NOVEL MECHANISMS OF STAT3 ACTIVATION

by

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Abstract

Stat3 (signal transducer and activator of transcription-3) is activated by a number of receptor and non-receptor tyrosine kinases, while a constitutively active form of Stat3 alone is sufficient to induce neoplastic transformation. Results presented in this thesis reveal that Stat3 can also be activated through homophilic interactions by the epithelial (E)-cadherin and cadherin-11, two members of the classical type I and II cadherin family of surface receptors, responsible for the formation of cell to cell junctions. Indeed, by plating cells onto surfaces coated with fragments encompassing the two outermost domains of these cadherins, we definitively demonstrate that cadherin engagement can activate Stat3, even in the absence of direct cell to cell contact. At the same time, levels of the extracellular signal regulated kinase (Erk)1/2, which is often coordinately activated by growth factor receptors and oncogenes, remain unchanged upon cadherin ligation. Most importantly, we report, for the first time, an unexpected surge in total Rac1 and Cdc42 protein levels, triggered by cadherin engagement, and an increase in Rac1 and Cdc42 activity, which is responsible for the Stat3 stimulation observed. Inhibition of cadherin interactions reduced Rac/Cdc42 and Stat3 levels and induced apoptosis, pointing to a significant role of this pathway in cell survival signalling, a finding which could also have important therapeutic implications.

To better understand the role of Rac/Cdc42 in the cadherin-mediated Stat3 activation, we compared Stat3 activity in mouse HC11 cells before and after expression of the mutationally activated, RacV12. We demonstrate a dramatic increase in protein levels and activity of both the endogenous Rac and RacV12 with cell density, which was due to inhibition of proteasomal degradation. Moreover, we clearly show that RacV12 expression can activate Stat3 through an increase in expression of members of the IL6 family of cytokines, known potent Stat3 activators. In fact, knockdown experiments indicate that gp130 receptor function, and Stat3 activation, are
essential for the migration and proliferation of Rac$^{V12}$-expressing cells, thereby demonstrating that the gp130/Stat3 axis represents an essential target of activated Rac in the regulation of both of these fundamental cellular functions.
Co-authorship

The data presented in this thesis result from collaborations with a number of researchers from Queen’s University, including Dr. Adina Vultur, Dr. Jun Cao, Dr. Mulu Geletu, Dr. Bruce Elliott, Dr. Peter F. Truesdell, Esther Carefoot, as well as Dr. Hélène Feracci and Dr. Sébastien Chevalier, from the Université Bordeaux 1, Centre de Recherche Paul Pascal, Pessac, France, and Dr. Lionel Larue, from the Institut Curie, Paris, France. The preface to each chapter describes the contribution of each author to the work presented. Most of the results shown have been published or are being submitted for publication. The full citation for each published manuscript is found at the beginning of each chapter.
Acknowledgements

I am forever grateful to my supervisor, Dr. Leda Raptis, for her mentoring, advice and support. Her depth of knowledge, as well as her enthusiasm and exceptional dedication to research, teaching and her students, represent to me a profound source of inspiration. I would like to thank Dr. Adina Vultur for showing me the ropes, Dr. Jun Cao, Marilyn Garrett, Dr. Mulu Geletu, Reva Mohan, Sam Greer, Chrystele Chaize and the past and present members of the Raptis lab, who have been a true joy to work with. I am most appreciative of the constructive advice, encouragement and reagents received from my committee members, Dr. Bruce Elliott and Dr. Graham Côté, as well as Dr. Peter Greer. Over the course of my degree, I have thoroughly enjoyed the opportunity to collaborate with Dr. Héléne Feracci and Dr. Olivier Courjean, who provided us with several nice batches of cadherin fragments, and Dr. Lionel Larue who sent us the mouse embryonic stem cells as well as precious lots of serum that caused much grief with customs. I owe many thanks to Jerry Dering, Chris Boer, Emilia Furmaniak-Kazmierczak and Karoline Fisher for their technical assistance and expertise. I would also like to acknowledge members of the Microbiology and Pathology departments, especially John Wu, Dr. Simon Weli, Dr. Sébastien Fraud, Dr. Ray Amith, Dr. Preethi Jayanth, Victoria Au, Jalna Meens, Sara Sharifpoor, Blerta Starova and Maike Bossert, for their continuing friendship, support and advice.

I am immensely grateful to God for granting me strength, perseverance, and a thirst for knowledge, and above all for blessing me with a truly exceptional family. This thesis is dedicated to Mark, whose patience, understanding, sacrifice and devotion have guided me through this degree, to my mother, Cynthia, for her continuing faith, support and more reasons than I could ever fit on this page, and to Anjali, my greatest accomplishment and the source of my inspiration.
and joy. I would also like to thank my grandparents, our dear aunt Maria Ralli, my aunt and uncle, friends and cousins, especially Geetha, for all their help, encouragement and prayers.

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<th>Definition</th>
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<tbody>
<tr>
<td>AJ</td>
<td>adherens junctions</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
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<td>β-gal</td>
<td>β-galactosidase</td>
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<td>BES</td>
<td>N,N'-bis[2Hydroxyethyl]-2-aminoethanesulfonic acid</td>
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<td>C/EBPδ</td>
<td>CCAAT/enhancer binding protein δ</td>
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<td>CAPE</td>
<td>caffeic acid phenethyl ester</td>
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<td>CBP</td>
<td>cAMP-response element-binding protein (CREB) binding protein</td>
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<tr>
<td>CHR</td>
<td>cytokine binding homology region</td>
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<tr>
<td>CLC</td>
<td>cardiotrophin-like cytokine</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CNF</td>
<td>cytotoxic necrotizing factor</td>
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<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
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<td>cadherin-specific repeats</td>
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<td>Cdc42/Rac-interactive binding</td>
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<td>elongation factor-2</td>
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<td>EC</td>
<td>extracellular region of classical cadherins</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>E-cadherin</td>
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<td>enhanced green fluorescent protein</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>EMT</td>
<td>epithelial mesenchymal transition</td>
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<td>ERK</td>
<td>extracellular signal regulated kinase</td>
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<td>ES</td>
<td>embryonic stem cell</td>
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<td>fluorescence activated cell sorting</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>FERM</td>
<td>a band four-point-one, ezrin, radixin, moesin)</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FNIII</td>
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<td>GAP</td>
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<td>GEFs</td>
<td>guanine nucleotide exchange factors</td>
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<td>glycogen synthase-3β</td>
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<td>human herpes virus-8</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>Hrs</td>
<td>HGF-regulated substrate</td>
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<tr>
<td>hSIE</td>
<td>high affinity mutant, sis-inducible-element</td>
</tr>
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<td>Hsp90</td>
<td>Heat shock protein 90</td>
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<td>IFN</td>
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<td>IκB kinase</td>
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<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<td>interferon regulatory factor</td>
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<td>ITO</td>
<td>indium-tin oxide</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>LEF/TCF</td>
<td>lymphoid enhancer factor/T-cell factor</td>
</tr>
<tr>
<td>LIF</td>
<td>leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LNCaP</td>
<td>lymph node carcinoma of the prostate</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinases</td>
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<td>M-cadherin</td>
<td>myotubule-cadherin</td>
</tr>
<tr>
<td>MgcRacGAP</td>
<td>male germ cell Rac1 GAP</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
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<td>Definition</td>
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<tr>
<td>MLk</td>
<td>mixed lineage kinase</td>
</tr>
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<td>NFkB</td>
<td>nuclear factor-κB</td>
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<td>nuclear localization sequence</td>
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<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
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<td>NSCLC</td>
<td>non-small cell lung carcinoma</td>
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<td>OB-cadherin</td>
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<td>oncostatin M</td>
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<td>p21-activated kinase</td>
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<td>poly (ADP-ribose) polymerase</td>
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<td>platelet-derived growth factor receptor</td>
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<td>pfu</td>
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<td>PI3-kinase</td>
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<td>PIAS</td>
<td>protein inhibitors of activated STATs</td>
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<td>POSH</td>
<td>plenty of SH3 domains</td>
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<td>pser</td>
<td>phosphorylated serine</td>
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<td>protein tyrosine phosphatases</td>
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<td>RA</td>
<td>retinoid acid</td>
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<td>RANK-L</td>
<td>receptor activator of NFκB (RANK) ligand</td>
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<td>retinal-cadherin</td>
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<td>Rho-GDI</td>
<td>Rho guanine nucleotide dissociation inhibitors</td>
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<td>Rm</td>
<td>Rhesus macaque rhadinovirus</td>
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<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<td>SLIM</td>
<td>STAT-interacting LIM protein</td>
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<td>Smurf</td>
<td>Smad-ubiquitin regulatory factor</td>
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<td>SOCS</td>
<td>suppressors of cytokine signaling</td>
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<td>serum response element</td>
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<td>serum response factor</td>
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<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td>SUMO</td>
<td>small ubiquitin-related modifier</td>
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<td>TBS</td>
<td>tris-buffered saline</td>
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<td>Transforming growth factor</td>
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<td>tumor necrosis factor</td>
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<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
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<td>vascular endothelial-cadherin</td>
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<td>VEGF</td>
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Chapter 1

Introduction
Human cancer invariably occurs through the accumulation of stepwise mutations that allow normal cells to effectively evade homeostasis and continue proliferating. The mitotic decision is a highly regulated process, controlled by the coordinated integration of molecular signalling networks. Cell surface receptor activation through ligand binding or the interaction between adhesion molecules can communicate the status of the extracellular environment to the nucleus, where target gene regulatory proteins become altered, thereby generating an appropriate response from the cell concerning proliferation, differentiation or apoptosis (Weinberg, 2007; Juliano, 2002). Cytoplasmic modulators of signal transduction pathways include small GTPases and tyrosine or serine/threonine kinases, or phosphatases, which are often aberrantly expressed in cancer. A number of different mitogenic pathways can often converge onto a limited set of transcription factors, the final switches that control the gene expression patterns that ultimately lead to malignancy (Yu and Jove, 2004). Hence, targeting a single transcription factor may block the effects of several upstream mutations that cause its persistent activation.

It has emerged over the last decade that the Signal Transducer and Activator of Transcription-3 (Stat3) can be a critical point of convergence for many intercellular pathways that are often exploited in malignancy (Yu and Jove, 2004; see Figure 2.1). Hyperactive Stat3 is present in a large number of cancers, and is required for tumor cell growth and survival, as well as angiogenesis, metastasis and immune evasion (Bromberg et al., 1999; Bowman et al., 2001, Yu and Jove, 2004; Yu et al., 2009). Stat3 inhibition in tumors was shown to induce apoptosis while having little effect upon normal tissues, possibly because tumor cells may have become irreversibly dependent on Stat3 signalling to sustain their growth and survival, while normal cells may be able to use alternate pathways to compensate for Stat3 loss (Yu and Jove, 2004). As a result, drugs inhibiting Stat3 may be specific for the tumor, with little effect upon normal tissues. It follows that a comprehensive understanding of Stat3 signalling mechanisms is indispensable for the development of effective anticancer therapies.
Cells in normal tissues or tumors have extensive opportunities for adhesion to their neighbors in a three-dimensional organization, and it recently became apparent that in the study of fundamental cellular processes it is important to take into account the effect of surrounding cells. The relevance of interactions observed in densely growing, cultured cells to human cancer is clearly evident from findings demonstrating a close correspondence of genes expressed in prostate cancer LNCaP cells cultured to high, but not low, densities, with genes associated with prostate cancer in vivo (Chen et al., 2006).

We recently discovered the existence of a novel pathway of activation of Stat3, brought about by cell density. This Stat3 activation was triggered by cell to cell adhesion and resistant to inhibition of a number of tyrosine kinases often activated in a variety of cancers. We now further examine the molecular details of this cascade, to enable the identification of prognostic markers and promote the validation of this pathway as a target for drug design, especially for the treatment of tumors which may be resistant to inhibition of many tyrosine kinase oncogenes known to be hyperactive in many cancers.
Chapter 2

Literature Review
1. Signal Transducers and Activators of Transcription (STATs)

*STAT activation pathways*

James E. Darnell and colleagues cloned the first members of the STAT family by purifying factors bound to promoters of interferon (IFN)-inducible genes (Fu et al., 1992). The IFN-α ligand-induced complex consisted of a 91 kDa protein identified as Stat1 and a 113 kDa protein termed Stat2, whereas IFN-γ stimulation seemed to trigger Stat1 homodimerization (Schindler and Darnell, Jr., 1995; Xu et al., 1996). Since then seven STAT family members have been identified in mammalian cells: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 (Kisseleva et al., 2002).

STATs are a family of latent transcription factors that become activated upon tyrosine phosphorylation. STAT activators include growth factor receptors with intrinsic kinase activity such as epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR), as well as cytokine receptors which are non-covalently associated with the JAK family of tyrosine kinases (Darnell, Jr., 1997; Murray, 2007; Yu and Jove, 2004). Cytoplasmic Src kinases and the Bcr-Abl oncoprotein were also shown to phosphorylate certain STATs independent of receptor engagement (Yu and Jove, 2004). Phosphorylated STATs dimerize via reciprocal phosphorylated tyrosine (ptyr)-Src-homology-2 (SH2) interactions and migrate to the nucleus where they bind target regions of DNA to regulate the transcriptional activation of genes involved in immune function, development, differentiation, proliferation and survival (Darnell, Jr., 1997; Murray, 2007; Yu and Jove, 2004). The classical model of Stat3 activation is presented in Figure 2.1.
Figure 2.1: Classical model of Stat3 activation:

Stat3 (red) is an important point of convergence for signalling pathways often activated in cancer. Binding of growth factors (a) or cytokines (b) to their receptors results in the stimulation of the intrinsic receptor tyrosine-kinase activity or that of receptor-associated kinases, such as JAKs (blue) or cSrc (pink), which may phosphorylate the cytoplasmic tail of the receptor. Cytoplasmic Stat3 then binds onto the activated receptor with its SH2 domain, is phosphorylated by the JAK kinases, dimerized through reciprocal SH2-ptyr interactions and translocates to the nucleus where it activates transcription of a number of genes involved in cell division or survival. Stat3 also downregulates p53 by direct binding to the p53 promotor (Niu et al., 2005). (c) Mutationally activated Src, or other non-receptor tyrosine kinases such as Abl or Btk autophosphorylate and activate Stat3 in a similar manner (Zhang et al., 2000).

Figure adapted from Yu and Jove, 2004.
Structure of STATs (Figure 2.2)

STAT proteins contain five structurally and functionally conserved domains and a variable carboxy (C)-terminal end. The amino (N)-terminal region is believed to be involved in the oligomerization of STAT dimers, which can promote cooperative binding to tandem DNA binding elements resulting in more efficient transcriptional control (Kisseleva et al., 2002; Bowman et al., 2001). The coiled-coiled region of STATs consists of four alpha helices which form a large hydrophilic surface that can interact with proteins involved in transcriptional regulation, for example the Interferon Regulatory Factor (IRF) family of DNA binding proteins which are important in IFN signalling responses (Kisseleva et al., 2002; Horvath et al., 1996). The DNA binding domain allows for direct association of STATs at various promoter regions. Most STATs recognize an 8-10 base pair consensus sequence of 5’ –TT (N₄₋₆)AA – 3’ referred to as the GAS (gamma activation sequence) element, as it was initially characterized as the IFN-γ activation sequence recognized by Stat1 homodimers (Xu et al., 1996). The SH2 domain can recruit STATs to specific ptyr motifs on ligand-activated receptors and also mediates STAT homo- or heterodimerization by recognizing, once phosphorylated, the preserved tyrosine, located approximately 700 residues from the N-terminus (Kisseleva et al., 2002).

