EGFR; (2) arsenite inhibition of the JAK and c-Src.

Key Words: EGFR, STAT3, STAT1, JAK1, c-Src, Arsenite

Introduction:

Signal transducer and activator of transcription (STAT) proteins were initially identified as a family of latent cytosolic transcription factors activated by non-receptor tyrosine kinases (i.e. JAKs and c-Src) downstream of interferon and other cytokines (11,14,25,29,33,44). Polypeptide growth factor receptors, such as epidermal growth factor receptor (EGFR), also activate STATs, JAK1, and c-Src (1,2,18,21,38,40,41,42,43,46). Polypeptide ligand binding induces growth factor receptor autophosphorylation and STAT association (13,42). The intrinsic kinase activity of the EGFR is known to be important for the activation of STAT proteins (10,16). The receptor-recruited STAT proteins are phosphorylated by the intrinsic kinase activity of the receptor on a conserved tyrosine residue (Y705 in STAT3 and Y701 in STAT1). However, the role of EGFR activated JAK1 and c-Src in STAT activation remains to be fully determined. Several lines of evidence indicate that the JAK kinases and c-Src work cooperatively with the EGFR in receptor autophosphorylation and in receptor-mediated STAT protein phosphorylation (18,19,22,27,30,31,32,35,37,39,41,43). Similarly, c-Src and JAK kinases may also mediate the activation of STATs by the platelet-derived growth factor receptor (PDGFR) (12,47,48). It has not yet been resolved whether or not the non-receptor tyrosine kinases JAK and c-Src are necessary for the distinct activation of different STAT isoforms in the EGFR pathway.

It has been observed that EGFR activation increases the activity of different STAT proteins (STAT1, STAT3, and STAT5) (2,13,21,22,23). *In vitro* and *in vivo* studies have shown that the activation of STAT1 and STAT3 has opposite biological consequences (29). STAT1 activation is associated with cell cycle arrest and cell growth

suppression while STAT3 activation is known to promote cell cycle progression and induce tumor growth *in vivo*. (5). The activation of EGFR can lead to two opposite biological consequences, cell transformation and cell cycle arrest (17,50). Specific inhibition of STAT3 activity in the EGFR pathway can significantly decrease EGFR-driven cell growth and cell transformation (20,28). Conversely, STAT1 activation is a key factor for the EGFR-dependent cell growth suppression (23). The underlying determining factor(s) for EGFR-induced activation of different members of STAT proteins (STAT3 and STAT1) and the determinants for the receptor-driven biological consequences (tumorigenesis or cell growth inhibition) remain to be determined.

In this study, we have examined the activation of STAT1 and STAT3 by EGFR. Our data demonstrate that, in the presence of arsenite, elevated EGFR activity resulted in an activity shift from a STAT3 to a STAT1 dominant pattern. We present evidence that the EGFR requires non-receptor tyrosine kinase JAK1 and/or c-Src for the full activation of STAT3 while the EGFR itself is sufficient for the activation of STAT1.

Materials and Methods:

Cells & cell transfection: The A431 squamous carcinoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The JAK1-deficient U4A cells (U4A *Jak1*$^-$) and U4A with stable expression of JAK1 (U4A *Jak1*$^+$) were the gifts from Dr. Michael David (University of California at San Diego, San Diego, CA) and have been described elsewhere (33). These cells were maintained with Dulbeco modified essential medium (DMEM), supplemented with 10% fetal bovine serum (FBS). The growth of U4A *Jak1*$^+$ cells was cultured with DMEM supplemented with 100 μg/ml

G418 (Invitrogen Corporation, Carlsbad, California, USA) to maintain the JAK1 stable expression. The human kidney 293T cells were a gift from Dr. Thomas Smithgall (University of Pittsburgh, Pittsburgh, PA). 293T cells were maintained in DMEM medium supplemented with 5% FBS.

The EGFR cDNA template was purchased from Upstate Biological Inc (Catalog #21-176) and re-constructed in pcDNA3.1/Zeo (+) (Invitrogen Corporation, Carlsbad, California, USA). For establishing U4A *Jak1*$^-$ and U4A *Jak1*$^+$ cells expressing wild-type EGFR (U4A *Jak1*$^-$*/EgfR* and U4A *Jak1*$^+$*/EgfR*), the U4A cells were transfected with full-length wild-type EGFR and then selected by 500 μg/ml Zeocin® (Invitrogen Corporation, Carlsbad, California, USA) for 20 days. As a mocking control, a stable cell line with empty vector (pcDNA3.1/Zeo+) was also established. The expression of wild type EGFR and JAK1 was confirmed by immunoblotting before cells were subjected to further experiments.

The EGFR/JAK2 chimeric construct was a gift from Dr. Osamu Miura (Tokyo Medical and Dental University, Tokyo, Japan) and has been described elsewhere (35). The EGFR/JAK2 was transiently expressed in 293T human kidney cells by transient transfection (Fugene 6, Roche Ltd.). After 48 hours, transfected 293T cells were subjected to arsenite exposure.

Reagents and cell treatment: Sodium arsenite (Sigma-Aldrich, St. Louis, MO, USA) was prepared in phosphate buffered saline (PBS, pH 7.4) as 100 X stock solution and used for a final concentration (0-400 μM). Recombinant transforming growth factor peptide (TGFα) (R&D Systems Inc., Minneapolis, MN, USA) was prepared in PBS (1000 X) and stored at –20 °C used at a final concentration (30 ng/ml).

For cell treatment, the A431 or U4A cells were subjected to serum-free starvation for 16 hours followed by 30 minutes of sodium arsenite treatment. For the co-stimulation by arsenite and TGFα, the serum-free starved cells were pretreated with sodium arsenite and followed by additional 30 minutes of treatment with TGFα (30 ng/ml). The transfected 293T cells were subjected to serum-free starvation for 2 hours prior to 1-hour exposure of arsenite.

Immunoprecipitation, co-immunoprecipitation & immunoblotting: Treated cells were collected in PBS and clarified (10 minutes, maximal speed, Eppendorf 5417C, 4 °C) in radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCL, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1% sodium deoxycholate), supplemented with 25 μg of aprotinin per ml, 50 μg of leupeptin per ml, 1 mM phenylmethylsufonyl fluoride, 20 mM NaF, 1 mM Na_3VO_4, and 50 μM Na_2MoO_4. Wild type EGFR, STAT1, STAT3, and c-Src were immunoprecipitated from clarified cell lysates with 1 μg specific polyclonal antibody against either EGFR or STAT3 or STAT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or c-Src (Upstate, Waltham, MA, USA) and 20 μl protein G agarose (Invitrogen Corporation, Carlsbad, California, USA) for two hours at 4 °C. Specific antibody recognizing the extracellular domain of the EGFR (Upstate, Waltham, MA, USA) was used for the immunoprecipitation of the chimeric EGFR/JAK2 protein from 293T cells. The immunocomplexes were washed three times with ice-cold RIPA buffer and final pellets were resolved with 7.5% SDS-PAGE gels. For immunoblotting analysis of the whole cell lysates, clarified whole cell lysate from treated cells was prepared by centrifugation and resolved with 7.5% SDS-PAGE gels. Resolved

proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane 0.45 μm, Millipore) and subjected to immunoblot.

The tyrosine phosphorylation of EGFR either from clarified whole cell lysate or from immunocomplex was analyzed by the antibodies recognizing individual tyrosine phosphorylation on EGFR cytoplasmic tail Y992, Y1068, Y1086, Y1148 and Y1173 (Biosource International, Camarillo, CA, USA). The phosphorylation of STAT3 and STAT1 was evaluated using antibody recognizing pY705 STAT3 (Cell Signaling Technology, Inc. Beverly, MA) or pY701 STAT1 (Cell Signaling). The corresponding protein levels were determined with the antibodies described above. For the experiment of chimeric EGFR/JAK2 from the expression in 293T cells, resolved chimeric protein was analyzed by anti-JAK2 (JH1 domain) (Santa Cruz). The tyrosine phosphorylation of the chimeric proteins was measured by anti-phosphotyrosine (PY99) (Santa Cruz). All immunoblots were visualized directly on PVDF membranes using the alkaline phosphatase (AP)-conjugated second goat antibody and colorimetric substrate BCIP/NBT.

***In vitro* kinase assay:** For *in vitro* immuno-complex kinase assays, the immunocomplexes of anti-EGFR from treated cells were resuspended in kinase assay buffer (50 mM HEPES, pH7.4; 10 mM $MgCl_2$). The aliquots of final immunocomplexes were incubated with [γ-^{32}P]-ATP (10 μCi, Pharmacia Corp. Peapack, NJ). For the kinase assay using recombinant EGFR purified from immunoaffinity chromatography (49), one unit of recombinant EGFR protein kinase (Calbiochem, San Diego, CA, USA) for each reaction was incubated with sodium arsenite (0, 200, 400 μM) *in vitro*. The proteins were labeled with [γ-^{32}P]-ATP (10 μCi, Pharmacia Corp. Peapack, NJ) for 30 minutes at room

temperature. All reactions were stopped by heating (95 °C X 5 minutes) in 2X SDS sample buffer. Phosphorylated proteins were resolved on 7.5% SDS-PAGE gels and visualized by autoradiography.

For the assessment of endogenous c-Src activity from treated cells, anti-c-Src immunocomplexes were prepared from JAK1-null U4A cells (with or without expression of the wild-type EGFR (U4A *Jak1*$^-$ or U4A *Jak1*$^-$*/EgfR*)). Each aliquot of the anti-c-Src immunocomplex from treated cells was incubated with additional recombinant substrate Sam68 (0.3 μg, Santa Cruz) and labeled by [γ-^{32}P]-ATP *in vitro*. For the assessment of direct action of arsenite on the c-Src kinase activity, the recombinant c-Src protein (purchased from Upstate) was incubated directly with different concentrations of arsenite. Each reaction (20 μl) contains purified c-Src (1 unit), recombinant substrate Sam68 (0.3 μg), and different levels of arsenite. The reaction was labeled by [γ-^{32}P]-ATP as described above.

Electrophoretic Mobility Shift Assay: We used whole cell extracts for the electrophoretic mobility shift assay (EMSA) to evaluate the STAT activity. Cell pellets were resuspended in 50 μL 2X high salt buffer (840 mM NaCl; 40 mM HEPES pH7.9; 2 mM EDTA; 2 mM EGTA and 40% Glycerol). After 3 cycles of freeze/thaw on ice, the samples were clarified by centrifugation (12,000 rpm x 15 minutes, 4 °C). The clarified cell extracts were incubated with [γ-^{32}P] end-labeled double-stranded oligonucleotides (*hSIE*, 5'-GTGCATTTCCCGTAAATCTTGTCTACA-3') for 20 minutes. The reactions were resolved on 5% non-denaturing polyacrylamide gels and visualized by autoradiography. The specificity of the identified STAT-probe co-migration was confirmed by co-incubation with unlabeled *hSIE* oligonucleotides (cold probe

competition); or with antibodies of against STAT3 (1 μg) or STAT1 (1 μg) for the "supershift" assay.

Results:

Activation of EGFR by arsenite: We employed an *in vitro* kinase activity assay to evaluate the effect of arsenite on the activity of EGFR. In A431 cells, TGFα treatment increased the activity of EGFR (Figure 1A, top row, compare lane 2 with lane 1). Arsenite treatment alone also elevated the EGFR activity (Figure 1A, top row, compare lane 3 to lane 1). Further, there is a synergy between TGFα and arsenite (Figure 1A, top row, compare lane 3 and 4). These results indicate that arsenite activates EGFR and the arsenite activation of EGFR is ligand-independent as well as ligand-synergistic.