The carboxy-terminal domain of STATs is variable among members and encodes a transactivation domain (Kisseleva et al., 2002). Stat1, Stat3, Stat4, Stat5a, Stat5b and Stat6 can become phosphorylated on a serine (ser) residue within this region (pser727 in the case of Stat1 and Stat3), and this is believed to be required for maximal transcriptional activation (Darnell, Jr., 1997; Wick and Berton, 2000). In fact, ser727 phosphorylation was shown to be required for full neoplastic transformation by the constitutively active form of Stat3, Stat3C (Bromberg et al., 1999). Stat1β and Stat3β are naturally occurring splice variants which lack the transactivation domain as well as this serine residue; they can often block the function of the full-length proteins in a dominant negative fashion (Caldenhoven et al., 1996; Bowman et al., 2001). Darnell’s group
Figure 2.2: Domain structure of Stat3

STAT proteins contain five structurally and functionally conserved domains: the N-terminal domain is involved in oligomerization of STAT dimers, the coiled-coiled domain mediates interaction with other regulatory proteins, the DNA binding domain enables direct association with STAT binding sites at promoter regions, the linker region connects the DNA binding and SH2 domains, and the SH2 domain binds phosphorylated tyrosine motifs of activated receptors. Approximately 700 residues from the N-terminal domain of STATs is a preserved tyrosine residue, tyr705 in the case of Stat3, which becomes phosphorylated by kinases and allows for STAT dimerization through reciprocal SH2-ptyr interactions. The carboxy-terminal transcriptional activation domain is variable among STAT family members and contributes to STAT specificity. This C-terminal domain also encompasses a site of serine phosphorylation, ser727 for Stat3, which is believed to be required for maximal transcriptional activity of all STAT members, with the exception of Stat2 (Kisseleva et al., 2002). Figure adapted from Yu and Jove, 2004.
claims that although serine phosphorylation of Stat1 and Stat3 may enhance gene expression by these STATs, is not required for their DNA binding (Wen and Darnell, Jr., 1997). Further work has demonstrated that ser727 phosphorylation of Stat1 may instead optimize transcriptional activity by increasing its binding affinity for nuclear coregulatory proteins such as MCM5, a member of the mini-chromosome maintenance family involved in initiating DNA replication (Zhang et al., 1998).

While tyrosine phosphorylation of STATs takes place at the plasma membrane, their phosphorylation on serine has been shown to occur in the cytoplasm (Darnell et al., 1997). Reports have claimed that the Mitogen Activated Protein Kinases (MAPKs) such as p38, c-Jun N-terminal kinase (JNK) or ERK can trigger serine phosphorylation of certain STATs, thus enabling cross-talk between signalling cascades (Decker and Kovarik, 2000). More specifically, Turkson et al. have demonstrated that Ras- and Rac1-mediated p38 and JNK signalling can lead to Stat3, ser727 phosphorylation, and this is required for Stat3 activation by the Src kinase (1999).

STAT proteins typically exhibit long half-lives, however their stability may be affected by ubiquitin-modification, which can lead to the degradation of polyubiquitinated STATs by the 26S proteasome (Ulane et al., 2003; Tanaka et al., 2005). E3 ubiquitin ligases are the largest group of enzymes involved in the ubiquitination process and act as the key determinants of reaction specificity by catalyzing the transfer of ubiquitin to lysine residues of the substrate protein (Finley, 2009). The V protein of Mumps virus plays an important role in host immune evasion, and this may be attributed to its ability to act as an E3 ligase for Stat1, Stat2 or Stat3, resulting in the suppression of cytokine signaling (Ulane et al., 2003; Horvath, 2004). Interestingly, by removing Stat3 from cells, Mumps virus has also demonstrated clear oncolytic properties and high potential for use in cancer therapy (Ulane et al., 2003). In the nucleus, Stat4 has been shown to associate with the STAT-interacting LIM protein (SLIM) E3 ligase which can result in STAT ubiquitination and degradation (Tanaka et al., 2005).
Nuclear trafficking of STATs

In order to exert their function as transcriptional regulators, STAT members must transit nuclear pore complexes (NPC) within the nuclear membrane. Proteins larger than 50 kDa, including most extracellular signal-activated transcription factors, generally achieve this by binding to soluble carriers of the karyopherin-β family (Reviewed in: Reich and Liu, 2006; Terry et al., 2007; Herrmann et al., 2007). In general, transcription factors, or binding partners of the transcription factor acting as chaperones, may associate with any of six characterized importin-α adaptor proteins (1-6), through a specific motif called the nuclear localization sequence (NLS). The importin-α complex then interacts with the karyopherin-β member, importin-β1, which mediates docking of the protein cargo at the NPC where translocation occurs through an energy-dependant active transport mechanism dependant on the NPC-associated Ran GTPase (Terry et al., 2007). While conventional NLSs, consisting of a single stretch or bipartite sequence of basic amino acids, have not yet been identified for all STATs, several of the IRFs and other interacting factors which bind to STATs have shown to contain known or putative NLS and may assist in directing them to the nucleus (Reich and Liu, 2006; Subramanian et al., 2001). Interestingly, in the case of Stat1, a conditional NLS has been uncovered, which can be recognized by importin-α5 member only following its tyrosine phosphorylation and dimerization (Sekimoto et al., 1996; McBride et al., 2002; Reich and Liu, 2006). In contrast, tyrosine phosphorylation is not a prerequisite for nuclear import of Stat3 (Liu et al., 2005). Unexpectedly, studies have revealed that Stat3 can enter the nucleus even in an unphosphorylated state and dynamically shuttles between cytoplasmic and nuclear compartments (Yang et al., 2005). Indeed, Stat3 was found to contain a constitutive NLS within its coiled-coiled domain which can be recognized by importins-α3 and -α6 regardless of its phosphorylation status (Liu et al., 2005). The role of unphosphorylated Stat3 in the nucleus is presently unclear. Controversial data have revealed that Stat3 with a mutation in tyr705 can retain transcriptional activity to affect a different subset of
genes than wild type Stat3 (Yang et al., 2005). Others have identified breast tumor kinase (btk) as a nuclear tyrosine kinase able to phosphorylate and activate Stat3 (Liu et al., 2006). Nuclear export of STATs is believed to be regulated by Nuclear Export Sequences (NES), which may be recognized by the exportin carrier, chromosome region maintenance 1 (CRM1; Reich and Liu, 2006).

The multifunctional role of Stat3

Stat3 was originally discovered as a mediator of the acute phase response, activated by Interleukin-6 (IL6) in the liver (Wegenka et al., 1993). Stat3 is ubiquitously expressed in most tissues and its phosphorylation can be brought about by activated receptors of cytokines, growth factors and hormones as well as through intracellular kinases (reviewed in: Groner et al., 2008; Yu and Jove, 2004; see Figure 2.1). The most common ligands known to activate Stat3 include IL6, IL10, EGF and PDGF (Groner et al., 2008).

Of all the Stat genes, only Stat3 knockouts display embryonic lethality, at the gastrulation phase, which argues for a crucial role in development (Takeda et al., 1997). Tissue-specific inhibition of Stat3 using the Cre-loxP recombination system adapted from the P1 bacteriophage has been used to further characterize Stat3 function (reviewed in: Akira et al., 2000; Levy and Lee, 2002). For this, paired loxP sites can be inserted into introns surrounding critical regions of Stat3, such as the site for tyrosine phosphorylation (Levy and Lee, 2002). Introduction of tissue specific promoters driving the expression of Cre recombinase enables targeted deletion of the DNA segment flanked by the loxP sites (Levy and Lee, 2002). This approach can be used to generate mice that are viable despite the absence of Stat3 in selected tissues. Expression of the Cre recombinase gene under the keratin-5 promoter has uncovered a role for Stat3 in epithelial tissues, more specifically, in hair and skin remodeling, as well as wound healing, likely in response to growth factors such as EGF and PDGF (Sano et al., 1999; Kisseleva et al., 2002). Further studies from DiGiovani’s lab demonstrating that skin-specific Stat3-deficient mice were
resistant to chemically-induced skin carcinogenesis offered support to their model indicating that Stat3 plays a critical role in the initiation, promotion and progression of epithelial carcinogenesis (Chan et al., 2004; Kim et al., 2007). Paradoxically however, studies where Cre was expressed under the β-lactoglobulin promoter have revealed that loss of Stat3 in the mammary gland can delay involution and epithelial apoptosis after lactation (Clevenger, 2004). In this case Stat3 may be inducing expression of CCAAT/enhancer binding protein δ (C/EBPδ), shown to activate pro-apoptotic genes such as p53 (Thangaraju et al., 2005).

Stat3 is also a key player in modulating immune responses. Stat3 deletion in T cells was shown to impair IL6-mediated T-cell survival (Takeda et al., 1998). Stat3 deficiency in macrophages and neutrophils can cause deregulated cytokine expression where IL10 production is reduced allowing for an increase in TNFα-mediated inflammation (Riley et al., 1999). Myeloid cell-specific Stat3 deficiency also enhances the susceptibility of these mice to bacterial infection resulting in endotoxic shock (Takeda et al., 1999). Furthermore, certain paramyxoviruses, such as mumps or measles, have evolved, as a means of escaping the host response to infection, the ability to target Stat3 for degradation, revealing a vital role for Stat3 in antiviral immunity (Horvath, 2004).

In cell culture systems, Stat3 is required to maintain the pluripotency of mouse embryonic stem cells by preventing their differentiation (Raz et al., 1999). Stat3 can however promote the differentiation of a variety of lines including lymphocytes, granulocytes and astrocytes (reviewed in: Hirano et al., 2000). Stat3 has also been linked to B lymphocyte proliferation by inhibiting the anti-apoptotic gene bcl-2, and a similar survival function has been attributed to Stat3 in motor and sensory neurons in vitro, as few of these cells, following Stat3 knockdown, survive in vivo (Heinrich et al., 1998; Alonzi et al., 2001; Levy and Lee, 2002). In sharp contrast, Stat3 was shown stimulate the growth arrest and terminal differentiation of
monocytes by downregulating \textit{c-myc} and \textit{c-myb} and upregulating \textit{junB} and \textit{IRF-1} (Yamanaka et al., 1996; Hirano et al., 2000; Levy and Lee, 2002).

In sum, Stat3 can induce a wide variety of responses including proliferation and growth arrest, cell division and differentiation, as well as survival and apoptosis. This may be due to the upregulation of a different set of Stat3 target genes in different cell types (Levy and Lee, 2002). Nevertheless, Stat3 seems to be a central mediator of key cellular functions and efficient control of its signalling is vital to maintaining homeostasis.

\textit{Regulation of Stat3 signalling}

Positive regulation of Stat3 is thought to occur through the MAPKs which mediate its phosphorylation on serine, thus enabling cross-talk between signalling cascades (Turkson et al., 1999; Kisseleva et al., 2002). Studies have also revealed an association between Stat3 and various proteins which facilitate transcription through chromatin modification, such as the transcriptional coactivators CREB binding protein (CBP) and p300 (Yuan et al., 2005). Further research has demonstrated that CBP/p300 can also acetylate Stat3 at lys685, and this can contribute to Stat3 dimerization and activation (Yuan et al., 2005).

To prevent overstimulation, three main classes of negative regulators of STAT signalling have been identified. These include: (1) protein tyrosine phosphatases (PTPs), (2) suppressors of cytokine signalling (SOCS), and (3) protein inhibitors of activated STATs (PIAS) (reviewed in: Chen et al., 2004).

Classical PTPs consist of the transmembrane receptor-like PTPs, and the intracellular PTPs, which include SH2 domain-containing PTP1b, and T-cell (TC)-PTP (Chen et al., 2004). The defining feature of all PTPs is the presence of a signature motif within the active site of their catalytic domain which contains a preserved cysteine (Burke and Zhang, 1998). This critical cysteine can mediate nucleophilic attack on substrate phosphotyrosine residues to promote their hydrolysis (Burke and Zhang, 1998). Studies have identified the tandem ptyr motif of Jak2
(pYpY^{1007/1008}) as a substrate of PTP1b (Myers et al., 2001). Further studies have shown that PTP1b activation in glioma cells can decrease pro-survival Stat3 signalling by dephosphorylating Jak2, its upstream activator, thereby priming these cells for apoptosis (Gu et al., 2003; Akasaki et al., 2006). Mouse embryonic fibroblasts isolated from TC-PTP knockout mice also show increased Jak2 and Stat3 activity, however in this case Stat3 may be a direct target of TC-PTP (Yamamoto et al., 2002).

SOCS are a family of eight proteins (SOCSs 1 through 7 and cytokine inducible SH2-containing protein (CIS)) that can be stimulated by the very cytokines that enhance STAT activation, to act as inhibitors of JAKs (reviewed in: Chen et al., 2000). Hence SOCS proteins can function in a classical negative feedback loop. Structurally, SOCS proteins consist of an N-terminal domain of variable length followed by a central SH2 domain and a C-terminal SOCS box (Chen et al., 2000). Both SOCS-1 and SOCS-3 have shown to concurrently inhibit Stat3 activation by binding to upstream JAK kinases through their SH2 domain (Chen et al., 2000). A kinase inhibitory region (KIR) at the N-terminus of these SOCS may act to suppress the enzymatic activity of JAKs by competing with the access of substrates and/or ATP into their catalytic pocket (Yasukawa et al., 1999; Chen et al., 2000). More recent studies have revealed that the SOCS box may function in ubiquitin-mediated proteasomal degradation by associating with factors to form an E3 ubiquitin ligase complex (reviewed in: Chen et al., 2000). Consistent with this notion, SOCS-1 was shown to target Jak2 for proteasomal degradation (Ungureanu et al., 2002). Interestingly, SOCS-1 and SOCS-3 are found to be methylated and silenced in certain types of cancer (Baus and Pfützner, 2006; Tischoff et al., 2007; Fernández-Mercado et al., 2008).

PIAS family members, which are encoded by four genes in humans, \textit{PIAS-1}, \textit{PIAS-2} (\textit{PIASx}), \textit{PIAS-3} and \textit{PIAS-4} (\textit{PIASy}), function to regulate the activity of several transcription factors, notably STATs (reviewed in: Rytinki et al., 2009). PIAS-3 can bind directly to phosphorylated Stat3 homodimers or Stat1:Stat3 heterodimers to prevent DNA recognition, possibly by recruiting co-repressor proteins to the complex (Chung et al., 1997). PIAS proteins
are also known to exhibit SUMO (small ubiquitin-related modifier)-ligase activity, similar that of an E3 ubiquitin ligase, however this may not play a role in the repression of Stat3 by PIAS-3 (Chung et al., 1997; Rytinki et al., 2009).

More recent evidence has identified Gene Associated with Retinoid IFN-Induced Immortality (GRIM)-19, a novel factor involved in Retinoid acid (RA)/IFN-β triggered apoptosis, as an inhibitor of Stat3 nuclear translocation and transcriptional activity (Zhang et al., 2003). Overexpression of GRIM-19 inhibits Stat3 through a mechanism involving direct binding. Interestingly, GRIM-19 expression is found to be lost in a number of different cancers and its re-expression in a cervical cancer line was shown to restore growth suppression in vivo through downregulation of Stat3 and its targets (Zhou et al., 2009).

Stat3 and cancer

Given that Stat3 has the intrinsic ability to promote cell proliferation and survival, it is not surprising that overexpression of Stat3 has been demonstrated in a large number of solid and blood tumors (reviewed in: Bromberg et al., 1999; Bowman et al., 2001; Yu and Jove, 2004; Germain and Frank, 2007). Indeed, immunohistochemical analysis has revealed the presence of nuclear, tyrosine phosphorylated Stat3 in 75% of primary breast and prostate cancers (Alvarez et al., 2005). While oncogenic mutations within the Stat3 gene itself have not been reported, hyperactive Stat3 can result from persistent signalling by upstream tyrosine kinases, which themselves have become overactive due to genetic or epigenetic alterations or through silencing or deregulation of its repressors (Yu and Jove, 2004).

While Stat3 tyr-705 phosphorylation is transient and tightly controlled in normal cells, generally peaking within 30 minutes after stimulation by cytokines or growth factors and declining in the following 30 minutes, Stat3 has been shown to be persistently phosphorylated in cells transformed by certain oncoproteins such as v-Src (viral sarcoma; Turkson et al., 1998). Data have shown that Stat3 is in fact required for full neoplastic transformation by v-Src, so that
dominant negative Stat3 can block v-Src-induced transformation by inducing cell cycle arrest and apoptosis (Turkson et al., 1998). Furthermore, Stat3 activation by v-Src is not affected by overexpression of SOCS-1 indicating that in transformed cells, hyperactive Stat3 may have become desensitized to normal negative regulatory mechanisms (Iwamoto et al., 2000).

Stat3 is also known to be activated by growth factor receptors such as EGFR, the dimerization of which may promote direct phosphorylation of Stat3 on tyr705 (Grandis et al., 1998). Several studies have shown that constitutive activation of this receptor through amplification, mutation or autocrine signalling, can lead to cellular transformation (reviewed in: Quesnelle et al., 2007; Normanno et al., 2006). Stat3 has been shown to play a major role in oncogenic signalling by the EGFR, by promoting the transcriptional activation of genes involved in cell cycle progression, cell survival and metastasis (Grandis et al., 1998, Vigneron et al., 2008).

Very recent evidence has emerged indicating that Stat3 may also promote cellular transformation by oncogenes which lack kinase activity. Gough et al. have shown that Stat3 could cooperate with activated (G12V) Ras to form colonies in soft agar and further demonstrated a requirement for Stat3 by Ras$^{V12}$ to generate subcutaneous tumors in nude mice (2009). This was demonstrated by the fact that Ras$^{V12}$-transformed, Stat3-deficient cells showed restricted colony formation and tumor growth when injected into mice compared with Ras$^{V12}$ cells alone. Interestingly, expression of Y705F Stat3 in shStat3 cells restored transformation by V12Ras and tumor formation, indicating a lack of requirement for tyrosine phosphorylation. Expression of various Stat3 deletion mutants revealed that pser-727 Stat3 plays a role in Ras-mediated transformation. Furthermore, cell fractionation experiments demonstrated that Stat3 localizes to the mitochondria in Ras$^{V12}$ cells. The data further indicate that mitochondrial Stat3 could support transformation by Ras specifically, but not vSrc, through the promotion of glycolytic and oxidative phosphorylation, exposing a novel, uncharacterized role of Stat3 (Gough et al., 2009).

The introduction of two cysteine residues in the SH2 domain of Stat3 has led to the generation of a constitutively active form of Stat3 (Stat3C), which dimerizes spontaneously and
shows increased DNA binding affinity than its wild type counterpart (Bromberg et al., 1999; Li and Shaw, 2006). Stat3C expression is sufficient to transform cells in culture and induce tumors in nude mice (Bromberg et al., 1999). Stat3C has also enabled the identification of several key Stat3-regulated genes. Targets of Stat3 include *c-myc, cyclin D1/D2, and hepatocyte growth factor (hgf)*, the expression of which can increase cell cycle progression and cell proliferation as well as motility (Bowman et al., 2001; Hung and Elliott, 2001). Stat3 activation can also promote cell survival through expression of members of the *bcl-2* family of anti-apoptotic genes, such as *mcl-1* (Epling-Burnette et al., 2001) and *bcl-xL* (Bromberg et al., 1999), as well as *survivin*, shown to block the mitochondrial-induced activation of caspases (Gritsko et al., 2006; Aoki et al., 2003). Stat3 also plays a role in preventing programmed cell death by inhibiting *p53* expression through direct binding to its promoter (Niu et al., 2005). Furthermore, Stat3 expression can promote angiogenesis by activating transcription of the vascular endothelial growth factor (*vegf*) gene; VEGF is the most potent angiogenic signal and can induce the formation of new blood vessels to facilitate the metastasis of cancer cells (Niu et al., 2002). Hyperactive Stat3 can restrain anti-tumor immune responses by inhibiting the expression of key mediators of T-cell mediated anti-tumor immunity and promoting the production of immunosuppressive factors such as IL10 (Yu et al., 2009). In addition, Stat3 promotes tumor-associated inflammation and the progression of inflammatory cancers, such as colorectal and gastric cancers, through the production of pro-inflammatory cytokines like IL6 (Wang et al., 2004, Yu et al., 2009).

Inhibition of Stat3 using various methods [dominant-negative Stat3β (Catlett-Falcone et al., 1999; Garcia et al., 2001), peptides (Turkson et al., 2001), antisense oligonucleotides (Niu et al., 2002), RNA interference (Konnikova et al., 2003), DNA decoy (Gu et al., 2008; Leong et al., 2003) or small molecule inhibitors (Turkson et al., 2004; Sun et al., 2005; Schust et al., 2006)] could induce growth arrest or apoptosis of a number of tumor cell lines derived from multiple myeloma, breast carcinoma, head and neck cancer, glioma, astrocytoma and hepatocellular carcinoma, amongst others. Data from a number of labs have shown that inhibition of
constitutive Stat3 signalling using in vivo model systems, can induce apoptosis of tumor cells with minimal effect on normal host tissues (reviewed in: Germain and Frank, 2007; Yu et al., 2009). These findings suggest that cancer cells depend on higher Stat3 levels for growth and survival. The effectiveness of Stat3 inhibitors in animal models has offered precedence for their further validation in the clinic, and trials are currently underway. Blocking Stat3 can also suppress angiogenesis and stimulate host immune responses. Hence targeting Stat3 in cancer can have a double benefit, by preventing the growth of tumor cells and by enhancing adaptive and innate immunity. While targeting a molecule which lacks enzymatic activity and does not bind low molecular weight ligands, such as growth factors, may be unconventional, the current problem facing anticancer therapy remains that oncogenic events in tumors can be highly diverse and unpredictable. Stat3 lies at the convergence of a number of common deregulated pathways in cancer and blocking this “central regulatory node” can overcome the upstream effects (Yu et al., 2009). Collectively, these findings point towards Stat3 as a leading target in cancer therapy. It follows that a comprehensive understanding of Stat3 signalling mechanisms is indispensable for promoting its anti-cancer drug development.

2. The Cadherin Family of Cell Adhesion Receptors

Multicellular organisms rely on the complex recognition properties of adhesion molecules for the selective aggregation of cells into fully functional, distinct anatomical structures. In addition to providing an essential role in the genesis and architecture of tissues, cell adhesion molecules are also dynamic units that can capture and integrate signals from the extracellular environment thereby affecting key processes such as cytoskeletal organization and cell proliferation (Jeanes et al., 2008; Takeichi 1990). Loss of adhesive properties has been shown to be associated with the aberrant morphogenetic effects of cancer such as the metastasis of tumour cells (Lodish et al., 2000).
**Structure of classical cadherins and mechanism of adherens junction formation**

The formation of cell-cell adhesion junctions is primarily modulated by cadherins, a calcium-dependent family of membrane-spanning glycoproteins. Over 100 different cadherins have been identified and can be subdivided into five groups: the classical cadherins, the desmosomal cadherins, the seven-pass transmembrane cadherins, the larger cadherins of the fat and daschous family and the protocadherins (reviewed in: Stemmler, 2008). The classical cadherins are the best characterized and are subdivided into type I and type II members. The term “classical” refers to the ability of the cytoplasmic domain of these cadherins to interact with β-catenin (Stemmler, 2008). Type I cadherins, which include epithelial (E)-cadherin, placental (P)-cadherin, neural (N)-cadherin, as well as the retinal (R)-cadherin and myotubule (M)-cadherin, are found in most tissues and are crucial to the developmental process (Yap et al., 1997; Stemmler, 2008; Kaufmann et al., 1999). E-cadherin is the major receptor involved in establishing and maintaining cell-cell or adherens junctions (AJ). Knockouts of the gene for E-cadherin die in utero due to defective formation of the first epithelial layer, the trophectoderm (Larue et al., 1994). N-cadherin is expressed in the early neuroepithelium and murine knockouts of this gene are viable only until day 10 of gestation, corresponding to the early stages of neural tube formation (Radice et al., 1997; Takechi 1990). P-cadherin was initially found to be expressed in mouse placenta during early pregnancy and also plays a crucial role in the terminal differentiation of the epidermis in mouse and human tissues (Takechi 1990). P-cadherin knockout mice are indeed viable and fertile, suggesting the possibility that other cadherins may substitute for its function. Together with N-cadherin, M- and R-cadherins are known to be involved in skeletal muscle differentiation (Kauffman et al., 1999).

Type I cadherins share low amino acid homology with type II cadherins, which include human cadherins-5 (also known as vascular endothelial (VE)-cadherin) to -12 (Yap et al., 1997). The functions of type II cadherins are often overlapping and mostly associated with the developing nervous system (Patel et al., 2006). Aside from neural development, cadherin-11
plays a role in the formation of bone and the synovial lining of joints, and is also known as osteoblast (OB)-cadherin (Okazaki et al., 1994). Cadherin-11 knockout mice display defects in bone growth and are also resistant to inflammatory arthritis (Okazaki et al., 1994).

Classical cadherins contain an extracellular region, a single pass membrane spanning region and a highly conserved cytoplasmic domain (Leckband and Prakasam, 2006; Figure 2.3). The extracellular region of classical cadherins is divided into five distinct domains (EC1-EC5) that each exhibit characteristic β-strand architecture (Boggon et al., 2002; Leckband and Prakasam, 2006). Aggregation assays have confirmed that calcium is indeed required at millimolar concentrations for AJ formation, which can be reversibly inactivated by EDTA (Leckband and Prakasam, 2006; Courjean et al., 2008). The binding of a total of twelve calcium ions to the interface between successive EC domains contributes to a more rigidified cadherin structure that is resistant to proteolytic degradation (Parsini et al., 2007; Boggon et al., 2002; Leckband and Prakasam, 2006). Mutations in the calcium binding sites of E-cadherin, such as the D103A mutation in the outermost pocket, have shown to decrease homophilic adhesion (Handschiuh et al., 2001; Courjean et al., 2008).

Two models of adherens junction assembly in classical cadherins exist, the first involves an initial cis interaction of receptors, followed by a trans binding between cadherin dimers. The current model suggests a zippering between trans homodimers on adjacent cells (Parsini et al., 2007; Perret et al., 2004; Leckband and Prakasam, 2006; Figure 2.4). Both models indicate that the EC1 domain of classical cadherins plays a critical role in homotypic recognition of their binding partner (Stemmler, 2008). In fact, adhesion is mediated by an exchange of the outermost
Figure 2.3: General structure of classical cadherins.

Left: Classical cadherins consist of an extracellular domain, a single-pass transmembrane region and a cytoplasmic domain, which interacts with catenins and the actin cytoskeleton. Right: The ectodomain of classical cadherins (represented here by C-cadherin from *Xenopus*) includes five tandem extracellular repeats (EC1-5). Three calcium ions (green) bind at each of the four EC domain junctions imposing rigidity to the structure. Figure adapted from Leckband and Prakasam, 2006.
\( \beta \)-strands in the EC1 domain, and the docking of a conserved tryptophan (W) side chain of one molecule into the hydrophobic pocket of its partner (Boggon et al., 2002; Chen et al., 2005; **Figure 2.5**). Type I cadherins mediate strong cell-cell adhesion through the binding of W2 within a site surrounding a His-Ala-Val (HAV) triple amino acid sequence in EC1 (Stemmler, 2008). Type II cadherins, on the other hand, have two W residues (W2 and W6) but lack the HAV motif within their much larger acceptor pocket, and the adhesive force is seven fold less with type II cadherins compared to type I (Patel et al., 2006; Chu et al., 2006). Mutation of W2 to Alanine (W2A) abolishes the adhesive capacity of several cadherins (Perret et al., 2002).

It has been suggested that both the EC1 and EC2 domains are sufficient for classical cadherin engagement. Indeed, results have shown that a fragment consisting of the two outermost domains of E-cadherin (E/EC1/2) can retain biological activity and be selectively recognized by E-cadherin expressing cells (Perret et al., 2002). Later studies however showed that at least EC1-3 are involved in maintaining strong adhesive bonds and constructs containing additional EC domains may be necessary to restore full activity (Leckband and Prakasam, 2006; Boggon, 2002). Accordingly, it is believed that the further interdigitation of cadherins may result in more tightly bound states (see Figure 2.4). Analysis of the binding properties of type I cadherins at the single molecule level has revealed two attachment subpopulations, one population maintaining attachments from 0.1 to 1 second and another from \( 10^2 \) to \( 10^5 \) seconds suggesting a role for cadherins in transient recognition as well as stable tissue formation (Perret et al., 2002; Perret et al., 2004; Pierres et al., 2007).
Figure 2.4: Proposed mechanisms for cadherin adhesion.

Lateral (cis) dimers may either (1) dissociate to form adhesive trans dimers or (2) remain dimerized, while binding to opposing cadherins. The trans adhesive complexes may further interdigitate to form additional bound states (right). Adapted from Leckband and Prakasam, 2006.
Adhesion between cadherins is mediated by the docking of the W2 side chain of one molecule into the hydrophobic pocket of the other molecule. This is the final step for the correct folding of EC1 allowing trans-interactions. Mutation of Trp to Ala (W2A) abolishes the binding capacity of several cadherins (Perret et al., 2002).
Cytoplasmic interactions of cadherins

Intercellular adhesion is strengthened by the clustering of cadherin receptors at adherens junctions and the association between cadherin complexes and the actin cytoskeleton (Yap et al., 1997). The cytoplasmic domain of cadherins is linked to underlying actin filaments through a group of proteins termed catenins. Classical cadherins encompass a carboxy-terminal, intracellular region for β- or γ-catenin (plakoglobin)-binding and a juxtamembrane region for p120 catenin (p120ctn) binding (Yap et al., 1997). β-catenin and γ-catenin bind to α-catenin, which is able to interact with actin binding proteins such as α-actinin, vinculin or ZO-1, as well as actin itself (Stemmler, 2008). Cadherin, β-catenin and α-catenin form the core cadherin-catenin complex and mutations affecting the expression or association of any of these proteins can lead to reduced cell-cell adhesion (Oyama et al., 1994; Stemmler, 2008). p120 catenin is a substrate of the Src kinase and may be recruited to the core cadherin complex to generally strengthen adhesions by clustering cadherins to specific sites on the cell surface (Yap et al., 1998; Reynolds et al., 1994). The small GTPases Rac, Rho, Cdc42, as well as Rap1, can also associate with cadherin adhesion complexes to regulate their assembly; the role of Rho family GTPases in AJ formation is discussed in section 3 below.

The adhesive interactions between cells are dynamic, so that normal morphological remodeling events linked to embryogenesis, motility and wound healing can involve controlled changes in cadherin function. In cultured cells, the depletion of extracellular calcium or sparse growth conditions can lead to the internalization of cadherin-catenin complexes through endocytic trafficking (Le et al., 1999). In response to extracellular factors, tyrosine phosphorylation of cadherins or catenins by receptor or non-receptor kinases also correlates with their disassembly at the membrane leading to decreased adhesion (Stemmler, 2008). Dissociation of β-catenin from cadherins can lead to its accumulation in the cytoplasm, where its levels are kept low through targeted destruction by the proteasome (Cavallaro and Christofori, 2004; Yap
and Kovacs, 2003). More specifically, cytoplasmic β-catenin associated with adenomatous polyposis coli (APC) can be sequentially phosphorylated by casein kinase (CK)II and glycogen synthase-3β (GSK-3β) leading to its ubiquitination and subsequent degradation (Gao et al., 2002; Cavallaro and Christofori, 2004). In tumor cells, the normal regulatory processes which control AJ assembly or disassembly can be perturbed.