To determine if arsenite can directly induce activation of the EGFR, we performed measurement of EGFR phosphorylation by direct incubation of recombinant EGFR protein with arsenite *in vitro*. The kinase labeling was conducted by [γ-^{32}P]-ATP with or without additional recombinant TGFα (Figure 1B). The direct incubation of arsenite with the EGFR protein resulted in increased receptor activity as judged by ^{32}P incorporation (Figure 1B, compare lane 1, 2, and 3). In addition, a synergistic effect of arsenite with ligand on the activation of EGFR was found (Figure 1B, lane 4, 5, and 6). Our results indicate that arsenite activation of EGFR does not require any additional factor. Arsenite can directly interact with EGFR and such interaction activates the EGFR.

Within the signal region of the EGFR, five major tyrosine autophosphorylation sites have been identified (3,13,36,43). To determine whether or not arsenite differentially affects the tyrosine phosphorylation at specific sites, we examined the

phosphorylation of each of the five tyrosine sites (pY992, pY1068, pY1086, pY1148, and pY1173). As shown in Figure 2, the activity of EGFR from treated A431 cells displayed a dose-dependent response to arsenite treatment. While there were slight differences in the sensitivity to treatment or immunoblotting, each of the five major tyrosine phosphorylation sites (Figure 2, rows from top to bottom in both panels) displayed the dose-dependent response patterns to the increasing levels of arsenite (compare lane 1-6 of each panel). Each of the five tyrosine sites showed further enhanced phosphorylation when co-stimulated by arsenite and TGFα (Figure 2, compare left panel to right panel). These findings confirm that arsenite is capable of activating EGFR and potentiating ligand-induced EGFR signaling. Furthermore, these results suggest that arsenite does not differentially affect individual tyrosine phosphorylation site within the signal region of the EGFR.

Distinct activation of STAT proteins by the EGFR: One of the downstream signaling pathways relaying EGFR signaling is the activation of STAT proteins. In treated A431 cells, we used EMSA to determine whether or not the arsenite-induced activation of EGFR results in activation of STAT3 and STAT1: As shown in Figure 3, TGFα stimulation in the absence of arsenite induced dominant STAT3 activation (Figure 3, lane 7). Co-stimulation with TGFα and arsenite increased the formation of STAT3:STAT1 heterodimers (Figure 3, compare lane 7, 8, 9 to lane 10, 11, and 12). With low levels of arsenite exposure, the formation of STAT3:STAT3 homodimers was slightly increased (Figure 3, compare lane 7, 8, and 9). Further increasing level of arsenite exposure eliminated the STAT3:STAT3 homodimers activity in EMSA whereas enhanced exposure to arsenite increased the formation complexes of STAT1:STAT1 and

STAT3:STAT1 (lane 10, 11 and 12). Thus, although arsenite synergistically activated the EGFR with TGFα, the co-stimulation resulted in a dose-dependent shift from a STAT3 dominant to a STAT1 dominant activity pattern. Arsenite alone produced no significant change in STAT activity (Figure 3, lane 1-6).

Increased association of the EGFR with STAT3 and STAT1 by arsenite: The activation of STAT3 by EGFR requires its interaction with the intracellular signal region of the EGFR (3,13,38). The phosphorylation of the intracellular signal region, particularly Y1068 and Y1086, provides the docking sites for phosphotyrosine (EGFR)-SH2 (STAT3) interaction and the receptor proximal recruitment of STAT3 (43). To rule out the possibility that the suppression of STAT3 activity in the activated state of EGFR is a result of arsenite selective blocking of STAT3 interaction with the EGFR, we conducted co-immunoprecipitation experiments to evaluate the physical association of STAT3 and STAT1 with the EGFR. After 16 hours serum-free starvation, A431 cells were stimulated with TGFα and arsenite (Figure 4, right panel); or, in a parallel experiment, treated with arsenite alone (Figure 4, left panel). As expected, TGFα stimulation induced EGFR activation (Figure 4, right panel, row 5, lane 1, from top to bottom). Co-stimulation by arsenite and TGFα increased the ligand-induced activity of the EGFR (right panel, row 5, compare lane 1 to lane 2, 3, and 4). Co-stimulation of EGFR by arsenite and TGFα could increase the association of EGFR with STAT3 (right panel, row 2) and STAT1 (right panel, row 4). The increased level of pY705 STAT3 (right panel, top row) and pY701 STAT1 (right panel, row 3) in the co-stimulated EGFR-associated immunocomplex was also found (compare lanes 1-4 in right panel). With arsenite alone, the association of STAT3 with the EGFR was slightly increased,

particularly with the exposure of higher concentrations of arsenite (left panel, row 2, compare lane 1-4). The association of STAT1with EGFR was also increased after exposure to arsenite (left panel, row 4, compare 1-4). The increased association of STAT3 and STAT1 with EGFR appears to be correlated with increased tyrosine phosphorylation of the EGFR (compare lane 1-4 in left and right panels). Although arsenite alone was sufficient to activate EGFR (left panel, bottom two rows, compare lane 1-4) and increased the association of STAT3 and STAT1 with the EGFR, no detectable tyrosine phosphorylation of STAT3 (left panel, row 1) or STAT1 (left panel, row 3) in the immunocomplex was found. Taken together, our results show that arsenite did not disrupt the specific interaction between EGFR and STAT3 or STAT1. Therefore, the distinct activation of STAT3 and STAT1 by the EGFR in the presence of arsenite is not a result of selective disruption of the physical association of STAT3 with the EGFR. Interestingly, in co-immunoprecipitation experiment (Figure 4), we found an unidentified protein (110~120 kDa) associated with the anti-EGFR immunocomplex and detectable by anti-STAT3 specific antibody (left and right panels, row 2, lane 4). Whether or not this unidentified protein plays a role in the selective inhibition of STAT3 requires further investigation.

It is known that the non-receptor tyrosine kinases c-Src and JAK can synergistically increase the EGFR-dependent activation of STAT3 (1,18) and the oncogenic activity of the EGFR (31). Other studies have shown that the JAK1 activity might be necessary for the c-Src-induced activation of STAT3 (8,51). In EGFR pathway, inhibition of JAK family tyrosine kinases partially blocks EGF stimulation-induced activation of STAT3; and inhibition of c-Src activity can block the JAK1 phosphorylation

in response to the EGF stimulation and EGFR-dependent STAT activity (18,22,37). These results indicate that c-Src mediates the activation of JAK1 by EGFR and suggest critical roles of JAK1 and c-Src in the maximal activation of STAT by the EGFR.

We have shown that arsenite can inhibit JAK kinase in cellular and subcellular systems (11). It is possible that, in EGFR pathway, arsenite can selectively inhibit JAK1 and/or c-Src while synergistically activate EGFR with the ligand. In order to examine these possibilities, with or without the presence of EGFR, we performed experiments to determine the effects of arsenite on JAK and c-Src kinase activity. In addition, we also examined if EGFR activation is sufficient for the activation of STAT3 or STAT1 and if JAK1 is essential for the EGFR-dependent activation of STAT proteins.

Selective inhibition of JAK kinase activity by arsenite. Previously, we have found that overexpression of wild-type JAK1 in JAK1-null Hela cells resulted in STAT3 activation (11) and no STAT1 activation was observed (unpublished data). Treatment by arsenite inhibits JAK1 kinase activity and blocks JAK1-dependent STAT3 tyrosine phosphorylation. In the same study, we found that arsenite can inhibit other JAK family members (Tel-JAK (JH1) fusion proteins), suggesting that arsenite can exert direct interaction with JAK catalytic domain. In the present study, we used an EGFR/JAK chimera, which is composed of the extracellular and transmembrane portions of the EGFR intracellularly fused to the JAK2 kinase domain (JH1 domain) (Figure 5A). Overexpression of the chimeric EGFR/JAK in 293T cells released JAK kinase activity and induced tyrosine phosphorylation (Figure 5B, top row, lane 1). Exposure to arsenite abolished its tyrosine kinase activity (lane 2). In a control experiment, arsenite did not inhibit the activity of wild-type EGFR in 293T cells (data not shown). This result

confirms our previous finding that arsenite can selectively inhibit JAK kinase activity while it synergistically activates EGFR. Our results demonstrate that arsenite is not a generic tyrosine kinase inhibitor.

Biphasic regulation of c-Src activity in the EGFR pathway by arsenite: To determine whether or not arsenite can affect c-Src activity, we first assessed the effect of arsenite on c-Src kinase activity using c-Src-containing immunocomplexes. To prepare anti-c-Src immunocomplexes, JAK1-null U4A cells with or without stable expression of the EGFR (U4A *Jak*$^-$ or U4A *Jak*$^-$*/EgfR*) were exposed to increasing concentrations of arsenite. The aliquots of c-Src-containing immunocomplexes were labeled by [γ-^{32}P] *in vitro* to analyze c-Src activity (Figure 6A). In the presence of EGFR (U4A *Jak*$^-$*/EgfR*, Figure 6A, lanes 1-5), co-stimulation with TGFα and low levels of arsenite increased c-Src autophosphorylation (Figure 6A, top row, compare lane 1, 2 and 3) and phosphorylation of tyrosine kinase substrate Sam68 (Figure 6A, row 2, compare lane 1, 2, and 3), indicating that low concentration of arsenite exposure enhances EGFR-dependent c-Src activity. However, exposure of cells to higher concentrations of arsenite (lane 4 and 5) resulted in a suppression of c-Src activity which leads to a decrease of both autophosphorylation of c-Src (top row) and phosphorylation of Sam68 (row 2). The results indicat that arsenite, at high levels, can block the EGFR-dependent activation of c-Src. We failed to see c-Src activity response to arsenite in the absence of EGFR expression (U4A *Jak*$^-$, Figure 6A, lane 6-10), suggesting that the elevated c-Src activity upon arsenite (lane 1-5) is EGFR-dependent. The biphasic effect of arsenite on the activity of c-Src in the presence of EGFR is correlated with the EGFR-dependent STAT3

activation (Figure 3), suggesting that the biphasic activity pattern of the STAT3 in response to arsenite-activated EGFR is functionally associated with c-Src activity.

To further determine the direct effect of arsenite on the c-Src tyrosine kinase activity, purified recombinant c-Src protein kinase was labeled by [γ-^{32}P]-ATP under the condition of direct incubation of c-Src with different levels of sodium arsenite (Figure 6B). The direct incubation of arsenite with c-Src resulted in decreased autophosphorylation of c-Src (top band) and Sam68 phosphorylation (bottom band). As shown in Figure 6B, the inhibitory effect of arsenite on the c-Src kinase activity is dose-dependent (compare lane 1 to 2, 3, and 4). Our data show that arsenite affects c-Src activity in two different ways. Arsenite can increase c-Src kinase activity through EGFR-dependent pathway but it also directly exerts inhibitory effect on c-Src kinase activity.

Requirement of JAK kinase for the activation of STAT3 by EGF receptor. To determine the specific role of JAK1 in the activation of STAT3 by the EGFR, we established stable cell line expressing the EGFR (U4A *Jak1*$^{-}$*/EgfR*) using JAK1-null U4A cells (U4A *Jak1*$^{-}$). We also established stable expression of the EGFR in JAK1-positive U4A cells (U4A *Jak1*$^{+}$ and U4A *Jak1*$^{+}$*/EgfR*). Using these established U4A cells, we analyzed the EGFR-dependent STAT1 and STAT3 tyrosine phosphorylation by immunoblotting (Figure 7). In the absence of JAK1, little or no response of STAT3 activity over basal background was induced by the stimulation of TGFα (Figure 7, left panel, row 3, lane 7) and co-stimulation by TGFα and arsenite did not change the activity of STAT3 (Figure 7, left panel, row 3, lane 8). Conversely, the activity of STAT1 was induced by TGFα stimulation under the same condition (Figure 7, left panel, top row, lane 7). In contrast to STAT3, the activity of STAT1 was enhanced by co-stimulation

with TGFα and arsenite without JAK1 (Figure 7, left panel, top row, compare lane 7 to 8). Co-expression of JAK1 and EGFR restored the induction of STAT3 activity by TGFα (Figure 7, right panel, row 3, lane 7) and co-stimulation by TGFα and arsenite further increased the phosphorylation of STAT3 (right panel, row 3, lane 8). However, the STAT1 response to the stimulation of TGFα in U4A cells expressing both JAK1 and EGFR (U4A *Jak1*$^{+}$*/EgfR*) was similar to JAK1-null cells (U4A *Jak1*$^{-}$*/EgfR*) (top row, compare lane 7 in left and right panels). Comparing the differences between the responses of STAT3 and STAT1 to the EGFR activation with or without JAK1 expression, we found that JAK1 presence is essential for STAT3 activation by EGFR and is required to maximize the activity of STAT3 coupled to EGFR activation. The result of increased STAT3 phosphorylation in response to the co-stimulation (Figure 7, right panel, row 3, lane 8) is correlated with the increased formation of STAT3:STAT1 heterodimers in JAK1-positive cells (A431 and U4A *Jak1*$^{+}$*/EgfR*, see EMSA results in Figure 3 and Figure 8B).

Further, we used EMSA to analyze the effects of arsenite on the activation of STAT1 and STAT3 by EGFR. In JAK1-null cells stably expressing EGFR (U4A *Jak1*$^{-}$*/EgfR*), we found that the JAK1 depletion significantly impaired the activity of STAT3 in response to the TGFα stimulation. The ligand-induced formation of STAT3:STAT3 homodimers and STAT3:STAT1 heterodimers were weakly detectable (Figure 8A, lane 7). However, in the absence of JAK1, it appears that the low levels of arsenite exposure still slightly increased the formation of STAT3:STAT3 and STAT3:STAT1 (Figure 8A, compare lane 7 to lane 8, 9 and 10). Our results indicate that the role of JAK1 in the EGFR activation of STAT3 is necessary but not exclusive. High levels of exposure to

arsenite suppressed the activity of STAT3:STAT3 (compare lane 7 - 12). In contrast, co-stimulation of arsenite and TGFα increased the activity of STAT1:STAT1 homodimers in a dose-dependent manner (Figure 8A, compare lane 7-12). In JAK1-null cells with EGFR (U4A *Jak1*$^{-}$*/EgfR*), costimulation by arsenite (high levels)) and TGFα did not enhance the activity of STAT3:STAT1 heterodimers (Figure 8A, lane 11 and 12, compare Figure 8A to Figure 8B and Figure 3). Without EGFR expression, no altered activity of either STAT3 or STAT1 was detected (Figure 8A, lane 1-6).

Next, we analyzed the activity of STAT1 and STAT3 in JAK1-restored U4A cells (U4A *Jak1*$^{+}$ and U4A *Jak1*$^{+}$*/EgfR*) (Figure 8B). With the EGFR expression, TGFα stimulation induced dominant formation of the STAT3:STAT3 homodimers and STAT3:STAT1 heterodimers (Figure 8B, lane 7). The ligand co-stimulation with low levels of arsenite enhanced the formation of STAT3:STAT3 homodimers and STAT3:STAT1 heterodimers (Figure 8B, compare lane 7 to lane 8, 9 and 10, arsenite < 200 μM). However, further increase in the concentrations of sodium arsenite abolished the formation of STAT3:STAT3 homodimers and promoted the formation of the STAT1:STAT1 homodimers (Figure 8B, compare lane 7-14). In addition to STAT1:STAT1, formation of STAT3:STAT1 heterodimers was also increased along with the increase of arsenite exposure (Figure 8B, compare lane 7-14). In the absence of EGFR, little or no effect of arsenite on the activity of STATs was found, indicating that the altered STAT1 and STAT3 activity by arsenite is an EGFR-dependent event (compare lane 1-6 with lane 7-12).

Discussion:

The main finding of this study is that arsenite-induced EGFR activation results in a shift from a STAT3 to a STAT1 activity dominant pattern. The dominant activity shift from STAT3 to STAT1 is a combined result that reflects the synergism of arsenite with ligand activation of the EGFR and the inhibition of JAK1 and c-Src by arsenite (see schematic model, Figure 9). Our data demonstrate that, in the absence of TGFα, arsenite can activate EGFR; they also show that co-activation of EGFR by arsenite and TGFα changes the activity ratio of STAT3 to STAT1 (compared to ligand alone). Our data define a previously unrecognized pathway for the differential activation of STAT1 and STAT3 by the EGFR. In this process, the JAK1 and c-Src kinases are essential for modulating the EGFR in the activation of STAT3. Arsenite can inhibit JAK tyrosine kinase activity *in vitro* and *in vivo* (11). The inhibition of EGFR/JAK2 (JH1) chimeric kinase by arsenite indicates that arsenite inhibition of JAK kinase is likely a result of direct action on the JH1 catalytic domain. In EGFR positive cells, c-Src displays a unique biphasic response to arsenite, reflecting both an EGFR activity-dependent activation and a direct inhibitory action of arsenite.

Although it has been suggested that activation of STAT proteins by the EGFR requires the cooperation of the EGFR intrinsic kinase activity with JAK1 and c-Src, our analysis demonstrates that the EGFR alone is sufficient for the activation of STAT1 while JAK1 and/or c-Src are necessary for the maximal activation of STAT3 downstream of the EGFR. Many investigators have shown that EGFR, JAK1, or c-Src alone can activate STAT3 (9,24,38,44,52) but other studies also indicate that the coordinated activation of EGFR together with the activity of c-Src and JAK1 may be necessary for STAT3 activation (18,37,39). Our data favor a model in which STAT3 and STAT1 are

the direct substrates of EGFR, but the receptor-dependent activation of non-receptor tyrosine kinases (JAK1 and c-Src) further enhances STAT3 activation by the EGFR (Figure 9). In this model, autophosphorylation of EGFR induced by ligand binding results in the association of STAT proteins and the activation of JAK1 and c-Src. Activated JAK1 and c-Src, in turn, increase the EGFR kinase activity and further facilitate receptor recruitment of STAT proteins, particularly STAT3.

Our data show that the presence of JAK1 and c-Src kinase activity is essential for the activation of STAT3 by EGFR. The inhibition of c-Src and JAK1 kinase by arsenite appears to be the direct inhibitory action of arsenite on the kinases. However, we can not rule out the possibility that JAK1 or c-Src can serve as the "adaptor protein" to facilitate the interaction of STAT3 with EGFR, which are disrupted by higher-level exposure to arsenite. It is also possible that arsenite-activated EGFR may trigger a feedback loop that negatively regulates the functions of JAK1 and c-Src; or that the interaction between EGFR and STAT proteins can be directly regulated by arsenite exposure.

Our findings suggest that JAK1 and/or c-Src serve as a switching point for the determination of EGF proliferative/apoptotic signals. Biologically, aberrant activation of EGFR is associated with malignant progression and poor prognosis (15). Tumorigenesis induced by EGFR activation is, in part, a result of increased activity of STAT3. Clinically, aberrant STAT3 activity and the EGFR have been targeted for therapeutical intervention (4,7,26,34,43,45). For EGFR ligand-dependent cell cycle arrest, it has been demonstrated that the activation of STAT1 is a key factor for the EGFR-induced cell growth suppression (5,6,23). Thus, the activity balance of STAT1 and STAT3 as downstream mediators in relaying EGFR signaling may determine the biological

consequences of EGFR activity. As a consequence, our results are important in understanding the underlying mechanism by which EGFR signaling switches from a proliferation (STAT3) orientation to an apoptotic/cell growth suppression (STAT1) orientation.

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Haiyan

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Figure 1. Effect of arsenite on the activation of EGFR. **A.** Immunocomplex of EGFR kinase activity assay. Anti-EGFR immunocomplexes were prepared from treated A431 cells and was assessed for the EGFR activity by an *in vitro* kinase assay (see Materials & Methods). Lane 1-4 of top row shows the incorporation of ^{32}P in the EGFR protein (lane 1: no treatment control; lane 2: TGFα alone; lane 3: arsenite alone; lane 4: costimulation of TGFα and arsenite). Bottom row is the immunoblot of EGFR protein as a measure of protein loading. **B**. Recombinant EGFR protein kinase activity assay. Recombinant EGFR protein (1 unit per reaction) was incubated directly with [γ-^{32}P]-ATP and different concentrations of arsenite *in vitro*. Lanes 1, 2, and 3 are recombinant proteins incubated with arsenite alone. Lanes 4, 5, and 6 are the EGFR co-incubated with arsenite and additional TGFα (30 ng/ml).

Figure 2. Effects of arsenite on phosphorylation of individual tyrosine site within the EGFR intracellular tail. Clarified whole cell lysates from treated A431 cells were resolved with 7.5% SDS-PAGE gel and subjected to immunoblotting. Each of the five major tyrosine autophosphorylation sites within the EGFR intracellular tail was analyzed using the antibodies respectively recognizing each tyrosine residue (pY992, pY1068, pY1086, pY1148 and pY1173). Samples in left panel were treated with arsenite only (lane 2-6) with no treatment control in lane 1. In right panel, lane 1 is the sample treated with TGFα only. Lanes 2-6 in right panel are cells co-stimulated by arsenite and TGFα. The level of the EGFR protein was assessed (bottom) as a loading control.

Figure 3. EMSA analysis of arsenic effects on the activity of STAT proteins in A431 cells. Whole cell extracts from treated A431 cells were assessed for STAT3 and STAT1 activity by EMSA (*hSIE* probe). Lanes 2-6 are the extracts with treatment by arsenite alone with no treatment control in lane 1. Lanes 8-12 are the extracts with co-stimulation by arsenite and TGFα with the treatment of TGFα alone in lane 7. Lane 13 and 14 are the "supershift" results using an extract co-stimulated by arsenite and TGFα (same as lane 12). Three active forms of STAT dimers co-migrated with the probe are indicated by arrows: the homodimers of STAT3:STAT3 (3:3), heterodimers of STAT3:STAT1 (3:1), and homodimers of STAT1:STAT1 (1:1).

Figure 4. Effects of arsenite on the association of STAT3 and STAT1 with the EGFR. The anti-EGFR immunocomplex from treated A431 cells were resolved with 7.5% SDS-PAGE gel and analyzed for the association of STAT1 and STAT3 with the EGFR by immunoblot. Left panel is the cells treated by arsenite alone with no treatment control in lane 1. Right panel is the cells co-stimulated by arsenite and TGFα with ligand stimulation only in lane 1. Top row: immunoblot of pY705 STAT3; Row 2: immunoblot of generic STAT3 protein; Row 3: immunoblot of phosphorylated pY701 STAT1; Row 4: immunoblot of generic STAT1 protein; Row 5: immunoblot of phosphorylated EGFR (pY1173); Row 6: immunoblot of generic EGFR as a control.