Regulation and role of E-cadherin in cancer

Germ line mutations in the E-cadherin gene (CDH1) can lead to a predisposition to gastric, lobular breast and colorectal cancers (Conacci-Sorrell et al., 2002). Furthermore, certain tumors or tumor cell lines which harbor mutations in one CDH1 allele often acquire deletions in the other one thereby characterizing E-cadherin as a classic tumor suppressor gene (reviewed in: Conacci-Sorrell et al., 2002; Hirohashi, 1998).

Adherens junctions are essential to the structural integrity of a tissue and can thereby restrict cell proliferation and motility (Conacci-Sorrell, et al., 2002; Oka et al., 1993). E-cadherin is expressed in normal epithelial tissues and loss of E-cadherin expression has been observed in a variety of epithelium-derived cancer cells and tumors in situ (reviewed in: Wheelock et al., 2001; Wheelock et al., 2008). This may occur through: (1) downregulation of E-cadherin expression following hypermethylation of its promoter or the binding of transcriptional repressors, (2) mutations in the coding region, as well as (3) the activation of pathways that prevent AJ assembly (Conacci-Sorrell et al., 2002). E-cadherin expression is negatively regulated by members of the zinc-finger-family of transcription factors, including Snail, Slug, Twist, SIP1 and EF1, which are able to bind to and repress transcription from the CDH1 promoter (Batlle et al., 2000; Wheelock et al., 2007). Indeed, examination of the levels of these transcription factors in various cancer cell lines reveals an upregulation correlating with E-cadherin repression (Batlle et al., 2000; Bolos et al., 2003). The EGF receptor, as well as Src, Fer and Abl non-receptor tyrosine kinases, which are often activated in cancer, can phosphorylate β-catenin, thereby disrupting its binding to
cadherin, or, in the case of Fer, α-catenin, leading to AJ disassembly (reviewed in: Roura et al., 1999; Nelson, 2008). EGFR, HGFR/Met and Src can also tyrosine-phosphorylate E-cadherin on its cytoplasmic tail (reviewed in Pece and Gutkind, 2002). More recent studies have identified Hakai as an E3 ubiquitin ligase able to interact with tyrosine-phosphorylated E-cadherin to promote its endocytosis and subsequent degradation in lysosomes (Fujita et al., 2002; Palacios et al., 2005; Shen et al., 2007). While the region in E-cadherin known to bind Hakai is conserved amongst all classical cadherins, the tyrosine residues which permit Hakai binding are only found in E-cadherin (Pece and Gutkind, 2002). Interestingly, expression of Hakai was recently shown to be upregulated in colon and gastric cancer (Figueroa et al., 2009).

The tyrosine phosphatase PTP1b, on the other hand, was shown to positively regulate N-cadherin-based adhesion by dephosphorylating β-catenin (Balsamo et al., 1996). Phosphorylation of serine residues in the cytoplasmic tail of cadherins by GSK-3β or CKII also results in increased adhesion by enhancing the binding of cadherins to β-catenin (Lickert et al., 2000). However, as discussed above, in the absence of Wnt signalling, serine phosphorylation of cytoplasmic β-catenin by GSK-3β or CKII kinases can lead to its ubiquitin-mediated degradation. Activation of the Wnt signalling pathway can repress GSK-3β activity, leading to the nuclear translocation of β-catenin, which may bind to Lymphoid Enhancer Factor/T-cell Factor (LEF/TCF) transcription factors leading to the expression of genes which play a role in tumor progression, such as c-myc and cyclin D1 (reviewed in: Cavallaro and Christofori, 2004; Yap and Kovacs, 2003). Similarly, dissociation of p120ctn can lead to its accumulation in the nucleus resulting in the activation of Kaiso, a recently characterized transcriptional repressor involved in cancer development (Daniel and Reynolds, 1999; Kim et al., 2004).

Certain receptor tyrosine kinases were shown to coimmunoprecipitate with cadherins, and cadherins may also in turn influence tyrosine kinase signalling. Qian et al. have reported that the growth inhibition observed in confluent cell cultures may occur as a result of the E-cadherin-
mediated suppression of RTKs, such as EGF or IGFR, or the activation of PTPs (Qian et al., 2004). They claim that the extracellular domain of E-cadherin may associate with RTKs to inhibit their ligand-mediated activation, more specifically by causing a decrease in their affinity for the ligand and reduced receptor mobility (Qian et al., 2004). This may explain why overexpression of E-cadherin in carcinoma cells can lead to a reversion to a normal epithelial phenotype (Vleminckx et al., 1991; Conacci-Sorrell et al., 2002). Paradoxically however, in other cell systems, interactions between EGFR or HGFR/Met and E-cadherin or FGFR and N-cadherin have shown to sustain receptor signalling through the ERK, MAPK pathway (Li et al., 2001; Pece and Gutkind, 2000; Hoschuetzky et al., 1994; Stemmler, 2008). Interestingly, FGFR also contains a HAV domain which mediates binding to N-cadherin, somewhat analogous to homophilic cadherin engagement (Williams et al., 2001).

Cadherin switching in cancer

Loss of E-cadherin is often concomitantly associated with a de novo gain of expression in the mesenchymal cadherins, N-cadherin and cadherin-11 (Stemmler, 2008; Conacci-Sorrell, et al., 2002; Oka et al., 1993; Hazan et al., 1997; Feltes et al., 2002 Pishivaian et al., 1999). While cadherin switching is common during normal embryonic development, it is the rate limiting step in the malignant progression of cancer cells, also known as the epithelial to mesenchymal transition, or EMT (Conacci-Sorrell, et al., 2002; Oka et al., 1993; Table 1.1). N-cadherin activation in cancer cell lines is associated with a highly invasive and motile phenotype (Hazan et al., 1997). Cadherin switching from E- to cadherin-11 has been observed in breast and prostate cancers and is linked with bone metastasis (Feltes et al., 2002 Pishivaian et al., 1999; Tomita et al., 2000; Chu et al., 2008). In sharp contrast, E-cadherin was also shown to promote tumorigenesis in tissues which do not normally express this adhesion molecule. Ovarian surface epithelial cells commonly express N-cadherin, and a switch from N- to E-cadherin has been reported to promote aberrant differentiation, characteristic of ovarian carcinogenesis (Wheelock
et al., 2008; Patel et al., 2003; Table 1.1). There is also strong evidence that once tumor cells reach distant metastatic sites, they may re-express E-cadherin to levels equal to or higher than that of the primary tumor (Kowalski et al., 2003).

### Table 1.1

**Cadherin switching during tumorigenesis**

(Wheelock et al., 2008)

<table>
<thead>
<tr>
<th>Switch</th>
<th>Example</th>
</tr>
</thead>
</table>
| From E-cadherin to N-cadherin | Melanoma  
TGFB-induced EMT in mammary epithelial cells  
Prostate cancer  
Breast cancer  
Pancreatic cancer |
| From E-cadherin to T-cadherin | Hepatocellular carcinoma |
| From E-cadherin to P-cadherin | Pancreatic cancer  
Gastric cancer |
| From E-cadherin to cadherin-11 | Prostate cancer  
Breast cancer |
| From E- and P- to N-cadherin | Oral squamous cell carcinoma |
| From N-cadherin to E-cadherin | Ovarian cancer |

3. **Rho Family GTPases**

Rho family GTPases are members of the RAS superfamily of small GTPases which cycle between an active GTP (guanosine 5’-triphosphate)-bound state and an inactive GDP (guanosine 5’-diphosphate)-bound form (reviewed in: Ellenbroek and Collard, 2007; Vega and Ridley, 2008). The first small GTPase to be discovered was Ras. While the ras gene is found to be the most frequently mutated in cancer, oncogenic transformation by aberrantly activated Ras has
been shown to depend on Rho family members (Yamamoto et al., 1999; Sahai et al., 2001; Bar-Sagi and Hall, 2000).

Regulation of Rho GTPases (Figure 2.6)

Ras and Rho GTPases have very low intrinsic GTP hydrolytic activity hence the cellular control of GTP/GDP cycling must be modulated by regulatory proteins (Ellenbroek and Collard, 2007). Rho GTPases remain inactive in the cytosol. The association of these GTPases to the cytoplasmic membrane following post-translational modifications by isoprenoid lipids (prenylation) at the C-terminus is a process critical for their activation (Vega and Ridley, 2008). Following membrane targeting, guanine nucleotide exchange factors (GEFs) can support the dissociation of GDP from Rho GTPases thereby favoring the uptake of GTP from the cytosol (Vega and Ridley, 2008). The Rho family of GEFs is very large compared to the Ras family of GEFs and includes at least 60 members, which may be either specific or non-specific towards the GTPase they act upon (Mackay & Hall, 1998). Rho GEFs are also referred to as Dbl family proteins, named after their founding member identified as a transforming protein from a human diffuse B-cell lymphoma (Dbl) cell line (Schmidt and Hall, 2002). All Rho GEFs contain a Dbl-homology (DH) domain, which is required for their catalytic activity, and a Pleckstrin-homology (PH) domain, which mediates membrane association but has also shown to modulate DH domain activation (Schmidt and Hall, 2002; Karnoub et al., 2004). Inactivation of small GTPases is promoted by GTPase activating proteins (GAPs), which increase the rate of bound GTP hydrolysis (Vega and Ridley, 2008). More than 80 known Rho GAPs exist, and amongst these is BCR, first identified as the translocation partner of the Abl tyrosine kinase in the Philadelphia chromosome, found in the majority of leukemias (Heisterkamp et al., 1993). Rho family GTPases are further negatively regulated by the effects of Rho guanine nucleotide dissociation inhibitors (Rho-GDIs; Mackay and Hall, 1998). Rho-GDIs may inhibit Rho GTPase signalling by: 1) preventing nucleotide dissociation and GDP/GTP exchange to antagonize GEF activity,
Rho GTPases are inactive when complexed to GDP and one of three known Rho-GDIs. Upon stimulation by extracellular factors, Rho is released from Rho-GDI and associates with the membrane through its C-terminal prenyl group. Rho-GEFs promote Rho-GTP exchange leading to the activation of various effector proteins. A Rho-GAP will then catalyze GTP hydrolysis and Rho-GDI will extract the GTPase from the membrane locking it once again in an inactive state.
2) by interfering with GTP hydrolysis and restricting GAP activity and 3) stimulating the release of Rho GTPases from the membrane, where they are active, to the cytosol (Karnoub et al., 2004). Hence Rho GTPases exist in a stable, cytosolic, inactive, GDP-bound state while complexed with Rho-GDIs. Cytokine and growth factor receptors, cell-cell (cadherin) and cell-matrix (integrin) adhesion receptors as well as G-protein coupled receptors may all activate Rho GTPases mainly via stimulation of Rho GEFs, and most GEFs contain additional domains such as SH2, SH3, serine/threonine or tyrosine kinase, Ras-GEF, Rho-GAP, PDZ or extra PH domains which may be involved in the interaction of GEFs with upstream modulators (Schmidt and Hall, 2002). By assisting in the membrane translocation of Rho GTPases and specifically promoting guanine nucleotide exchange and activation, Rho GEFs may disrupt the cytosolic complex of Rho-GDI (Bokoch et al., 1994; Schmidt and Hall, 2002). In their GTP-bound form, Rho GTPases are able to bind to various effectors to regulate a number of cellular responses in the cytoplasm and the nucleus including actin cytoskeletal rearrangement, cell cycle progression, proliferation and apoptosis (Ellenbroek and Collard, 2007; Karnoub et al., 2004). RhoA, Rac1 and Cdc42 are the prototype Rho GTPase members, most known for their ability to regulate the assembly of focal complexes associated with actin stress fibers, lamellipodia and filopodia, respectively (Ellenbroek and Collard, 2007).

**Structural features of Rho GTPases (Figure 2.7)**

For Rho GTPases, as for Ras and other small GTPases, the exchange of GDP for GTP is accompanied by conformational changes in two N-terminal regions termed switch regions I and II (Ihara et al., 1998; Bishop and Hall, 2000). In Rac1, switch I and switch II span amino acids 25-49 and 59-76, respectively (Karnoub et al., 2004). It is interesting to note that these two regions are essentially identical in RhoA, Rac1 and Cdc42 with the exception of position 38 in switch I which is aspartic acid (D) in Rac1/Cdc42 and glutamic acid (E) in RhoA (Bishop and Hall, 2000). The Cdc42/Rac-interactive binding (CRIB) motif, present in many Rac and Cdc42 effectors, may
preferentially recognize D38, preventing its association with Rho (Bishop and Hall, 2000). Indeed, while the switch I region is important for the interaction of Rho GTPases with effector molecules, and has been termed the effector domain, point mutations to this site inhibit the binding of some but not all downstream targets (Karnoub et al., 2004). For example, while binding of the Rac/Cdc42 effector p21-activated kinase (PAK) is prevented by a tyrosine to cysteine substitution at residue 40, the same mutation does not affect the association of Rac with mixed lineage kinase (Mlk)-2 and -3 (Bishop and Hall, 2000). Mutagenesis and structural studies have also identified a role for the switch I region in proper GTPase function, indicating that it constitutes a docking site for GEFs and GDIs (Karnoub et al., 2004). However, while GDIs may contact the C-terminal end of switch I, they primarily associate with the switch II region (Hakoshima et al., 2003). In contrast to the Ras family, Rho GTPases possess a 13 amino acid insert region close to the C-terminus (Karnoub et al., 2004). This insert region has also been implicated in some but not all effector interactions. For instance, the insert region of Rac1 and Cdc42 is necessary for IQGAP binding, however it is not required for their association with any of the known CRIB-containing effectors (Bishop and Hall, 2000).

As mentioned above, Rho proteins, like Ras GTPases, undergo posttranslational modification at the C-terminus which contains a CAAX tetrapeptide motif (cysteine (C) followed by two aliphatic amino acids (AA) and a terminal amino acid (X)) (Roberts et al., 2008). For RhoA, Rac1 and Cdc42, the first step involves covalent addition of isoprenoid moieties to the cysteine residue by geranylgeranyltransferase I (Roberts et al., 2008). Next, the AAX residues are removed by proteolysis and a methyl group is added to the prenylated cysteine by a carboxymethyltransferase (Roberts et al., 2008).

Characterization of the biological roles of Rho GTPases has been facilitated by the generation of activated and dominant-negative mutants, as well as bacterial toxins. Amino acid substitutions of either valine for glycine at codons 12 in Rac1/Cdc42, or 14 in RhoA, as well as leucine for glutamine at codons 61 in Rac1/Cdc42, or 63 in RhoA, can prevent intrinsic and GAP-
Rho GTPases share significant sequence identity with Ras proteins. The switch I and switch II domains adopt a conformational change upon binding to GTP and are involved in the association of these GTPases with various effectors. In contrast to Ras, Rho GTPases contain a 13 amino acid insert domain which may also regulate effector binding. The CAAX motif at the C-terminal end, along with another upstream membrane targeting motif, enables membrane association of these GTPases, a process critical for their activation. Constitutively activating (G12V, Q61L) and dominant negative (T17N) mutations have been identified in Rho GTPases that have enabled the characterization of their role in various biological processes. Figure adapted from Karnoub et al., 2004.
induced GTP hydrolysis resulting in GTP-bound proteins which may be activated independently of ligand binding (Bishop and Hall, 2002). On the other hand, substitution of asparagine for threonine at codons 17 in Rac1/Cdc42, or 19 in RhoA, can allow these mutants to bind GEFs with higher affinity than their wild type counterparts (Feig, 1999). Hence, these mutants display a dominant negative phenotype by competing with the endogenous GTPases for GEF binding, thereby blocking downstream signalling from effectors (Feig, 1999; Figure 2.7).