Figure 5. Effect of arsenite on the activity of JAK kinase. **A**. Schematic structure of EGFR/JAK chimera. The fusion protein is composed of an extracellular/transmembrane portion of EGFR and a kinase domain (JH1) of JAK2 tyrosine kinase as the intracellular

tail. Receptor dimerization is mediated by the EGFR portion, which induces the intracellular tail autophosphorylation by the intrinsic tyrosine kinase activity of t JAK2 kinase (JH1) domain. **B**. The anti-EGFR (anti-extracellular portion of the EGFR, Upstate) immunocomplexes were prepared from transfected 293T cells and immunoblotted with anti-JAK2 antibody recognizing the carboxyl epitope within the JH1 domain (bottom row). The tyrosine phosphorylation of the chimeric protein was immunoblotted with anti-phosphotyrosine antibody (PY99) (top row). Left lane is the control without arsenite treatment; right lane is the result treated with 400 μM sodium arsenite for 1 hour.

Figure 6. Regulation of c-Src activity by arsenite. **A.** Anti-c-Src immunocomplexes were prepared from treated JAK1-null cell lines with or without expression of EGFR (Materials & Methods). The c-Src-containing immunocomplexes were assessed for the c-Src tyrosine kinase activity by kinase labeling with [γ-^{32}P]-ATP *in vitro*. Each reaction contains an aliquot of c-Src immunocomplex, recombinant substrate Sam68 (0.3 μg), and [γ-^{32}P]-ATP. The reactions were resolved with 7.5% SDS-PAGE gels and the ^{32}P incorporation into the c-Src (top row) and Sam68 (second row) was visualized by autoradiography. Lanes 1-5 are JAK1-null cells with expression of EGFR (U4A *Jak1*$^{-}$*/EgfR*); and lane 6-10 are the mocking control of JAK1-null stable cell line (U4A *Jak1*$^{-}$). Lane 1 (U4A *Jak1*$^{-}$*/EgfR*) and lane 6 (U4A *Jak1*$^{-}$) are cells treated with TGFα alone. Blot of c-Src protein is shown at the bottom as the control. **B.** Recombinant c-Src protein was co-incubated *in vitro* directed with sodium arsenite and the kinase activity was labeled by [γ-^{32}P]-ATP. Each reaction contains recombinant c-Src (1 unit), recombinant Sam68 (0.3 μg), [γ-^{32}P]-ATP and different amount of arsenite. ^{32}P incorporation into the c-Src and

Sam68 were visualized by autoradiography. Top band: ^{32}P incorporation in c-Src; bottom band: ^{32}P incorporation in Sam68.

Figure 7. Requirement of JAK1 in the activation of STAT3 by EGFR. Immunocomplexes of anti-STAT3 and anti-STAT1 from cells with or without JAK1 and EGFR were assessed for the activity of STAT3 and STAT1. Left panel is the result from JAK1-null U4A cells with EGFR (U4A *Jak1*$^{-}$*/EgfR,* lane 5-8); or without EGFR (U4A *Jak1*$^{-}$, lane 1-4). Right panel is the result of JAK1-restored U4A cells with EGFR (U4A *Jak1*$^{+}$*/EgfR,* lane 5-8); or without EGFR (U4A *Jak1*$^{+}$, lane 1-4). In both left and right panels, lane 1 and 5 are cells with no treatment (controls); lane 2 and 6 are cells treated with arsenite alone; lane 3 and 7 are cells treated with TGFα alone; and lane 4 and 8 are cells treated by arsenite and TGFα. From top to bottom, row 1: blot of pY701 STAT1; row 2: blot of generic STAT1; row 3: blot of pY705 STAT3; row 4: blot of generic STAT3.

Figure 8. Analysis of STAT activity by EMSA. **A**.EMSA result of STAT activity from JAK1-null U4A cells. JAK1-null U4A cells with EGFR (U4A *Jak1*$^{-}$*/EgfR,* lane 7-12) or without EGFR (U4A *Jak1*$^{-}$, lane 1-6) were stimulated by arsenite and TGFα. Lane 1 and 7 are cells treated with TGFα alone. Lane 13 and 14 are the "supershift" result of reaction incubated with additional 1μg STAT1 (lane 13) or STAT3 (lane 14). Three active STAT dimers co-migrated with [γ-^{32}P]-labeled probe (*hSIE*) are indicated by arrows. Bottom panels are immunoblots of the STAT1 (top row) and STAT3 (bottom row) for the control purposes. **B.** EMSA result of STAT activity from JAK1-restored U4A cells. Similarly,

JAK1-restored U4A cells with EGFR (U4A *Jak1*$^{+}$*/EgfR*, lane 7-12) or without EGFR (U4A *Jak1*$^{+}$, lane 1-6) were stimulated by arsenite and TGFα. Lane 1 and 7 are cells treated with TGFα alone. Lane 13 and 14 are the "supershift" results. Western blots for the STAT3 and STAT1 of the extracts are shown at the bottom (top row: immunoblot of STAT1; bottom row: immunoblot of STAT3).

Figure 9. Schematic model of arsenite-mediated distinct activation of STAT3 and STAT1 by EGFR. Ligand binding induces EGFR dimerization. Dimerized EGFR results in autophosphorylation of EGFR and activation of downstream non-receptor tyrosine kinases JAK1 and c-Src. Activated JAK1 and c-Src further enhance EGFR interaction with STAT proteins and receptor-proximal recruitment. The recruited STAT proteins are phosphorylated by EGFR intrinsic kinase and can be further enhanced by orchestral activity from activated JAK1 and c-Src. The functional cooperation between EGFR intrinsic kinase activity and JAK1/c-Src is necessary for maximal activation of STAT3. Arsenite synergistically increases ligand-induced EGFR activity. However, arsenite exerts direct inhibitory action on JAK1 and c-Src. Inhibition of JAK1 and c-Src subsequently blocks the STAT3 activation by the EGFR. In contrast, EGFR is sufficient for activation of STAT1. As a consequence, the combined result of arsenite upregulation of EGFR activity and inhibition of JAK1 and c-Src leads to the activity shift from STAT3 to STAT1 dominant in response to the activation of EGFR.

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Figure 1

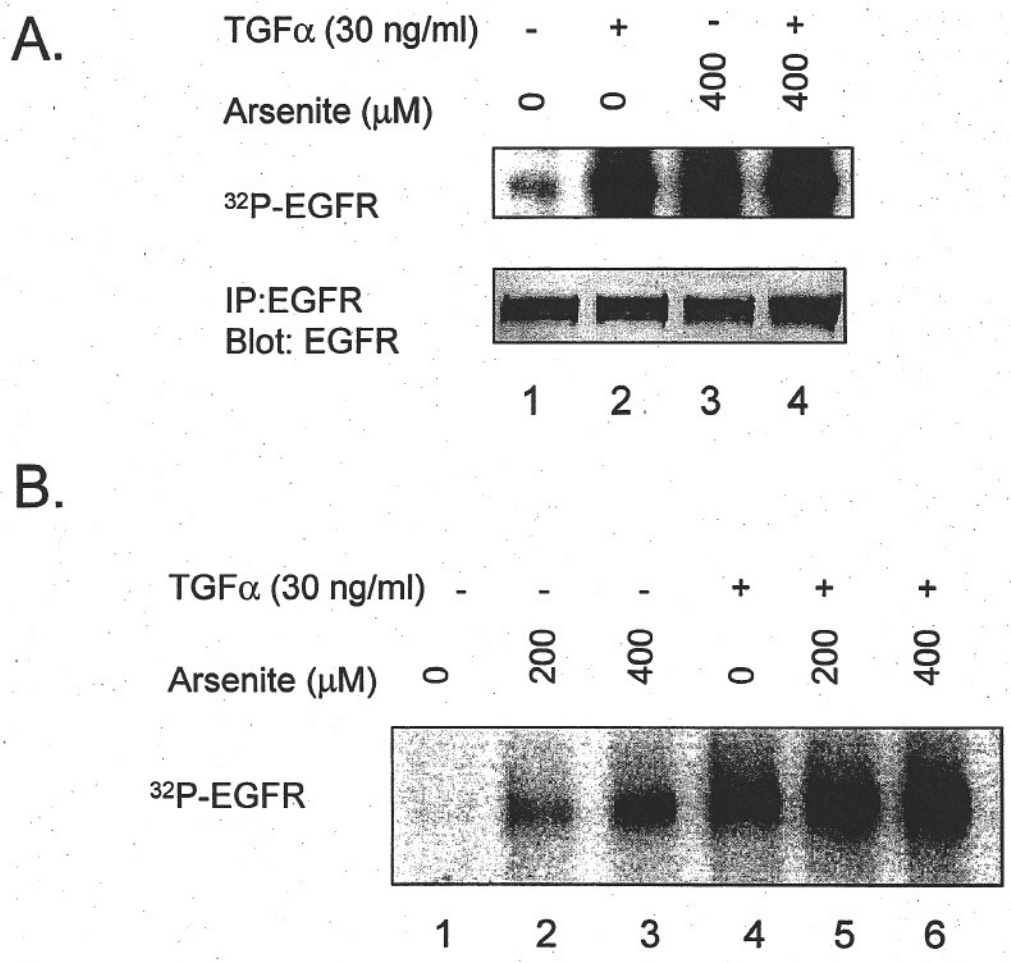
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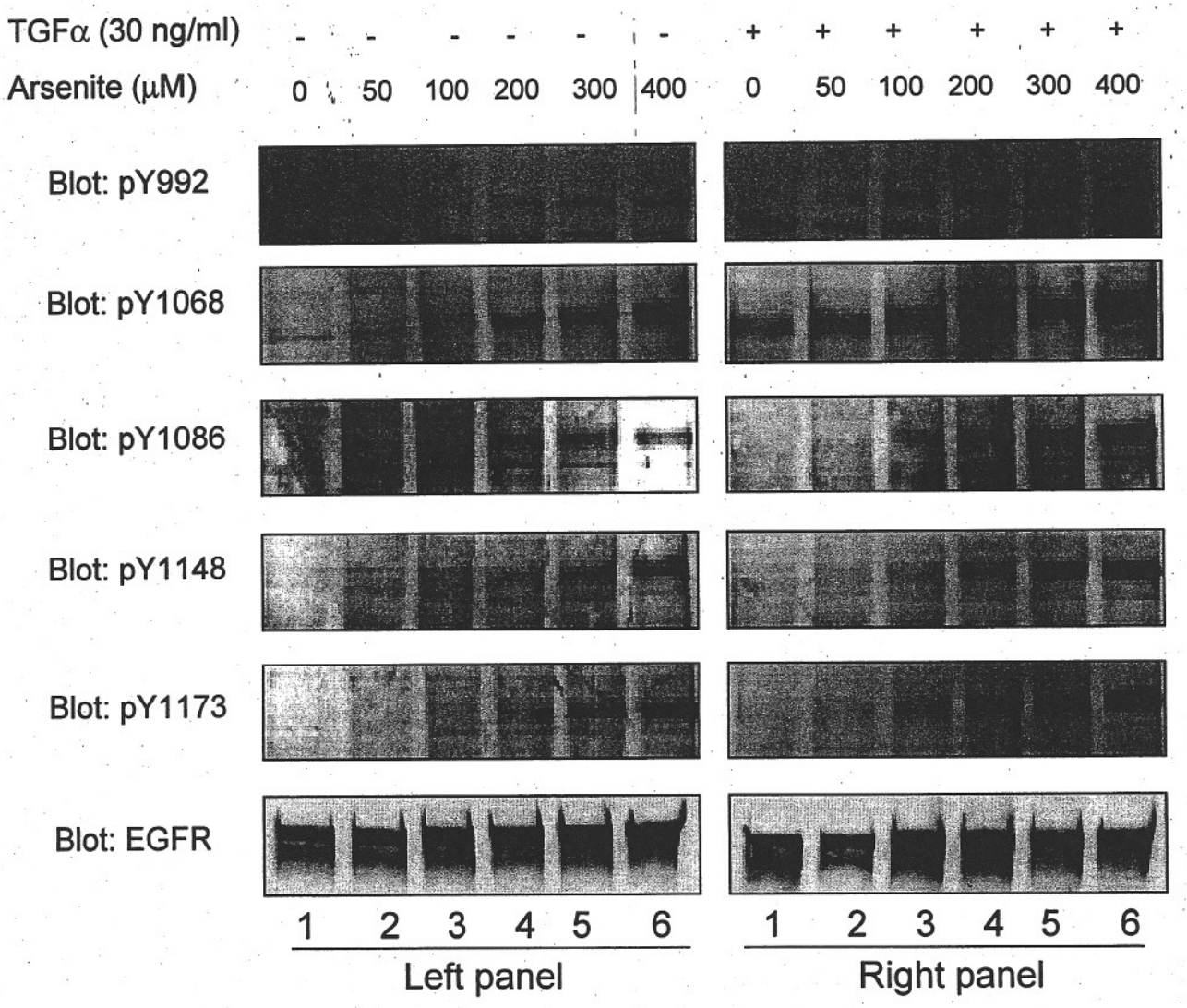
Figure 2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Blot: pY1086
Blot: pY1148
Blot: pY1173
Blot: EGFR
1 2 3 4 5 6
Left panel
1 2 3 4 5 6
Right panel

Figure 3

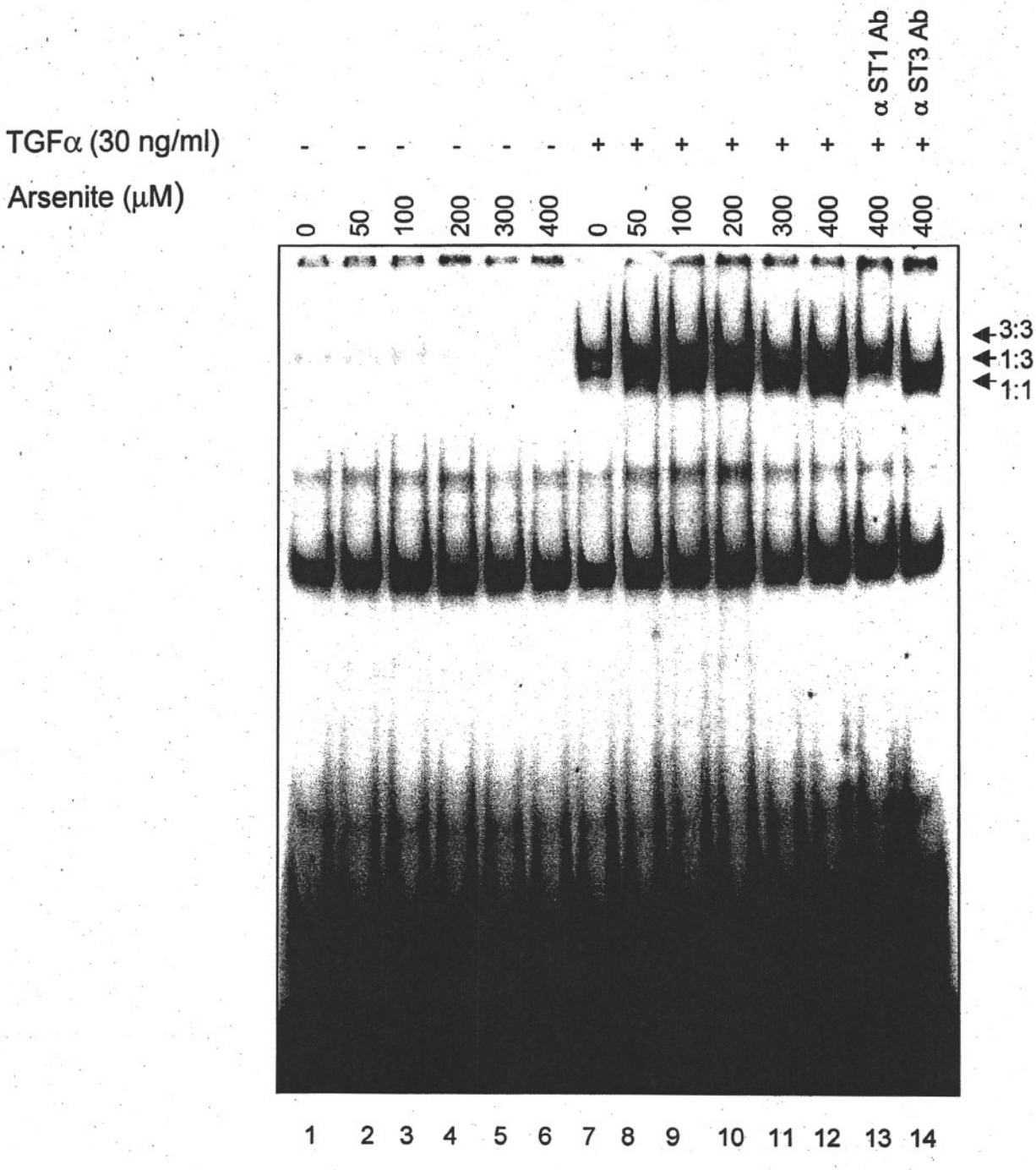

TGFα (30 ng/ml)
Arsenite (μM)
α ST1 Ab
α ST3 Ab
0 50 100 200 300 400 0 50 100 200 300 400 400 400
- - - - - - + + + + + + + +
3:3
1:3
1:1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4

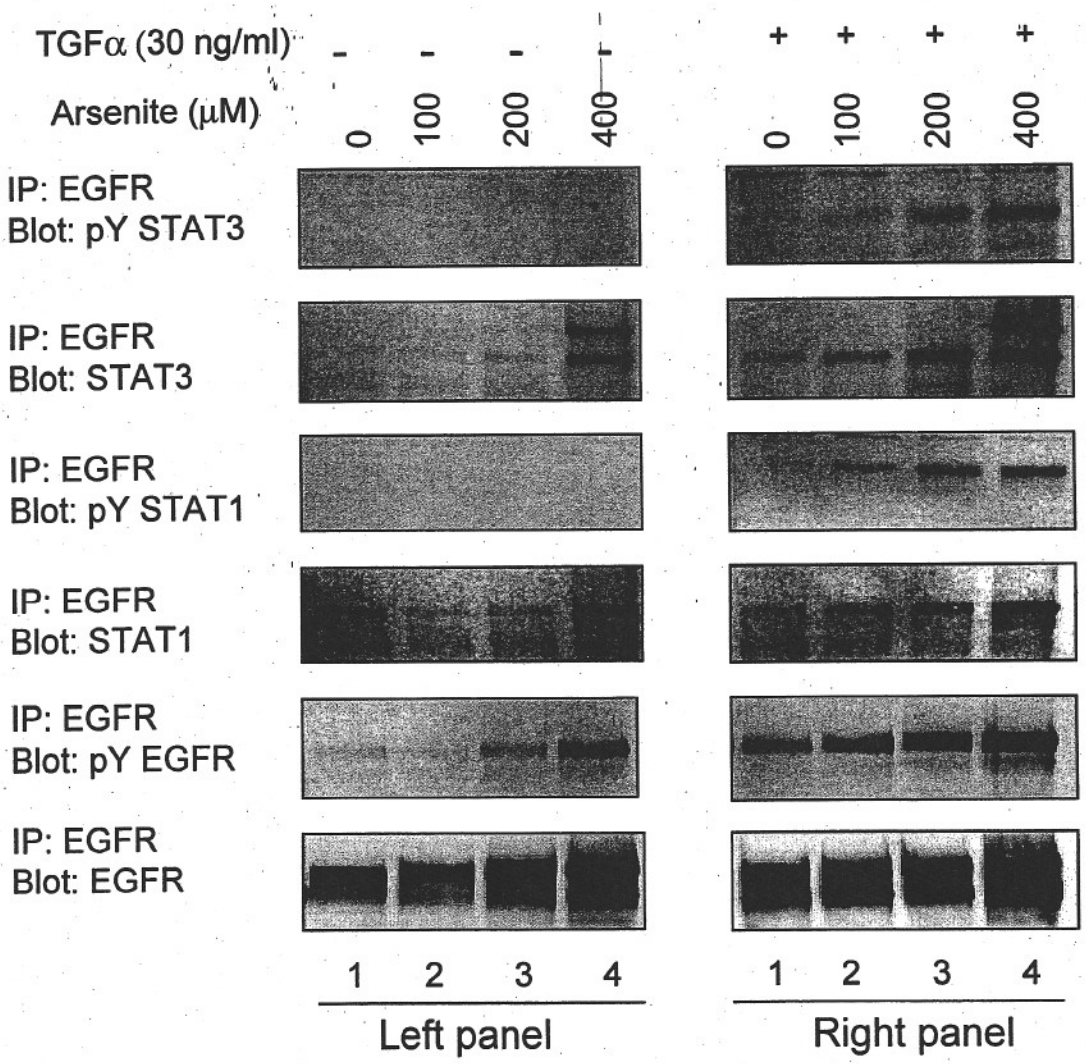

TGFα (30 ng/ml)
- - - -
+ + + +
Arsenite (μM)
0 100 200 400
0 100 200 400
IP: EGFR
Blot: pY STAT3
IP: EGFR
Blot: STAT3
IP: EGFR
Blot: pY STAT1
IP: EGFR
Blot: STAT1
IP: EGFR
Blot: pY EGFR
IP: EGFR
Blot: EGFR
1 2 3 4
1 2 3 4
Left panel
Right panel