Certain bacterial toxins are also able to exert negative effects on Rho GTPases. The C3 transferase from *Clostridium botulinum* can inactivate RhoA, and other subtypes RhoB and RhoC, through ADP-ribosylation at asparagine 41 (Wilde and Aktories, 2001). It has been suggested that this modification may impair the ability of Rho proteins to interact with GEFs and may also sequester it in the cytoplasm by preventing its release from GDIs (Wilde and Aktories, 2001). On the other hand, the toxin B from *Clostridium difficile* is known to inhibit the activity of most, if not all, Rho GTPase members (Just et al., 1994; Bishop and Hall, 2000). In this case toxin B may modify Rho GTPases by glucosylation at threonine 37, thereby inhibiting effector binding and also efficient cycling between the cytosol and membrane (Genth et al., 1999).

*Rho GTPases and cell-cell adhesion*

Rho GTPases are key regulators of cell morphology and motility through their intimate association with the actin cytoskeleton. Incidentally, Rac1 and Cdc42 proteins can co-localize with E-cadherin at cell-cell AJs and are believed to contribute to the stabilization of cadherin receptors (reviewed in: Fukata and Kaibuchi, 2001; Nelson, 2008). Calcium chelation, which leads to the rapid disassembly of E-cadherin complexes, has also shown to promote the translocation of these GTPases to the cytosol in Madin-Darby Canine Kidney (MDCK) cells (Kovacs et al., 2002a). Further data has revealed an increase in active Rac-GTP levels in epithelial cells plated on surfaces coated with an E-cadherin fragment indicating that Rac activation is a specific consequence of cadherin ligation (Kovacs et al., 2002a). Indeed, a number
of labs have provided evidence of a positive feedback loop enabling cadherin assembly to
increase the activity of Rac1 and Cdc42, which may in turn stabilize adherens junctions
(reviewed in: Fukata and Kaibuchi, 2001; Braga et al., 1999).

Cadherins may communicate with Rho GTPases through phosphatidylinositol-3 (PI3)
kinase signalling pathways (Sander et al., 1998). Cell to cell contact can bring about the
association of PI3-kinase with E-cadherin containing complexes, possibly through catenins which
may serve as docking proteins, and its subsequent activation (Pece et al., 1999; Rivard, 2009;
Larue and Bellacosa, 2005). This may lead to the recruitment of GEFs such as Tiam1, which is
Rac-specific, to the membrane, through binding of its PH domain to the 3-phosphorylated
phosphoinositides (PIP3) products of PI3-kinase (Sander et al., 1998; Rivard, 2009; Figure 2.8).
While inhibition of PI3-kinase prevented activation of Rac1 by E-cadherin, it did not abolish it
completely indicating that other mechanisms may exist for E-cadherin to activate Rac1 and other
GTPases (Kovacs et al., 2002a). Apart from E-cadherin, other cadherin family members may
also activate Rho GTPases, as Tiam1 has been implicated in the activation of Rac1 by the type II
cadherin, VE-cadherin, in endothelial cells (Lampugnani et al., 2002).

Rho GTPases may also function in stabilizing cadherin junctions. In fact, Tiam1 is also
known to inhibit cell scattering brought about by HGF in MDCK cells, likely by increasing E-
cadherin based adhesion (Hordijk et al., 1997). In addition, Rac1 and Cdc42 have shown to
reinforce cadherin mediated cell-cell contacts by sequestering IQGAP, a downstream effector of
these GTPases, which is known to negatively regulate E-cadherin adhesion by binding and
displacing β- and α-catenin from the cell adhesion complex (Sander and Collard, 1999; Briggs
and Sacks, 2003; see Figure 2.9). Furthermore, cadherins may act to strengthen AJs by
expanding sites of newly formed cell-cell contacts (reviewed in Yap and Kovacs, 2003). Indeed,
activated Rac1 and Cdc42 may stimulate the catalytic activity of the Arp2/3 actin nucleator
complex, which may be recruited to the membrane upon cadherin ligation, thereby enabling
cadherins to mark sites for actin assembly (Kovacs et al., 2002b).
Figure 2.8: Activation of Rac1 by E-cadherin involves PI3-kinase

In the absence of cell-cell adhesion, Rac1-GDP exists in an inactive complex bound to GDI. Rac1 has been shown localize to sites of nascent cell contact, however the specific manner in which Rho GTPases can be targeted to the membrane is yet unknown. Calcium chelation or treatment of epithelial cells with an E-cadherin blocking antibody leads to cytosolic Rac1 (and Cdc42). E-cadherin engagement can result in the activation of PI3-kinase, which may lead to the membrane translocation of the Rac1-GEF Tiam1, or other potential GEFs with PH domains that recognize the PIP3 products of PI3-kinase. Inhibition of PI3-kinase activity can inhibit, at least in part, the activation of Rac1.

(Yap and Kovacs, 2003; Braga, 2000; Fukata and Kaibuchi; 2001)
Figure adapted from Fukata and Kaibuchi, 2001.
Unlike Rac1 and Cdc42, RhoA is found to localize in the cytosol of confluent cells and might instead influence adhesive activity through its effects on the actin cytoskeleton (Takaishi et al., 1997). While some studies have demonstrated its activation upon N-cadherin engagement, others have revealed RhoA-GTP levels to generally decrease following E-cadherin engagement, and in confluent epithelial cells (Charrasse et al., 2002; Yap and Kovacs, 2003; Fukata and Kaibuchi, 2001). In line with the latter findings, Noren et al. have identified a RhoA-specific GAP, p190RhoGAP, which is activated upon cadherin engagement, through a mechanism dependant on its tyrosine phosphorylation by Src kinases (2002). Incidentally, crosstalk between Rac1 and RhoA GTPases has also been demonstrated and studies have revealed that Rac1, which is generally activated at sites of cell-cell contact, may antagonize RhoA through activation of Rho-GAPs, such as p190 (Bustos et al., 2008), or the inhibition of Rho-GEFs.

*Rho GTPases and Stat3*

By targeting a variety of different effectors, Rho GTPases have shown to play a role in cell migration as well as cell cycle progression and proliferation (Benitah et al., 2004). The overexpression of Rho proteins in human tumors suggests that Rho GTPases are involved in carcinogenesis. High levels of Rac1, Cdc42 and RhoA have been found in breast and testicular cancers amongst others (Ellenbroek and Collard, 2007). In contrast to Ras proteins, where direct mutational activation is prevalent in a number of cancers, the evidence associating Rho GTPases with cancer is more indirect and may occur through aberrant signalling from upstream signalling components such as receptor tyrosine kinases in which genetic or epigenetic alterations are more common. Studies over the last decade have indicated that the role of Rho GTPases in tumor initiation, tumor progression and metastasis may be linked to their ability to affect gene transcription (Benitah et al., 2004).
Figure 2.9: Role of the Rac1 and Cdc42 effector, IQGAP, in E-cadherin-mediated cell-cell adhesion.

When Rac1 and Cdc42 are inactive in the cytosol, IQGAP can negatively regulate cadherin activity by binding to β-catenin, thereby displacing α-catenin from the complex. Active, GTP-bound Rac1 or Cdc42 can sequester IQGAP, to allow the association of the E-cadherin complex with α-catenin and the actin cytoskeleton, leading to strong cell-cell adhesion. E: E-cadherin; β: β-catenin; α: α-catenin.

Figure adapted from Fukata and Kaibuchi, 2001.
RhoA, Rac1 and Cdc42 can modulate the expression of several transcription factors, including nuclear Factor (NF)κB, elongation factor-2 (E2F), Serum Response Factor (SRF), as well as Stat3 (Benitah et al., 2004). In addition to tyr705, Stat3 requires phosphorylation at ser727 for maximal transcriptional activity (Wen et al., 1995). In vitro kinase assays using purified full length Stat3 as the substrate showed that Rac1 mediates ser727 phosphorylation of Stat3 in vSrc transformed fibroblasts through activation of the stress-activated serine/threonine MAP kinases, p38 and JNK (Turkson et al., 1999). The Rac1/Cdc42 effector, p21-activated kinase (PAK) shows high homology to an upstream regulator of the pheromone MAP kinase pathway in yeast and therefore might link Rac1 and Cdc42 activation to JNK activity (Mackay & Hall, 1998). Reports have claimed that although constitutively active PAK can stimulate p38 and JNK activity, Rac1 mutants which do not bind PAK can still stimulate JNK activation suggesting the possibility of other effectors (Aznar and Lacal, 2001). One such candidate is the Rac effector POSH (plenty of SH3 domains), which has been shown to lead to JNK activation as well as nuclear translocation of NFκB in Cos-1 cells (Aznar and Lacal, 2001; Tapon et al., 1998). POSH was also revealed to have a scaffolding role by forming a multiprotein complex linking together Rac1 and modulators of the JNK cascade, MLK3 and MEKK4 which may act as MAPK kinases (Mackay & Hall, 1998; Aznar and Lacal, 2001; Tapon et al., 1998).

Independent studies have demonstrated that Rho GTPases could also effectively phosphorylate Stat3 at tyr-705, leading to its activation (Debida et al., 2005; Aznar and Lacal, 2001; Pelletier et al., 2003; Simon et al., 2000; Faruqi et al., 2001). Controversial studies by Simon et al. have suggested that a direct association between Rac1 and Stat3 could lead to tyrosine and serine phosphorylation of the latter (Simon et al., 2000). On the other hand, Faruqi et al. have reported that Stat3 activation by Rho GTPases may occur indirectly through transcriptional activation of IL6 and IL6 receptor genes by NFκB, thus forming an autocrine activation loop (Faruqi et al., 2001). Once secreted, the IL6 cytokine is the most potent activator of the Stat3 response. Rac1 and Cdc42-mediated tyrosine 705 phosphorylation of Stat3 was also
shown to occur via G-protein coupled receptor (GPCR)-mediated Jak2 signalling in two peaks of activation, the second more sustained response requiring autocrine IL6 production (Pelletier et al., 2003). A more recent paper has disproved any stable association between active RhoA, Rac1 or Cdc42 and Stat3 and further demonstrates that Rho GTPases can also effectively induce Stat3 activation independently of IL6, suggesting the possibility of yet unappreciated mechanisms of Stat3 activation by the Rho proteins (Debidda et al., 2005).

4. The gp130/JAK/Stat3 axis

Cytokines and the JAK-STAT pathway

The JAK-STAT pathway was first discovered through the study of cytokines, more specifically interferon-induced signal transduction (Imada and Leonard, 2000; Schindler and Plumlee, 2008). The large hematopoetin subfamily of cytokines which trigger signalling to STATs now include IFN-α, -β and -γ; Interleukins 2-7, 10-13, 15, 19-24, 27 and 35; growth hormone, erythropoetin, prolactin, thrombopoetin and other polypeptides (Darnell, Jr., 1997; Schindler and Plumlee, 2008).

Like growth factors and hormones, cytokines are important mediators of intercellular communication. Unlike hormones which are preformed and stored in glands, cytokines can be rapidly synthesized and are secreted by cells following an immune stimulus, particularly during infection or inflammation (Heinrich et al., 1998). They are pleiotropic molecules, affecting many different target cells by binding to their matching cell surface receptor in an autocrine or paracrine manner. Cytokines are also redundant in that similar responses can be achieved by several different members of this large family. These molecules have been classified on the basis of (1) their actions as pro- or anti-inflammatory cytokines, (2) according to the receptors used or (3) their three-dimensional structure (Heinrich et al., 1998).

While lacking intrinsic enzymatic activity themselves, cytokine receptors are found to be non-covalently associated with the JAK family of kinases (Jak1, Jak2 and Jak3 and tyrosine
 kinase-2 (Tyk2); Imada and Leonard, 2000). Cytokine binding to its receptor can lead to the activation of JAKs, which phosphorylate themselves and the receptor at specific tyrosines that may be recognized by the SH2-domain of STATs or other SH2-containing adaptor proteins (Imada and Leonard, 2000). Cytokine signalling can ultimately modulate gene transcription through the activation of two major transduction pathways: (1) the JAK-STAT pathway and (2) the MAP kinase cascade (Heinrich et al., 1998). Signalling through these pathways can result in the production of more cytokines, their specific receptors or a suppression of their own effect through feedback inhibition (Heinrich et al., 1998).

**Janus kinases (JAKs)**

The connection between JAKs and cytokine signalling was first observed following the analysis of Tyk2 mutant cell lines, where it was shown that Tyk2 was required for interferon signalling (Velazquez et al., 1992). Members of this family now include Jak1, Jak2, Jak3, as well as Tyk2 (O'Shea et al., 2004). JAKs are fairly large kinases of about 120-140kDa, that associate with a membrane-proximal, proline-rich domain of cytokine receptors (Kisseleva et al., 2002; Imada and Leonard, 2000). Jak1, Jak2 and Tyk2 are expressed in many tissue types and bind several cytokine receptors whereas Jak3 is found predominantly in myelocytic or lymphocytic lineages and selectively binds the common gamma cytokine (γc)-receptor subunit which associates with the interleukins 2, 4, 7, 9, 15 and 21 (Schindler and Darnell, Jr., 1995; O’Shea et al., 2004). That is to say, many different cytokines can activate the same JAKs. JAKs contain seven conserved regions, termed Janus Homology domains 1-7 (Imada and Leonard, 2000; Ortmann et al., 2000; **Figure 2.10**). The presence of a pseudokinase domain in JH2 in addition to a kinase domain in JH1 is what makes this family unique. In fact, the name “Janus” kinase refers back to the Roman mythological god of gates and doorways and implies that JAKs are two-faced with reference to these domains (Imada and Leonard, 2000). While the JH1 and JH2 regions are structurally similar, in terms of their function the pseudokinase domain lacks catalytic activity,
although it appears to be involved in the regulation of the kinase domain (reviewed in: Imada and Leonard, 2000; O’Shea et al., 2004). Other studies have suggested that the JH2 region serves as a docking site for STATs (Fujitani et al., 1997). The amino terminal JAK region (JH3-JH7) is less well conserved and contains a FERM (a band four-point-one, ezrin, radixin, moesin) region that is crucial for receptor association, as well as an SH2-like domain in JH4 of unknown function (Kisseleva et al., 2002; O’Shea et al., 2004; Schindler and Plumlee, 2008).

*IL6-type cytokines and the gp130 receptor*

Binding of cytokines of the IL6 family, consisting of IL6, IL11, IL27, Leukaemia Inhibitory Factor (LIF), Oncostatin M (OSM), ciliary neurotropic factor (CNTF), cardiotropin-1 (CT-1) and cardiotropin-like cytokine (CLC), to the common gp130 receptor subunit can trigger Stat3 phosphorylation via Jak1, Jak2 or Tyk2 (Heinrich et al., 2003). Members of the IL6 family all share a common structure consisting of four long chain alpha-helix bundles (reviewed in: Hirano et al., 2000; Heinrich et al., 2003). Aside from their role in inflammation and the acute phase response to infection, IL6 cytokines are also involved in haematopoiesis, liver and neuronal regeneration, embryonal development and fertility (Hirano et al., 2000). Aberrant signalling from these cytokines has been shown to contribute to the initiation and progression of several diseases such as rheumatoid arthritis, inflammatory bowel disease, osteoporosis, multiple sclerosis as well as a number of different cancers (Heinrich et al., 2003). IL6 in particular is found to be overexpressed in many tumor derived cell lines, breast and lung cancers as well as metastases (Scheller and Rose-John, 2006; Selander et al., 2004; Garcia et al., 2001; Gao et al., 2007).

Receptors involved in the recognition of IL6 type cytokines include the non-signalling α-receptors (IL6R, IL11R and CTNF-R) and the signal transducing receptors (gp130, LIFR and OSMR). IL6, IL11, and CTNF must first bind to their respective α-receptors, via their site I and
Figure 2.10: Domain structure of JAKs.

All JAKs contain seven JH domains (JH1-JH7). JH1 represents the catalytic domain and JH2 the pseudo-kinase domain, which may serve to regulate kinase activity. JH4 includes an SH2-like domain of unknown function. JH4-JH7 comprise a FERM domain which mediates binding to cytokine receptors. JAKs autophosphorylate at multiple sites, including two key tyrosine residues (pY) in the activation loop of the kinase domain.

Figure adapted from Schindler and Plumlee, 2008.
II regions, in order to recruit signalling receptor subunits (Wang et al., 2009; Heinrich et al., 2003). The CTNFα receptor is unique in that it can recognize CTNF, CT-1 and CLC (Wang et al., 2009; Figure 2.11).

All IL6 family cytokines possess a site III receptor binding site which recognizes gp130 (Wang et al., 2009). IL6 and IL11 can signal through gp130 receptor homodimers, while the rest of the IL6 family cytokines can signal via gp130-LIF heterodimers (LIF, CT-1, CTNF, OSM and CLC) or through gp130-OSMR heterodimers (OSM) (Heinrich et al., 2003; Wang et al., 2009). Gp130 has also shown to engage the more recently identified IL-27 in conjunction with the T cell cytokine signalling receptor (TCCR) (Pflanz et al., 2004). Interestingly, two viral homologs of IL6 have been found in Human Herpes Virus-8 (HHV-8) and Rhesus macaque rhadinovirus (Rm), which bind to gp130 alone (Wang et al., 2009; Figure 2.12). Alternatively spliced isoforms of interleukin receptors have also been identified, soluble IL6 and IL11 receptors may lack the cytoplasmic domains and function as antagonists by competing with native IL6 or IL11 but failing to transmit signals.

The extracellular region of gp130 consists of six domains (D1-D6) and is characterized by an Immunoglobulin (Ig)-like region at the N-terminus (D1), a cytokine binding homology region (CHR; D2 and D3) and three Fibronectin type III (FNIII) domains (D4-D6; Wang et al., 2009; see Figure 2.12). Both the Ig and CHR sites are necessary for the binding and full activation of gp130 (Wang et al., 2009). Binding of an IL6 family cytokine to its receptor results in phosphorylation and activation of the gp130-associated JAKs (Jak1, Jak2 and Tyk2) (Hirano et al., 2000). The JAKs then phosphorylate gp130 at specific tyrosines which act as binding sites for the SH2 domains of (1) Stat3, (2) the protein tyrosine phosphatase SHP-2 and (3) SOCS-3 (Figure 2.13). Tyr759 of the gp130 receptor can recruit SHP-2, which itself can be phosphorylated by JAKs (Jak1) to attract the SH2 domain of Grb2 resulting in activation of the MAP kinase pathway, or the scaffolding protein Gab1 which can associate with p85, the regulatory subunit of PI3-kinase (Hirano et al., 2000). Tyr759 is also known to bind SOCS-3, a
Figure 2.11: Receptor complexes of the IL6-type family of cytokines.

The seven members of the IL6-type family of cytokines are represented by dark grey circles. Signalling receptors include gp130 (dark pink), OSMR and LIFR (light pink). The non-signalling α-receptor subunits for IL6, IL11, CT-1 and CNTF are shown in light grey. Soluble isoforms of these receptors exist which lack the cytoplasmic domain; these may compete for cytokine binding in an inhibitory manner.

Figure adapted from Heinrich et al., 2003
Figure 2.12: Extracellular structure of the gp130 receptor, which binds IL6-type cytokines (Wang et al., 2009).

The extracellular region of the gp130 receptor is characterized by an Ig-like region (IgD) at the N-terminus (D1), a CHR region for cytokine binding (D2 and D3) and three FNIII repeats (D4-D6). Both the IgD and CHR sites are necessary for the binding and full activation of gp130. IL6-type cytokines with known three-dimensional structure are shown with cylinder representations of the four helix bundles. Viral homologs of IL6 have been identified in Human Herpes Virus-8 (HHV8) and Rhesus macaque rhadinovirus (Rm) (Wang et al., 2009).
known inhibitor of Stat3 signalling. Mutation of the tyr759 binding site has been shown to result in enhanced Stat3 signalling suggesting a negative regulatory role for SOCS-3 and SHP-2 in gp130/JAK/Stat3 signalling (Hirano et al., 2000; Fairlie et al., 2003).

Stat3 can bind to any one of four YXXQ motifs in the carboxy-terminus of the gp130 receptor, these contain tyrosines 767, 814, 905 or 915. Subsequently, Stat3 phosphorylation at tyr705 by JAKs results in its homodimerization or heterodimerization with Stat1 (Hirano et al., 2000). IL6, which binds to gp130 homodimers can specifically trigger Stat3:Stat3 binding (Darnell, Jr., 1997).

**IL6 and cancer**

The link between the IL6/JAK/Stat3 axis and survival signalling was first demonstrated in 1996 through data indicating that Stat3 is needed for gp130-induced proliferation in a pro-B cell line (Hirano et al., 2000). While gp130 can activate MAPK and PI3K pathways as well as Stat3, it was revealed that tyr759 of this receptor was essential for cell cycle progression but not for preventing apoptosis. In fact, the tyrosines involved in Stat3 activation were required for bcl-2 induction and cell survival (Hirano et al., 2000).

Further studies have indicated that the specific gp130 ligands, IL6, OSM and LIF, can play a role in the differentiation and growth inhibition of a variety of cultured cells (Selander et al., 2004). However, IL6/gp130 signalling in particular can trigger constitutive activation of Stat3 in a number of tumors and tumor-derived cell lines including myeloma, head and neck squamous cell carcinoma (HNSCC), breast and prostate cancers, cholangiocarcinoma, and non-small cell lung carcinoma (NSCLC) (Catlett-Falcone et al., 1999; Sriuranpong et al., 2003; Gao et al., 2007). Recently activating mutations in the gp130 receptor have been identified, enabling Stat3 binding in the absence of ligand; this was shown to occur in 60% of inflammatory hepatocellular adenomas (Rebouissou et al., 2009). Persistent IL6/JAK/Stat3 activation can also result from feedforward signalling as Stat3 can transcriptionally increase IL6 expression (Yu et
Upon gp130 activation, receptor associated JAKs may phosphorylate tyrosines (Y) 759, 767, 814, 905 or 915 of human gp130. Y759 is a binding site for the SH2 domain of SHP-2, which can result in MAPK signalling or PI3-kinase activation. Y759 also recognizes the SOCS-3 inhibitor of cytokine signalling. Y767, Y814, Y905 and Y915 all have the conserved YXXG motif which contains a glutamine at position +3 of tyrosine; any of these tyrosines are able to bind the SH2 domain of Stat3. (Hirano et al., 2000)
Indeed high levels of serum IL6 have been detected in colorectal, liver, lung and breast cancer patients and in the latter this correlates with a more advanced tumor stage, metastases and an overall poor prognosis (Sullivan et al., 2009). In myeloma, as well as other cancer types, IL6 production has also been observed in non-transformed stromal cells suggesting both autocrine and paracrine mechanisms of Stat3 activation (Yu et al., 2009).

Inhibition of gp130 through dominant negative mutants was reported to downregulate tumor invasion and angiogenesis by blocking constitutive Stat3 signalling in the breast cancer line MDA-MB-231 (Selander et al., 2004). Similarly, treatment of NSCLC-derived lines with anti-gp130 or anti-IL6 antibodies reduced Stat3 activity and decreased in vitro and in vivo growth of H2355 cells (Gao et al., 2007). Gao et al. further demonstrated that these tumor-derived lines produced high levels of IL6, which was secreted into the growth medium and could trigger Stat3-ptyr705 phosphorylation in normal MCF-10A cells (Gao et al., 2007). In addition, tissue microarray samples of lung adenocarcinomas revealed a positive correlation between pStat3-positive samples and high IL6 levels (Gao et al., 2007). These data taken together identify the gp130/JAK/Stat3 axis as a critical marker in carcinogenesis and drugs inhibiting this pathway may be valuable therapeutic targets for the malignancies which exploit it.
5. Previous results from our lab: cell to cell adhesion activates Stat3

Interactions with neighboring cells can profoundly influence signal transduction pathways, many of which are activated or inactivated by phosphorylation at specific tyrosine residues (Juliano, 2002; Stemmler, 2008). Dense cell cultures, albeit only two-dimensional, may in part mimic some of the physiological stress signals that occur in fast-growing tumors. Indeed, many of the cells in tumor nests lack direct interaction with surrounding connective tissue and they may depend on survival signalling generated by cell-cell contacts.

We recently discovered that cell density causes a dramatic increase in Stat3 activity in breast carcinoma as well as normal epithelial cells or fibroblasts (Vultur et al., 2004; Vultur et al., 2005). Stat3 activation levels peaked at approximately 1-2 days post-confluence, when cell-cell contacts were highest and cell-matrix contacts were minimal. The density-dependent Stat3 activation was substantially greater than that brought about by serum or EGF stimulation (Vultur et al., 2004). Treatment of postconfluent cells with a calcium chelating solution (EGTA/EDTA) abrogated Stat3 tyr705 phosphorylation, DNA binding activity and transcription, yet when the dispersed cells were allowed to aggregate on agar-coated petris, Stat3 activity was significantly increased, indicating that cell-cell adhesion is likely the cause behind the Stat3 activation in dense cultures.

Exploration of the nature of the kinase(s) involved revealed that this Stat3 activation was independent of Src/Fyn/Yes, Fer, Abl, EGFR and the Insulin Growth Factor-1 Receptor, often activated in a variety of cancers. In addition, cell density did not lead to activation of the Ras/Raf/Erk pathway, which is downstream of a number of receptor and non-receptor tyrosine kinases. These results indicate that, individually, these kinases may not play a role in activating Stat3 in our system. Stat3 activation was partially suppressed following inhibition of JAKs with the JAK-selective inhibitor, AG490. Most significantly, inhibition of Stat3 in postconfluent cells resulted in a dramatic increase in apoptotic cell death indicating that this pathway constitutes a strong survival signal (Vultur et al., 2004). By elucidating the underlying mechanism of this
novel cascade we may be able to identify the tumors that rely on it for growth and survival; this may lead to the further validation of this pathway as an important target for anti-cancer drug design.
Hypothesis and Objectives

Given our previous findings, our aim was to determine the upstream signals involved in triggering the density-mediated Stat3 activation. Cadherins are the primary mediators of calcium-dependent intercellular adhesion, and cadherin engagement has been shown to trigger signalling to the Rho family of GTPases, notably Rac1 and Cdc42. Independent studies have shown that Rho GTPases can lead to Stat3 activation in a JAK-dependent manner, possibly by promoting transcription of the IL6 cytokine, a key transducer of Stat3 signalling.

Our hypothesis was that cell-cell adhesion by cadherins could activate Rho family GTPases, which would lead to a potent activation of Stat3, through the transcriptional upregulation of IL6 family cytokines.

More specifically our objectives were:

1. To identify the type of cadherin(s) involved in the density-mediated, Stat3 activation.
2. To determine the nature of the Rho family GTPase(s), which are activated by the cadherin(s).
3. To examine the role of the IL6 family of cytokines in the density-dependent Stat3 signalling.

While investigating the mechanism of the density-mediated Stat3 activation, we discovered that certain common transfection techniques such as lipofectamine or calcium phosphate precipitation could upregulate Stat3 tyr-705 phosphorylation and activity. Our observations prompted us to generate cell lines stably expressing specific genes or their mutants for all subsequent studies where Stat3 function was examined.

Most significantly, our results uncovered a novel pathway of Stat3 activation triggered by homophilic interactions between E-cadherin or cadherin-11, prototype members of type I and II classical cadherins, in different cell systems. Stat3 activation was preceded by an increase in
Rac1 and Cdc42 activity which could, in turn, promote the expression of members of the IL6 family of cytokines, responsible for the Stat3 stimulation observed. Unexpectedly, in addition to this cadherin-induced activation of Rac1/Cdc42, we demonstrated, for the first time, a dramatic increase in the endogenous levels of total Rac1/Cdc42 proteins at high cell densities. Our results revealed that cadherin engagement could inhibit the ubiquitination and degradation of these GTPases, leading to a further increase in their activity. Inhibition of cadherin engagement induced apoptosis, indicating that this pathway plays a crucial role in cell survival signalling.

Upon further examination of the mechanism of Stat3 activation by Rac1/Cdc42, we also observed increased stability of mutationaly activated forms of Rac1 (Rac\textsuperscript{V12} or Rac\textsuperscript{L61}) and Cdc42 (Cdc42\textsuperscript{V12}) with cell density which was due to the inhibition of their modification by ubiquitin. Moreover, we clearly showed that Rac\textsuperscript{V12} expression can activate Stat3 through an NF\kappa B-mediated increase in mRNA of members of the IL6 family of cytokines. In fact, knockdown experiments indicated that gp130 receptor function, and Stat3 activation, are essential for the migration and proliferation of Rac\textsuperscript{V12}-expressing cells, thereby demonstrating that the gp130/Stat3 axis represents an essential target of activated Rac in the regulation of both of these fundamental cellular functions.
Significance

Cells in normal tissues or tumors have extensive opportunities for adhesion to their neighbors in a three-dimensional organization, and it has recently become apparent that in the study of fundamental cellular processes it is important to take into account the effect of surrounding cells. Indeed, the relevance of interactions observed in densely growing, cultured cells to human cancer is clearly evident from findings demonstrating a close correspondence of genes expressed in lymph node carcinoma of the prostate (LNCaP) cells cultured to high, but not low, densities, with genes associated with prostate cancer in vivo.

The aim of my project is based on previous data from our lab demonstrating that cell density can activate Stat3 independently of a number of known tumor-promoting tyrosine kinases, including the Src family, IGF1-R, Abl, Fer and EGFR, often activated in a variety of cancers. Most importantly, this density-dependant Stat3 activation constitutes a potent survival signal, which could be exploited by cancer cells. Hence, given that Stat3 is associated with malignant cell proliferation, cell survival, angiogenesis, and metastasis, our identification of a novel mechanism of Stat3 activation triggered by cadherin engagement and the upregulation of Rac1/Cdc42 GTPases has obvious implications in the development of targeted therapies. This pathway could be a prognostic marker, as well as a promising predictive target for the treatment of tumors which may be resistant to inhibition of many tyrosine kinase oncogenes known to be hyperactive in many cancers.
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Chapter 3

Transfection techniques affecting Stat3 activity levels

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Author contributions:

RA performed most of the experiments, AV and LR conducted the electroporation work.
Transfection techniques, such as calcium-phosphate or liposome-mediated gene transfer, are commonly used for the examination of the effect of a gene upon cellular phenotype and biochemical properties. We previously demonstrated that cell to cell adhesion causes a dramatic increase in Stat3 activity. Given that the opportunities for cell to cell adhesion could be altered due to the presence of the DNA-containing complexes, we examined the effect of the calcium-phosphate transfection procedure upon Stat3 activity levels. The results revealed a dramatic increase in Stat3 phosphorylation at the critical tyr705 site and Stat3 activity following calcium-phosphate transfection. This increase was noted even in the absence of DNA and was not due to the mere presence of calcium ions. In contrast, DNA introduction through electroporation or infection with a retroviral vector did not affect Stat3 activity, while cationic lipids such as lipofectamine or Fugene6 had a less pronounced effect than calcium-phosphate transfection. These results indicate that caution is required in the interpretation of results with regard to activity of Stat3 following certain commonly used transient transfection regimens.
Introduction

The signal transducer and activator of transcription-3 (Stat3) protein is activated by cytokines as well as growth factors such as EGF or PDGF through their receptors, or the non-receptor tyrosine kinase Src (reviewed in Yu and Jove, 2004). Stat3 is invariably latent in the cytoplasm and, subsequent to binding to an activated receptor through its SH2 domain, becomes activated through phosphorylation by the receptor itself or by the associated JAKs, or cSrc tyrosine kinases (Wang et al., 2000; Bromberg et al., 1998; Turkson et al., 1998). Phosphorylation of a single critical tyrosine residue, tyr705, activates Stat3 by stabilizing the association of two Stat3 monomers through reciprocal SH2-ptyr interactions to form a dimer which migrates to the nucleus and binds specific DNA sites to initiate transcription from a number of genes. Intense interest in Stat3 signalling was sparked by the discovery that inappropriate activation of Stat3, along with Stat5, occurs with high frequency in a number of human cancers, while constitutively high Stat3 activity is required for the growth and survival of many tumor-derived, cultured cells (Song and Grandis, 2000; Coffer et al., 2000; Niu et al., 2002; Yu and Jove, 2004).