Figure 5

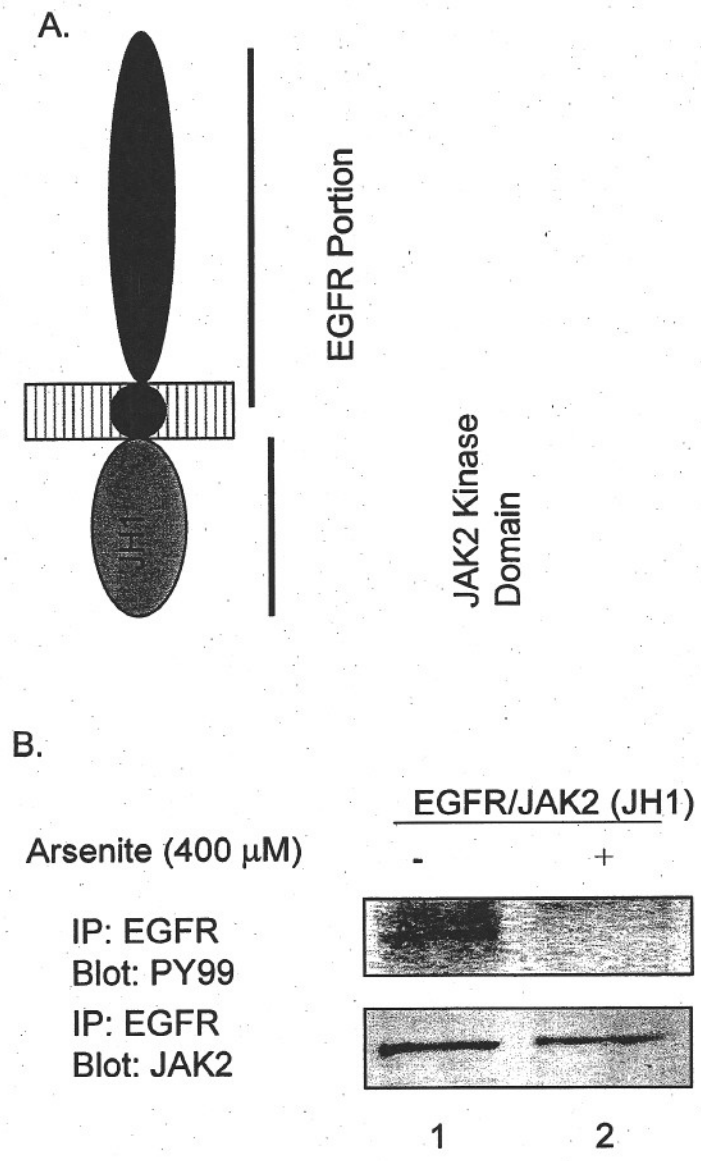

A.
EGFR Portion
JAK2 Kinase Domain
B.
EGFR/JAK2 (JH1)
Arsenite (400 μM)
-
+
IP: EGFR
Blot: PY99
IP: EGFR
Blot: JAK2
1
2

Figure 6

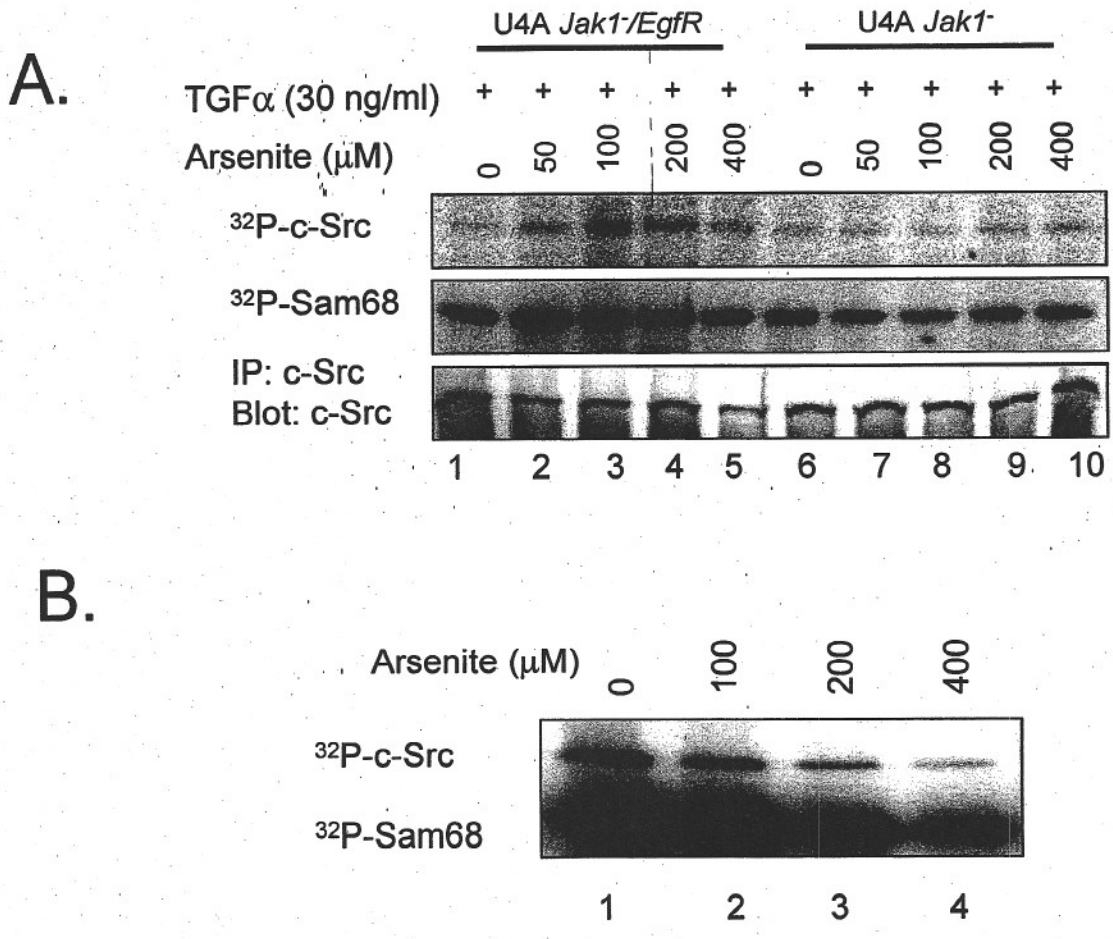

A.
U4A Jak1-/EgfR
U4A Jak1-
TGFα (30 ng/ml)
Arsenite (μM)
0
50
100
200
400
32P-c-Src
32P-Sam68
IP: c-Src
Blot: c-Src
1 2 3 4 5 6 7 8 9 10
B.
Arsenite (μM)
0
100
200
400
32P-c-Src
32P-Sam68
1 2 3 4

Figure 7

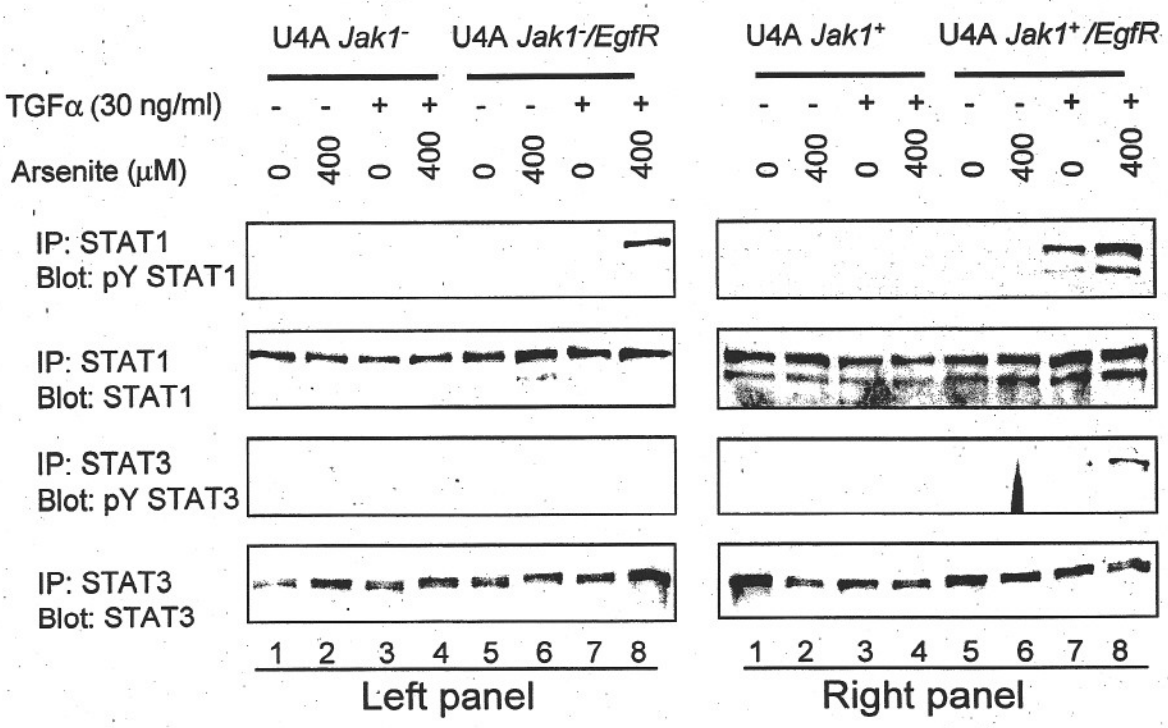
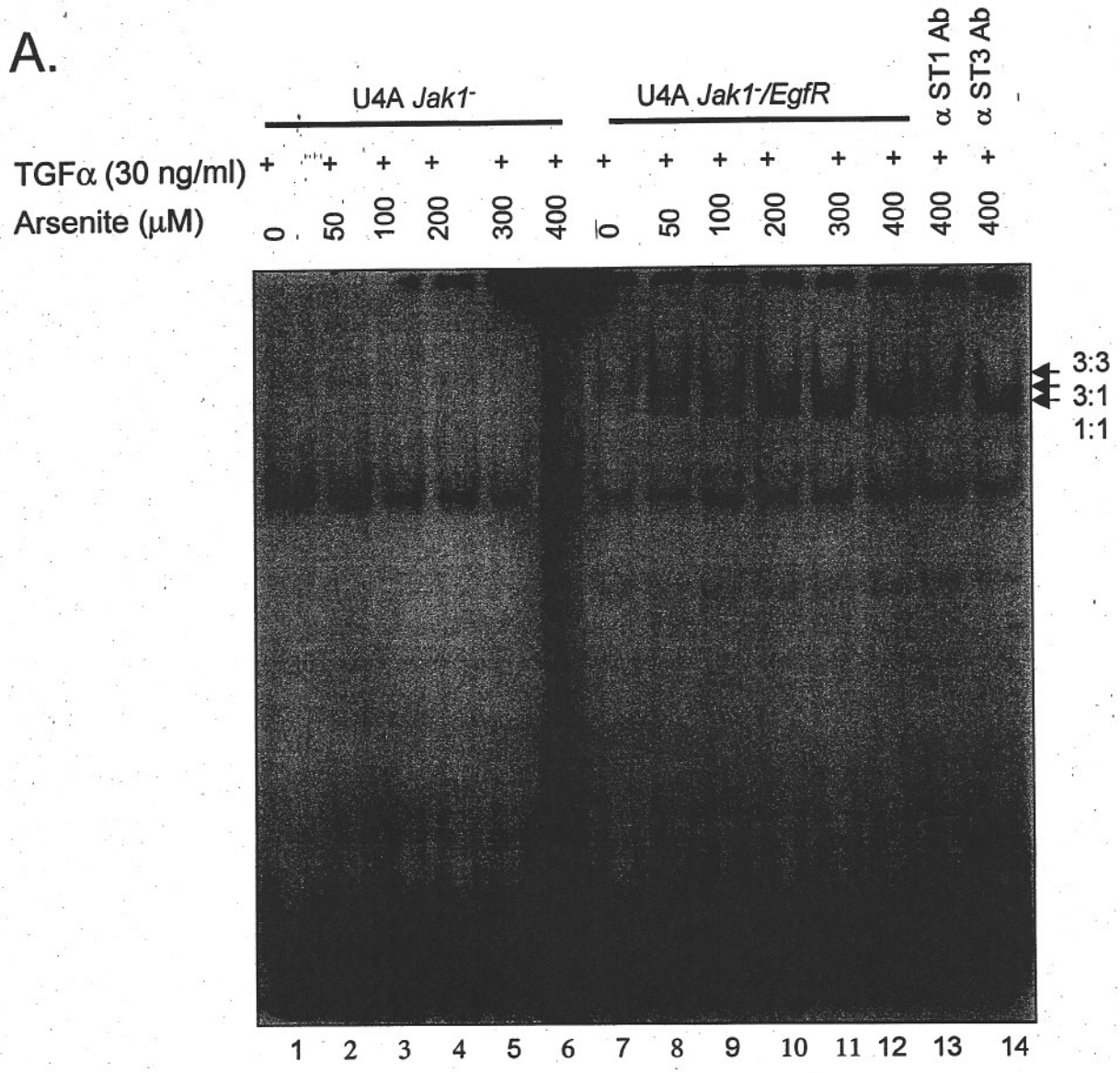

U4A Jak1-
U4A Jak1-/EgfR
U4A Jak1+
U4A Jak1+/EgfR
TGFα (30 ng/ml)
Arsenite (μM)
IP: STAT1
Blot: pY STAT1
IP: STAT1
Blot: STAT1
IP: STAT3
Blot: pY STAT3
IP: STAT3
Blot: STAT3
1 2 3 4 5 6 7 8
Left panel
1 2 3 4 5 6 7 8
Right panel

Figure 8

A.


U4A Jak1-
U4A Jak1-/EgfR
α ST1 Ab
α ST3 Ab
TGFα (30 ng/ml)
Arsenite (μM)
0
50
100
200
300
400
3:3
3:1
1:1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

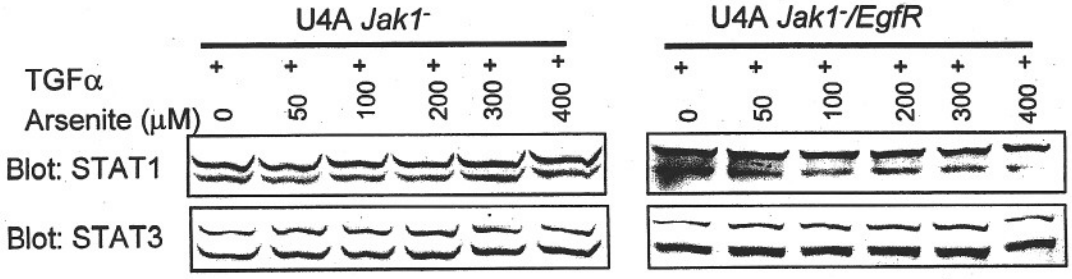
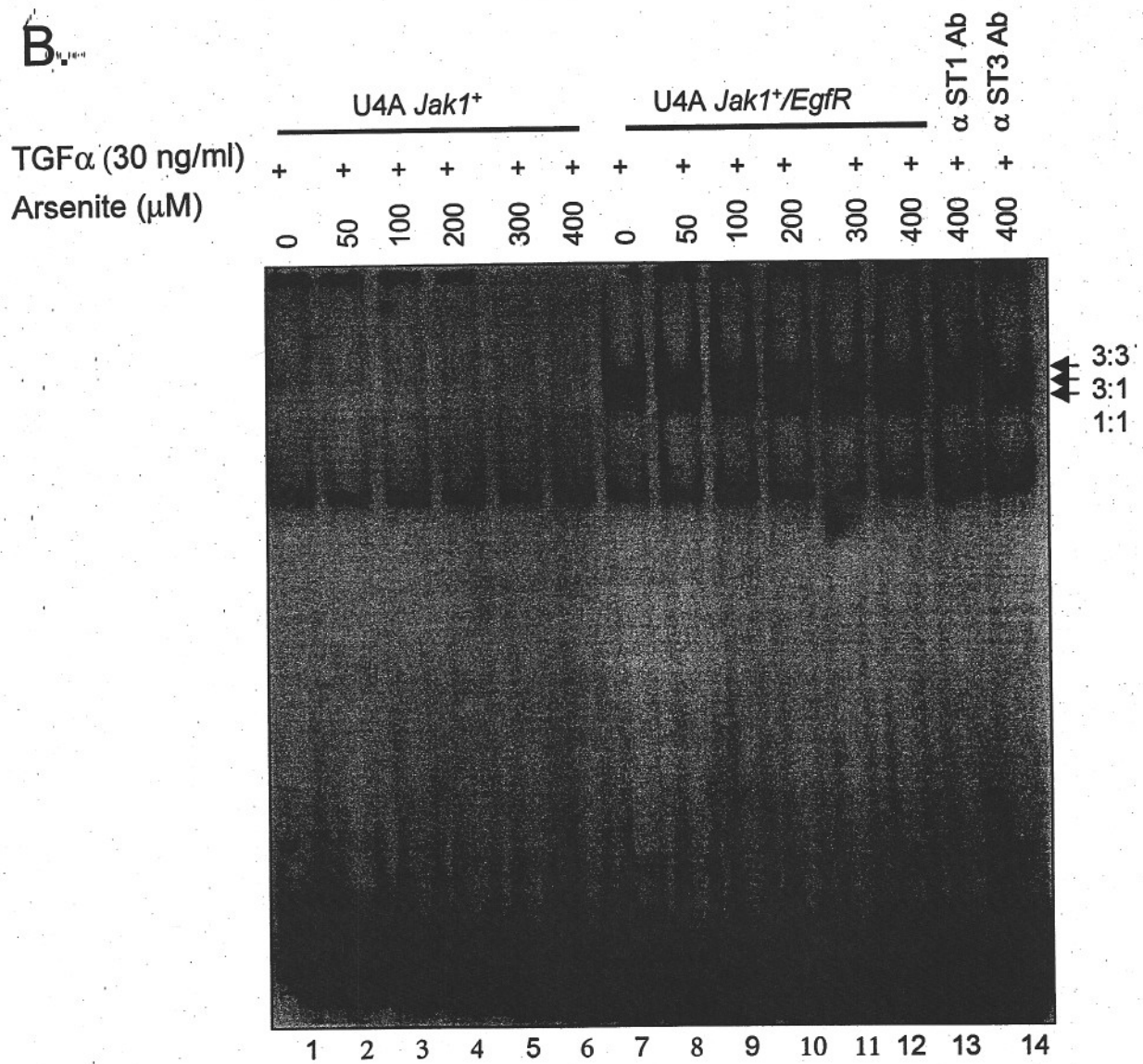

U4A Jak1-
U4A Jak1-/EgfR
TGFα
Arsenite (μM)
0
50
100
200
300
400
Blot: STAT1
Blot: STAT3

Figure 8

B.


U4A Jak1+
U4A Jak1+/EgfR
α ST1 Ab
α ST3 Ab
TGFα (30 ng/ml)
Arsenite (μM)
0 50 100 200 300 400 0 50 100 200 300 400 400 400
3:3
3:1
1:1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

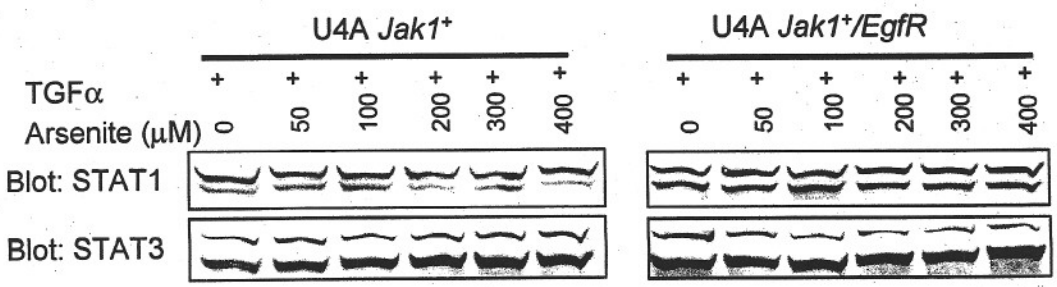
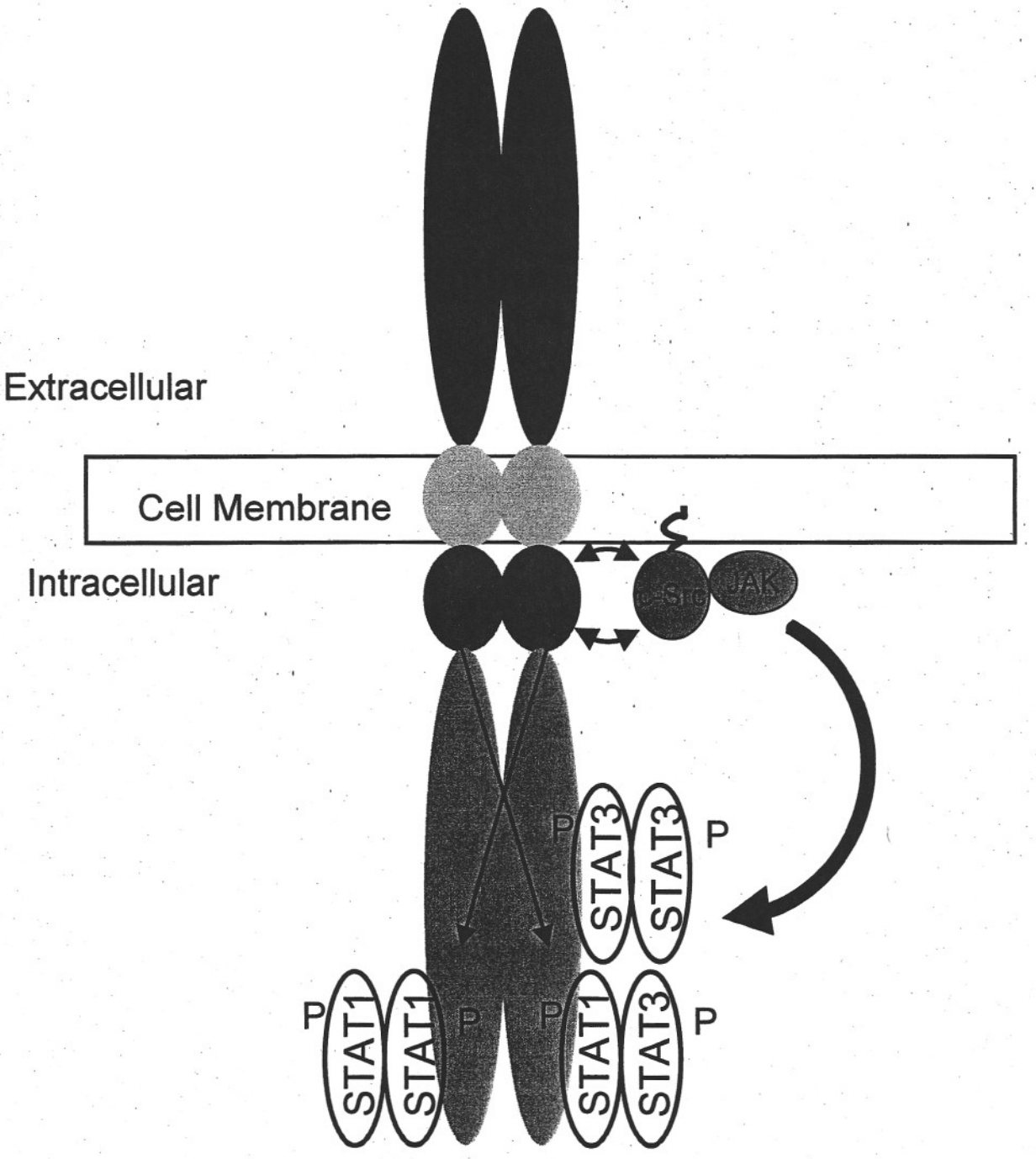

U4A Jak1+
U4A Jak1+/EgfR
TGFα
Arsenite (μM)
0 50 100 200 300 400 0 50 100 200 300 400
Blot: STAT1
Blot: STAT3

Figure 9


Extracellular
Cell Membrane
Intracellular
JAK
P
STAT3
STAT3
P
P
STAT1
STAT1
P
P
STAT1
STAT3
P

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Jaks and Stats: Development to Disease