The investigation of Stat3 function has often been accomplished through the introduction of a variety of genes, such as activated forms or dominant negative mutants of different signal transducers, through DNA transfection into cultured cells. In many cases, the experiments involved transient expression of these genes, followed by examination of their effect upon Stat3 activity at 48-72 hours after transfection (Turkson et al., 1999; Turkson et al., 1998). A very commonly used transfection method is the calcium-phosphate precipitation technique (Graham and van der Eb, 1973); a precipitate is formed between calcium and phosphate ions and the added DNA, the coarseness of which can be adjusted through precise control of the pH. The mixture is added to the culture through the medium and the grains are taken up by the cells.
We previously demonstrated that cell to cell adhesion causes a dramatic increase in Stat3 activity levels in carcinoma as well as normal epithelial cells or fibroblasts (Vultur et al., 2004). This is unique to Stat3 since no activation of other pathways, often coordinately activated with Stat3 by growth factors or oncogenes, such as the Ras/Raf/Erk1/2 or PI3-kinase/Akt, was observed under these conditions (Vultur et al., 2004). Since the opportunities for cell to cell adhesion could be altered due to the presence of the calcium phosphate/DNA precipitate, we examined the effect of this transfection procedure upon Stat3 activity levels. The results revealed a dramatic increase in Stat3-tyr705 phosphorylation and consequently Stat3 activity following calcium phosphate transfection, as determined by measurement of phosphorylation of the critical tyrosine 705 residue, or transcriptional activity using a Stat3-specific reporter. This increase was noted even in the absence of DNA and was not due to the mere presence of calcium ions. In contrast, DNA introduction through electroporation or infection with a retroviral vector did not affect Stat3 activity, while cationic lipids such as Lipofectamine or Fugene6 had a less pronounced effect than calcium phosphate transfection. These results indicate that caution is required in the interpretation of results regarding activity of Stat3, following certain commonly used, transient transfection regimens.
**Materials and Methods**

**Cell lines and culture techniques**

Mouse NIH 3T3, 10T½ or rat F111 fibroblasts (Raptis et al., 2000) were grown in plastic 3 cm dishes in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% calf serum, in a 7% CO₂ incubator. For calcium phosphate transfection, cells were transfected with the β-gal plasmid (Turkson et al., 1998), using the technique of Chen and Okayama (Chen and Okayama, 1987) with some modifications. When they reached confluences of 15%, 35% or 60%, the DNA was prepared by dissolving a dried pellet of 4 μg DNA with 135 μl sterile H₂O and adding 15 μl 2.5 M CaCl₂·6 H₂O. The precipitate was formed through the addition of 150 μl BES buffer [50 mM BES (N,N’-bis[2Hydroxyethyl]-2-aminoethanesulfonic acid), Sigma, 280 mM NaCl, 1.5 mM Na₂HPO₄·2H₂O] and incubation at room temperature for 2 or 10 min, when opalescence appeared. The suspension was added to the cells and following a 4 hr incubation at 37°C and every day thereafter, the medium was changed to DMEM with 10% calf serum.

For Lipofectamine transfection, 8 μg β-gal DNA in the form of ethanol precipitate were resuspended with 290 μl of serum-free DMEM and 10 μl of Plus reagent added (Life Technologies Inc). In a separate tube, 15 μl of Lipofectamine reagent were added to 285 μl DMEM. The two tubes were mixed, incubated for 15 min at room temperature and the mixture added to NIH 3T3 cells at 35% or 60% confluence, growing in 6 cm petris and covered with 3 mls of serum-free DMEM, according to the manufacturer’s protocol. Cells were placed in the incubator for 3-4 hrs, followed by a medium change to DMEM containing 10% calf serum.

For retroviral infection, NIH 3T3 cells growing at confluences of 15%, 35% or 60% in 6 cm dishes were infected with the vector MSCV (murine stem cell virus, 10⁶ pfu/ml) which codes for the enhanced green fluorescence protein (EGFP), in the presence of 8 μg/ml polybrene.
(McLemore et al., 2001). Following incubation at 37°C for 4 hours, the medium was changed to DMEM with 10% calf serum.

**Western blotting**

Total cell lysates were obtained three days following transfection, when cells were at 30%, 70% or 100% confluence, respectively. Proteins were extracted using 50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10mM Na₂P₂O₇, 100 mM NaF, 2 mM Na₃VO₄, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100 (Raptis et al., 2000). 50 μg of clarified cell extract were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk for at least one hour followed by an overnight incubation in primary antibody.

Immunodetection was performed using antibodies against the tyrosine-705 phosphorylated, i.e. activated form of Stat3 (Biosource International); or phosphorylated Erk1/2 (Biosource), followed by alkaline phosphatase-conjugated goat secondary antibodies (Biosource) as described (Vultur et al., 2004). The bands were visualized using enhanced chemiluminescence (ECL), according to the manufacturer’s instructions (PerkinElmer Life Sciences, Cat.# NEL602). As a control for protein loading, parallel blots were probed with the mouse monoclonal anti-heat shock protein 90 (Hsp90) antibody (Stressgen).

**Luciferase Assays**

vSrc-transformed NIH 3T3 cells were transfected with the pLucTKS3 reporter plasmid which harbors seven copies of a sequence corresponding to the Stat3-specific binding site in the C-reactive gene promoter (termed APRE, TTCCCGAA) upstream from a firefly luciferase coding sequence (Turkson et al., 1998) with G418-resistance co-selection. Individual clones were grown
up and screened for firefly Luciferase activity, measured in total cell extracts according to the manufacturer’s protocol (Promega, Cat. # E4030). As a control, pLucTKS3-expressing cells were stably transfected with a different reporter, pRLSRE, which contains two copies of the serum response element (SRE) of the c-fos promoter, subcloned into the Renilla luciferase reporter, pRL-null (Promega), and Zeocin-resistance co-selection (Turkson et al., 2001). The firefly and Renilla luciferases utilize different substrates and thus can be assayed independently in the same lysates using this kit. Cells were subject to calcium phosphate transfection as above and Stat3 activity assessed.

**In situ electroporation**

The electroporation equipment was provided by Ask Science Products Inc., Kingston, Ont. Canada. The conditions described before (Raptis et al., 2004; Raptis et al., 2003) were used with some modifications. Briefly, cells were grown on conductive and transparent indium-tin oxide (ITO)-coated glass slides with a cell growth area of 15x7 mm. When 35 or 60% confluent, the oligonucleotide solution (10-30 μg/ml, in calcium-free DMEM) was added to the cells and the electrode set placed on the slide. The optimal voltage and capacitance settings determined before were used (Raptis et al., 2004; Raptis et al., 2000) to ensure efficient incorporation of the DNA without damage to the cells (six pulses of 30 volts from a 2 μF capacitor).
Results

**Calcium phosphate transfection activates Stat3**

As shown in Fig. 3.1A (lanes 1, 5 and 9), cell density caused an increase in Stat3-ptyr705 levels in untreated cells, as expected based on our previous results (Vultur et al., 2004). The addition of the calcium phosphate/DNA precipitate caused a further increase in Stat3 tyr705 phosphorylation. This increase was evident in cells that were at a confluence of 30% at the time of harvest, as well as in 100% confluent cells, at which time Stat3 tyr705 phosphorylation reached approximately half the levels in NIH 3T3 cells transformed by the potent Stat3 activator, vSrc (Fig. 3.1A, lane 12 vs 13). Stat3 activation was caused by the precipitate itself, since it was strong even in the absence of DNA (lanes 3, 7 and 11 vs 4, 8 and 12), while Stat3 levels gradually waned to background by 6 days following transfection. When the DNA preparation was allowed to stand for only 2 minutes at room temperature following addition of the BES buffer, the precipitate was finer and Stat3 activation less pronounced (lanes 2, 6 and 10 vs 3, 7 and 11), while the efficiency of gene expression was reduced (~10% vs 18%, respectively), as revealed by staining for β-galactosidase (not shown). The addition of lower amounts of DNA resulted in a finer precipitate and slightly reduced Stat3 levels. Similar results were obtained with mouse 10T½ or rat F111 fibroblasts, or using pUC19 plasmid DNA (not shown), indicating that the calcium phosphate transfection procedure can have a profound effect upon Stat3 tyrosine phosphorylation levels in cultured cells. At the same time, examination of Erk1/2 activity levels showed no differences between transfected and control cells (Fig. 3.1B), indicating that the calcium phosphate effect is specific for Stat3.

The vSrc oncoprotein is known to activate Stat3, as part of the process of vSrc-mediated malignant transformation (Turkson et al., 1998). As a result, vSrc expression increases transcription from a Stat3-specific binding element (termed APRE, TTCCCGAA, derived from
Figure 3.1

**Calcium-phosphate transfection activates Stat3.**

**A-C: Stat3 tyr-705 phosphorylation.**

NIH 3T3 cells were subject to the Calcium phosphate transfection procedure in the presence or absence of DNA (β-gal plasmid), as indicated. The transfection mix was allowed to stand for 2 or 10 minutes before addition to the cells, which were harvested three days later, when they were at a confluence of 30%, 70% or 100%, as indicated. Lanes 1, 5, 9: Untransfected cells. Lane 13: NIH 3T3 cells transformed by vSrc, harvested at 100% confluence. Detergent lysates were resolved by gel electrophoresis and Western immunoblots were probed for, A, the tyr705 phosphorylated form of Stat3; B, the phosphorylated form of Erk1/2; and C, Hsp90, as indicated. 40 µg of lysate were loaded in all cases. Numbers at the left refer to molecular weight markers. Arrows point to the position of Stat3 ptyr705, Erk1/2 or Hsp90, respectively. Cell confluence was estimated by microscopic observation and quantitated by imaging analysis of live cells under phase contrast using a Leitz Diaplan microscope and the MCID-elite software (Imaging Research, St. Catharine’s Ont.).

**D: Stat3 Transcriptional activity.**

vSrc-transformed, NIH 3T3 cells, stably expressing a plasmid encoding the Stat3-dependent luciferase reporter, pLucTKS3, were subjected to the Calcium phosphate transfection procedure. Untransfected cells served as controls, as indicated. Three days later, when cells reached a confluence of 30% (□) or 100% (■), Luciferase activity was examined in cytosolic extracts, as light emission. Values shown in each panel represent arbitrary luciferase units, means plus standard deviations of at least 3 experiments, each performed in triplicate.

the C-reactive protein gene promoter), driving a luciferase reporter construct (pLucTKS3, see Materials and Methods), when co-expressed through transfection into mouse NIH 3T3 fibroblasts. Previous results indicated that, although vSrc-transformed cells have higher Stat3 activity than the parental NIH 3T3 line when sparse, cell density causes a further increase, in an additive manner (Vultur et al., 2004). To examine whether, in addition to tyr705 phosphorylation, the calcium phosphate treatment can also increase Stat3 transcriptional activity, we measured Stat3-specific transcription in vSrc-transformed NIH 3T3 cells and Firefly Luciferase activity was measured in total cell extracts (see Materials and Methods).

As shown in Fig. 3.1D, cells treated with the calcium phosphate precipitate had ~2.5 times higher Stat3 transcriptional activity levels compared to untreated controls. As a further control, given that cell to cell adhesion is often mediated through cadherin engagement, which is known to require calcium, we examined whether calcium ions alone might be able to increase
Stat3 activity. Cells were grown in DMEM containing 1.8 mM CaCl₂ (the normal concentration) or 3, 5, 7.5, 10, 20 or 40 mM CaCl₂. While up to 5 mM CaCl₂ had no significant effect upon Stat3 phosphorylation or activity levels, the highest concentration was visibly inhibitory (not shown), indicating that the increase in Stat3 activity seen following calcium phosphate transfection is not due to increases in free calcium concentration.

**Lipofectamine transfection activates Stat3**

Another commonly used transfection technique is the use of cationic lipids, such as Lipofectamine. As shown in Fig. 3.2A, Lipofectamine treatment (see Materials and Methods) brought about an increase in Stat3 tyrosine phosphorylation. Contrary to calcium-phosphate transfection, this increase was slightly more pronounced in the absence of added DNA (lanes 2 vs 3 and 5 vs 6). Addition of 8 μg β-gal DNA reduced the levels almost to the background of untreated cells (lanes 1 vs 3 and 4 vs 6). Similar results were obtained with Fugene6 (Roche) while the efficiency of gene expression was ~20% with both treatments. The above findings taken together indicate that treatment with cationic lipids can affect Stat3 activity.

**Retroviral infection does not affect Stat3 phosphorylation**

Retroviral infection is an effective way of gene transfer. To examine its potential effect upon Stat3 activity, NIH 3T3 cells were infected with the retroviral vector MSCV which codes for the enhanced green fluorescence protein (EGFP, see Materials and Methods). Examination of EGFP expression by fluorescence microscopy three days later indicated that for all three sets more than 80% of the cells had been transduced with this vector. As shown in Fig. 3.2B, examination of Stat3 activity levels three days following infection, at which time cells were 30%, 70% or 100% confluent, revealed an increase with cell density, as observed before (Vultur et al., 2004). However, no differences between infected and uninfected cells were noted, indicating that, unlike transfection, retroviral infection itself does not increase Stat3 phosphorylation.
Figure 3.2

Effect of Lipofectamine and retroviral infection upon Stat3 activity

A: Lipofectamine transfection results in increased Stat3 phosphorylation

NIH 3T3 cells were transfected with Lipofectamine and harvested three days later, when they reached a confluence of 70% or 100%, as indicated. Lane 7: NIH 3T3 cells transformed by vSrc, harvested at 100% confluence. Detergent lysates were resolved by gel electrophoresis and Western immunoblots were probed for, top panel, the tyr705 phosphorylated form of Stat3; bottom panel, Hsp90, as indicated. 40 μg of lysate were loaded in all cases. Numbers at the left refer to molecular weight markers. Arrow points to the position of Stat3 ptyr705 or Hsp90, respectively.