Robert D. Schreiber, David E. Levy and John J. O'Shea

Fairmont Chateau Whistler • Whistler, British Columbia, Canada

April 15 - 20, 2004

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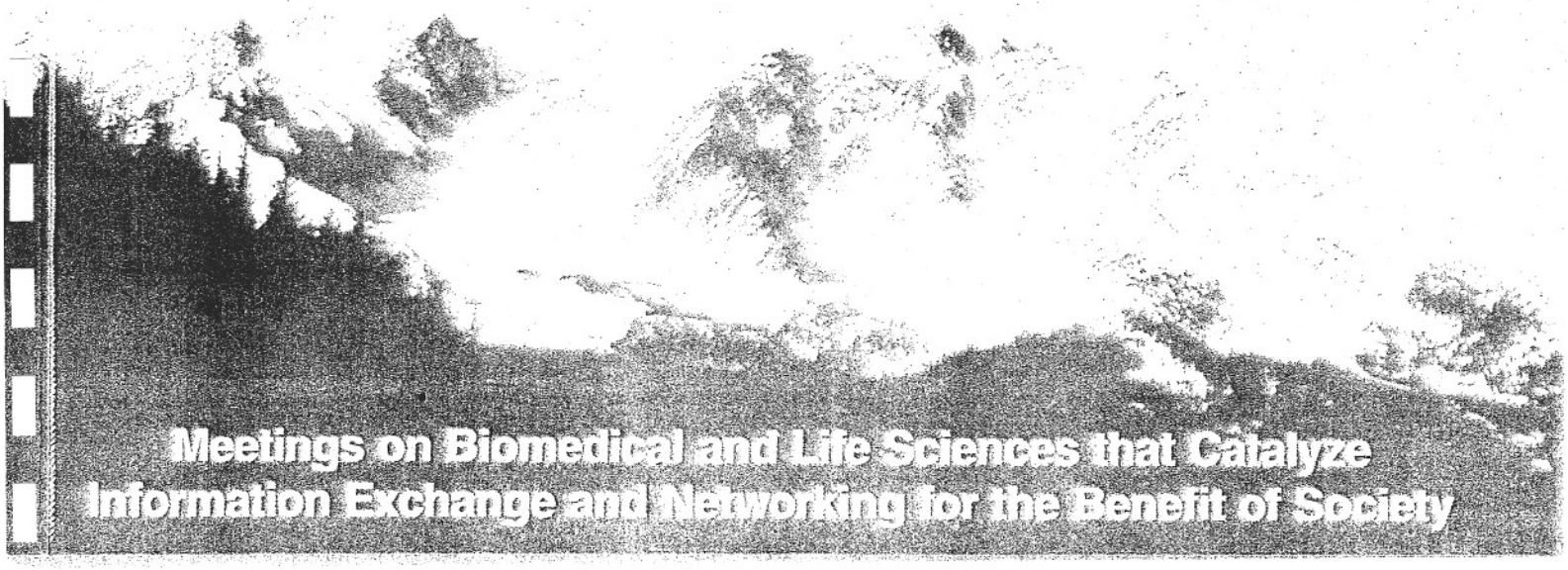

Jaks and Stats: Development to Disease

Robert D. Schreiber, David E. Levy and John J. O'Shea

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Summary of Meeting

The study of the Jak-Stat signaling pathway has now reached an extremely sophisticated level. Nevertheless, significant new insights continue to rapidly emerge involving either novel pathway components or novel actions of known pathway components that have profound influences on signaling through both the Jak-Stat pathway as well as other signaling pathways. Recent work has also shown that Jak and Stats are evolutionarily conserved signaling molecules that regulate a diverse group of fundamental processes ranging from stem cell renewal and embryogenesis to organogenesis and development of hematopoietic and lymphoid cells. Furthermore their roles in preventing or promoting infectious, malignant and autoimmune diseases have now become areas of intense study and exciting new findings are being made in these areas that have wide applicability to several fields of biology.

The goals of this meeting are as follows:
- Identify novel interactions between members of the Jak-Stat pathway not only with one another but also with receptors and components of other signal transduction pathways with special emphasis on positive and negative acting mechanisms that regulate Jak-Stat pathway signaling.
- Define the roles of the Jak-Stat pathway and its components in development of primitive and higher organisms with special emphasis being placed on model genetic organisms and on the hematopoietic and immune systems.
- Discuss the various protective versus disease-promoting effects of the Jak-Stat signaling pathway in infectious and neoplastic diseases.

Thursday, April 15

3 - 7pm	Registration	Macdonald Foyer
6:15 - 7:15pm	Welcome	Macdonald Foyer
7:15 - 7:30pm	Orientation	Macdonald AB
7:30 - 8:30pm	**KEYNOTE ADDRESS**	Macdonald AB

James E. Darnell Jr., Rockefeller University (001)
Integrating STATs in Transcriptional and Developmental Function

201 STAT2 nuclear trafficking

Gregg Banninger and Nancy C. Reich, Department of Pathology, Stony Brook University, Stony Brook, NY, USA, 11794

STAT2 is a transcription factor that is critical to a cell's innate response to virus infection. Localization is often crucial for the proper function of a protein. STAT2 resides primarily in the cytoplasm and interacts constitutively with a non-STAT protein, interferon regulatory factor-9 (IRF-9). Our studies demonstrate that IRF-9-STAT2 complex can shuttle in and out of the nucleus. IRF-9 contains a constitutive NLS that targets the complex to the nucleus, but a strong C-terminal NES within STAT2 rapidly exports the complex back to the cytoplasm. This leads to a cytoplasmic pool of STAT2-IRF-9 to allow for a rapid response to extracellular signals. In response to type I interferons (IFN-α/β/ω) STAT1 and STAT2 become tyrosine phosphorylated and dimerize. Dimerization leads to the creation of an additional NLS and targets the STAT1-STAT2-IRF-9 complex to the nucleus, where it binds DNA and activates transcription. Nuclear STAT2 is subsequently redistributed to the nucleus, allowing for continued response to extracellular signals. We are currently investigating the biological significance of STAT2-IRF-9 shuttling.

202 IRF-7 Plays a Key Role in the BRCA 1/Interferon γ Mediated Apoptotic Response

Buckley N. E., Mullan P.B., Andrews, H.N., McWilliams S., Quinn, J.E., Kennedy, R.D., Johnston, P.G., and Harkin, D.P.
Dept. Oncology, Queen's University Belfast, Belfast, Northern Ireland. BT9 7AB

BRCA1 encodes a tumour suppressor gene that is mutated in the germline of women with a genetic predisposition to breast and ovarian cancer. BRCA1 has been implicated in a number of important cellular functions including DNA damage repair, transcriptional regulation, cell cycle control, and more recently, ubiquitination. Using an Affymetrix U95A microarray IRF-7 was identified as a BRCA1 target and it was also shown to be synergistically up-regulated by BRCA1 in the presence of interferon-γ (IFN-γ), but not interferons -α or -β. This was accompanied by a synergistic induction of apoptosis. Transient transfection of three IRF-7 isoforms, IRF-7 A, B and C caused a decrease in cell proliferation compared to empty vector control implying that IRF-7 may play a role in the observed apoptosis. We have shown that there is protection from the BRCA1/IFN-γ mediated growth suppression when cells are treated with siRNA directed specifically against IRF-7 compared to a scrambled control.
It has been shown that BRCA1 can interact with STAT-1 to differentially activate transciption of a subset of IFN-γ target genes. We have shown, using STAT-1 specific siRNA and the pharmacological STAT-1 inhibitor, MTA, that STAT-1 plays a key role in the synergistic induction of IRF-7 by BRCA and IFN-γ.

This work has been funded by the European Social Fund and Cancer Research UK

203 Deacetylase activity is essential for interferon-stimulated gene transcription, acting at a step downstream of ISGF3 assembly on target promoters

Hao-ming Chang, Matthew Paulson, Michelle Holko, Bryan R. Williams, Isabelle Marié, and David E. Levy
Departments of Pathology and Microbiology and NYU Cancer Institute, New York University School of Medicine, New York NY 10016; Rockefeller University, New York NY 10021; Department of Cancer Biology, The Cleveland Clinic, Cleveland OH 44195

IFN-stimulated gene expression is mediated by the ISGF3 transccription factor complex, composed of tyrosine phosphorylated Stat1 and Stat2, in conjunction with the DNA binding protein, IRF9. While Stat2 has been shown to contain the majority of the transactivation potential of ISGF3, the mechanisms of target gene activation are incompletely defined. We report a requirement for a histone deacetylase (HDAC) activity for ISGF3 function. Trichostatin A (TSA), a general inhibitor of HDACs, impairs interferon-stimulated gene (ISG) expression, suggesting that deacetylase activity is required in this transcriptional process. Microarray studies demonstrated the generality of the inhibition of ISG expression by TSA. Our data showed that TSA does not impair STAT1 STAT2, or IRF9 abundance or stability, IFN-stimulated phosphorylation, or nuclear retention. Electrophoresis mobility shift assays similarly detected no impairment of ISGF3 integrity or DNA binding ability in TSA-treated cells. Recruitment of ISGF3 to chromatin in vivo was further confirmed by chromatin immunoprecipitation (ChIP) assays that showed that ISGF3 assembly was not abrogated by TSA. However, the transactivation capability of STAT2 was impaired by TSA, as demonstrated by reporter assays using Gal4-Stat2 fusions. Additional structurally unrelated HDAC inhibitors, such as valproic acid and HC toxin, also inhibited ISGs expression, demonstrating that the inhibition of ISG expression is mechanism based, requiring the catalytic activity of HDAC(s).

Inhibition of ISG expression by impaired HDAC activity resulted in a sevelley compromised antiviral state, as demonstrated by the inability of IFN to block hepatitis C viral replication without active HDAC function. Other inducible transcriptional processes also required HDAC activity, including induction of some IFN-γ target genes dependent on phosphorylated Stat1 and induction of virus-stimulated genes, dependent on activated IRF proteins. These results suggest that the requirement for deacetylase activity may be a general mechanism for regulating rapidly stimulated gene expression.

204 Distinct Activation of the Stat1 and Stat3 by the Epidermal Growth Factor Receptor

Haiyun Cheng, M. Lieberman, Baylor College of Medicine, Houston, Texas 77030

Ligand binding to the epidermal growth factor (EGF) receptor triggers various signal transduction pathways including activation of signal transducer and activator of transcription (STAT) factors as well as non-receptor tyrosine kinase JAK1 and c-Src. In this report, we show that the activation of different STAT isoforms (STAT3 & STAT1) by the EGF receptor involves different mechanisms. Physiologically, STAT3 is the major STAT isoform associated with the EGF receptor activation after ligand (TGFα or EGF) stimulation. Exposure to arsenite induced EGF receptor activation. However, co-stimulation with arsenite and TGFα or EGF resulted in a complete shift from a STAT3 to a STAT1 dominant activity pattern. Despite the fact that arsenite has synergism to the ligand-induced activation of EGF receptor, the activity of downstream JAK1 and c-Src is downregulated by high-level arsenite treatment. Here, we provide evidence that non-receptor tyrosine kinases are necessary for a maximal activation of STAT3 in the EGF receptor pathway. The shift of STAT3 to STAT1 activity in the activated EGF receptor pathway is a combined result of arsenite-activation of the EGF receptor and arsenite-inhibition of JAK1 and c-Src. Our study defines the molecular basis for the distinct activation of different STAT isoforms in the EGF receptor pathway and reveals two independent roles of the arsenite in the EGF receptor pathway: (1) arsenite activation of the EGF receptor; (2) arsenite inhibition of the JAK and c-Src.

The regulation of STAT distinct activation by the epidermal growth factor receptor is still active. Our recent discovery is novel and revealing the important regulatory pathway for the activation of STAT proteins. Because of its potential implications in further understanding of EGFR-STAT signal pathway; and most importantly, new strategy for the designs of therapeutics in the treatment of cancer or infectious diseases, the project is certainly innovative and NIH fundable. We have submitted to the NIH a full application (R21) on June 1st, 2004 and are expecting enthusiastically the evaluation.

Haiyun Cheng, M.D., Ph.D.
Assistant Professor
Pathology
Baylor College of Medicine

July 18, 2004