B: Retroviral infection does not affect Stat3 tyrosine phosphorylation

NIH 3T3 cells were infected with the MSCV retroviral vector and harvested three days later, when they reached a confluence of 30%, 70% or 100%, as indicated. Lane 7: vSrc-transformed NIH 3T3 cells, harvested at 100% confluence. Detergent lysates were resolved by gel electrophoresis and Western immunoblots were probed for, top panel, the tyr705 phosphorylated form of Stat3; bottom panel, Hsp90, as indicated. 40 μg of lysate were loaded in all cases. Numbers at the left refer to molecular weight markers. Arrow points to the position of Stat3 ptyr705 or Hsp90, respectively.
In situ electroporation does not affect Stat3 phosphorylation or transcriptional activity

Electroporation in situ has been extensively employed for the introduction of peptides (Raptis et al., 2000; Boccaccio et al., 1998), proteins (Nakashima et al., 1999), radioactive nucleotides (Boussiotis et al., 1997; Raptis et al., 2003; Tomai et al., 2003), oligonucleotides (Bardelli et al., 1998; Gambarotta et al., 1996), as well as a variety of prodrugs and other nonpermeant molecules (Marais et al., 1997; reviewed in Raptis et al., 2004). We previously demonstrated that electroporation of a decoy double-stranded oligonucleotide, corresponding to the hSIE, Stat3 DNA binding site (high affinity mutant, sis-inducible-element, derived from the c-fos promoter 5’-GTGCATTTCCCGTAAATCTTGTCTA-3’(Turkson et al., 1998) into vSrc-transformed cells which stably express the pLucTKS3 plasmid caused a potent reduction in the levels of luciferase activity obtained 24 hrs after electroporation of the hSIE decoy, indicating that this oligonucleotide can dramatically reduce transcription, presumably by dislodging the activated Stat3 from its endogenous binding site on the APRE element (Raptis et al., 2003). At the same time, an unrelated control oligonucleotide (FIRE sequence: AGCGCCTCCCCGCGCCGGG (Turkson et al., 1998), which does not bind Stat3 did not lead to a reduction (Raptis et al., 2003). To compare the effect of electroporation with transfection concerning Stat3 activation, we repeated the experiment with the control oligonucleotide, or with the tracking dye, Lucifer yellow alone in DMEM lacking calcium and compared the results with nonelectroporated cells serving as controls.

As previously demonstrated (Raptis et al., 2004), incorporation of the tracking dye, Lucifer yellow indicated that essentially 100% of the cells had been permeated under these conditions, while immunostaining with antibodies against activated forms of the stress-activated kinases (SAPK/JNK or p38\textsuperscript{\text{mapk}}) indicated the absence of stress to the cells (Brownell et al., 1998). Following pulse application and a 24 hr incubation (see Materials and Methods), Stat3 tyr705 phosphorylation levels were examined. As shown in Fig. 3.3B, electroporation with or without the control oligonucleotide did not affect Stat3 activity levels (lane 1 vs 2 and 3, or lane 4 vs 5
**Figure 3.3**

*In situ* electroporation does not affect Stat3 phosphorylation or transcriptional activity

**A: In situ electroporation does not affect Stat3 phosphorylation**

vSrc-transformed, NIH 3T3 cells were plated on ITO-coated slides at a confluence of 35% or 60%. 30 μg/ml of the control oligonucleotide were introduced by electroporation (2 μF, 30V, 6 pulses) and 24 hours later, when 75% or 100% confluent, detergent lysates were resolved by gel electrophoresis and Western immunoblots probed for, top panel, the tyr705 phosphorylated form of Stat3; bottom panel, Hsp90, as indicated. Untreated cells served as controls, as indicated. Arrow points to the position of Stat3 ptyr705 or Hsp90, respectively.

**B: In situ electroporation does not affect Stat3 transcriptional activity**

vSrc-transformed, NIH 3T3 cells were plated on ITO-coated slides at a confluence of 50%. Increasing amounts of the hSIE decoy oligonucleotide (■), or the same amounts of a control...
oligonucleotide (□) as indicated were introduced by electroporation (2 μF, 30V, 6 pulses) and 24 hours later Luciferase activity was examined in cytosolic extracts, as light emission. Untreated cells (■), or cells electroporated with the tracking dye, Lucifer yellow (▨) served as controls, as indicated. Values shown in each panel represent arbitrary luciferase units, means plus standard deviations of at least 3 electroporation experiments, each performed in triplicate.

and 6). Similarly, examination of luciferase activity in cytosolic extracts showed that electroporation did not engender a change to Stat3 activity; luciferase values obtained were essentially the same as for parallel, nonelectroporated cultures of identical confluence, or cultures electroporated with the tracking dye alone (Fig. 3.3C). At the same time, cells electroporated with the decoy oligonucleotide had progressively lower luciferase activity levels, indicating that this decoy can effectively compete with the APRE site which was already occupied by Stat3, activated by vSrc in the transformed cells. This indicates that efficient interruption of transcription can be achieved with this technique, under conditions that Stat3 activity is not affected by the procedure. Similarly, electroporation of the β-gal plasmid had no effect upon Stat3 activity (not shown).
Discussion

Transfection techniques utilizing calcium phosphate or cationic lipids are widely employed for the study of Stat3 function in signal transduction. Given the importance of precise Stat3 activity measurement, the observation that these procedures can have a profound effect upon Stat3 activity levels, indicates that caution is required in the interpretation of results regarding activity of Stat3 following transient transfection. Our results demonstrate that electroporation or retroviral infection have no detectable effect, indicating that these could be alternative approaches for Stat3 studies.
References


Chapter 4

Cadherin engagement promotes cell survival
via Rac/Cdc42 and Stat3

These data are included in the following manuscripts:

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Feracci, H. and Raptis, L. (2009). Cadherin engagement promotes survival via Rac/Cdc42 and

Arulanandam, R., Geletu, M., Chevalier, S., Feracci, H. and Raptis, L. Cadherin-11 engagement
activates Stat3 and promotes survival of mouse fibroblasts. In preparation.

Author contributions:

RA performed the bulk of the work in this chapter. JC helped with the RT-PCR experiments. AV
produced preliminary data and discussed results. MG performed the siCad11 experiments. As
collaborators, LL from the Institut Curie, Paris, France, provided the ES cell lines and HF and SC
from the Universite Bordeaux 1, Centre de Recherche Paul Pascal, Pessac, France, generously
provided the recombinant cadherin fragment constructs. As collaborators from the Cancer
Research Institute at Queen’s, EC and BEE performed the tissue immunostaining and PFT
generously provided primary epithelial cell lysates. All authors contributed insightful advice and
helped revise the manuscripts.
Abstract

Stat3 (signal transducer and activator of transcription-3) is activated by a number of receptor and non-receptor tyrosine kinases, while a constitutively active form of Stat3 alone is sufficient to induce neoplastic transformation. In the present report we demonstrate that Stat3 can also be activated through homophilic interactions by the epithelial (E)-cadherin and cadherin-11, two members of the classical type I and II cadherin family of surface receptors, responsible for the formation of cell to cell junctions. Indeed, by plating cells onto surfaces coated with fragments encompassing the two outermost domains of these cadherins, we clearly demonstrate that cadherin engagement can activate Stat3, even in the absence of direct cell to cell contact. Most importantly, our results also reveal for the first time a dramatic surge in total Rac1 and Cdc42 protein levels triggered by cadherin engagement, and an increase in Rac1 and Cdc42 activity, which is responsible for the Stat3 stimulation observed. Inhibition of cadherin interactions using a peptide, a soluble cadherin fragment or genetic ablation induced apoptosis, pointing to a significant role of this pathway in cell survival signalling, a finding which could also have important therapeutic implications.
Introduction

Stat3 is found to be overexpressed in a number of carcinomas and tumor cell lines [reviewed in (Yu and Jove, 2004; Germain and Frank, 2007)]. The fact that a constitutively active form of Stat3 alone is sufficient to induce transformation points to an etiological role for Stat3 in neoplasia (Bromberg et al., 1999). Stat3 can be stimulated by cytokines and receptor tyrosine kinases, as well as the non-receptor tyrosine kinase Src (Yu et al., 1995). Stat3 is invariably latent in the cytoplasm and, subsequent to binding to an activated receptor through its SH2 domain, becomes activated through phosphorylation by the receptor itself or by the associated JAKs or Src family kinases. Phosphorylation at the critical tyrosine-705 activates Stat3 by stabilizing the association of two monomers through reciprocal SH2-phosphotyrosine interactions. The Stat3 dimer then migrates to the nucleus where it binds to target sequences, leading to the transcriptional activation of specific genes, such as myc, bcl-xL, cyclin D, survivin, hgf and others (Yu and Jove, 2004).

In addition to providing structure and integrity to the cell, adhesion molecules can play a key role in the initiation of signalling through multiple intracellular pathways which involve protein phosphorylation. The formation of cell to cell adhesion junctions is primarily modulated by the calcium-dependent family of cadherin receptors, and leads to a reorganization of the underlying actin cytoskeleton. Classical cadherins contain an extracellular domain organized into five distinct cadherin-specific repeats (EC) which display calcium ion binding, a membrane spanning region and a highly conserved cytoplasmic domain, which enables association with the cytoskeletal network through catenin interactions. Classical cadherins are subdivided into type I and type II (Halbleib and Nelson, 2006); type I cadherins have a conserved HAV motif in the most distal EC and include E-cadherin, P-cadherin and N-cadherin, which are found in most tissues and are crucial to the developmental process, while type II cadherins, such as cadherin-11,
lack this motif in their hydrophobic acceptor pocket. E-cadherin represents the best characterized classical cadherin; it is responsible for the formation and maintenance of epithelial structures and is abundant in cultured cells of epithelial origin (Jeanes et al., 2008). Although it is generally thought that cadherin expression results in a tight cell association, mesenchymal cells, which are loosely associated, express mesenchyme-specific cadherins such as cadherin-11. Cadherin-11 was originally identified in mouse osteoblasts (Okazaki et al., 1994), but it was later found to be expressed in a variety of normal tissues of mesodermal origin, such as areas of the kidney and brain (Hoffmann and Balling, 1995), as well as in cultured fibroblasts (Orlandini and Oliviero, 2001), and recent results showed that cadherin-11 promotes the metastasis of prostate cancer cells to the bone (Chu et al., 2008).

One of the signalling targets of cadherin engagement is the Rho family of proteins, which consists of a number of small GTPases acting as intracellular molecular switches cycling between the active, GTP-bound state and the inactive, GDP-bound form (Etienne-Manneville and Hall, 2002). The Rho proteins, Rac1, Cdc42 and RhoA are best known as master regulators of the actin cytoskeleton and promote the formation of lamellipodia, filopodia and stress fibers, respectively. Previous work has indicated that following ligation, the juxtamembrane domain of cadherins interacts with p120 catenin, which can activate Rac1 and Cdc42, possibly by binding to Vav2, an exchange factor for these GTPases, although the precise mechanism is not clearly established (Jaffer and Chernoff, 2004).

We and others recently demonstrated a dramatic increase in the activity of Stat3 triggered by cell confluence in a variety of cell lines (Vultur et al., 2004; Onishi et al., 2008; Steinman et al., 2003; Su et al., 2007; Kreis et al., 2007; Vultur et al., 2005). Given the generally accepted, positive role of Stat3 in proliferation, the Stat3 activity increase observed in post-confluent cells, at a time when cells do not divide, was a surprising observation. In this communication we definitively demonstrate that E-cadherin or cadherin-11 engagement alone activates Stat3, even in the absence of direct cell to cell contact. This activation occurs through an increase in the activity
of Rac1 and Cdc42. Unexpectedly, we also found that cadherin engagement induces a dramatic increase in total Rac1 and Cdc42 protein levels, which points towards a novel mechanism of Rac1 and Cdc42 upregulation. We demonstrate that this event causes a further increase in Rac1, Cdc42 and Stat3 activity, and constitutes a potent survival signal.
Materials and Methods

Cell lines and culture techniques

The normal mouse mammary epithelial line HC11 is a prolactin-responsive cell clone originally isolated from the COMMA-1D mouse mammary epithelial cell line derived from a female Balb/c mouse in mid-gestation (Ball et al., 1988). HC11 cells transformed by an activated Y527F-Src have been described previously (Wojcik et al., 2006). Normal mouse Balb/c 3T3, Balb/c 3T3 transformed by vSrc and mouse 10T1/2 fibroblasts have been previously described (Vultur et al., 2004). ES cell cultures were grown as before (Larue et al., 1996). Cell confluence was estimated visually and quantitated by imaging analysis of live cells under phase contrast using a Leitz Diaplan microscope and the MCID-elite software (Imaging Research, St. Catharine’s Ont.). For the isolation of mouse breast epithelial cells, mammary glands at day 12-14 of pregnancy were aseptically removed and transferred to a culture dish. The digestion of tissue and epithelial cell growth was adapted from (Medina and Kittrell, 2000). These cells grow in islands, so that it is difficult to obtain sparse cultures.

JAK-inhibitor-1 (EMD Biosciences, Gibbstown, NJ) and Toxin B (Sigma, St. Louis, MO) were added at the indicated concentrations. Cell viability was assessed by trypan blue exclusion and by replating cells in medium lacking the inhibitors. IL6 was purchased from R&D Systems (Minneapolis, MN).

Conditioned medium was prepared by adding 3 mls of serum-free RPMI to a 10 cm plate of HC11 cells grown to 3 days post-confluence.

Cadherin recombinant fragments and peptides

The plasmid constructs coding for the first two extracellular domains of E-cadherin (E/EC12) or cadherin-11 (11/EC12), fused with a C-terminal hexahistidine tag were previously
described (Perret et al., 2002). Cadherin fragment expression was induced by the addition of IPTG for 2 h. Cell pellets were resuspended in lysis buffer: 4 M urea, 50 mM Na$_2$HPO$_4$ pH 7.8, 20 mM Imidazole and 20 mM $\beta$-mercaptoethanol. Purification was carried out by Ni-affinity chromatography as previously described (Perret et al., 2002). Purified preparations of each cadherin fragment yielded only one band upon analysis by 15% SDS-PAGE and Coomassie blue staining. Purified fragments were attached to plastic petris as follows: petris were treated with poly-DL-lysine (Sigma, 0.1 mg/ml in H$_2$O, for 1 hour at room temperature), followed by glutaraldehyde treatment (Sigma, 2.5% in PBS, for 1 hour at room temperature). The cadherin fragments were subsequently added at the indicated concentrations for 2 hours, followed by glycine treatment (0.2 M in PBS, overnight). Petris were incubated with serum-free medium for one hour, prior to the addition of cells in complete medium. Growth of cells on the E/EC12 or cadherin-11 fragments did not cause any detectable change in morphology. Peptides were synthesized by CPC (San Jose, CA) and used at the indicated concentrations.

**Gene expression**

Rac1$^{N17}$ was expressed with a myc-tag at the carboxy-end by plasmid transfection. Expression was verified by blotting against the myc-tag using the 9E10 antibody (Sigma). Rac1 shRNA was purchased from Open Biosystems (Huntsville, AL, Cat# RMN 1766-97047533), in the retroviral vector pSM2c. For gp130 knockdown, a mouse pSM2 retroviral target gene shRNA set (Cat#. RMM4530-NM_010560) was purchased from Open Biosystems. A cadherin-11 shRNA set was also purchased from Open Biosystems (Cat#. RMM4530-NM_009866). Cells infected with the pSM2 constructs were selected for puromycin resistance. For the complete shRNA sequences see Appendix, Table A.4.
**Lentivirus vector production**

293T cells were plated at a density of $4 \times 10^6$ cells per 10-cm culture dish. The cells were co-transfected by calcium phosphate co-precipitation with 15 μg of pLKO1-Stat3 shRNA (Mission shRNA TRCN0000020842 and TRCN0000020840) or pLKO1-control shRNA (Sigma) and 10 μg of pPACK packaging plasmid mix (SBI, Mountain View, CA). The culture medium was replaced with fresh medium after 6 h. The supernatant was collected 16 h after the transfection and stored at −80°C. To determine the vector titers, $10^5$ HT1080 cells were seeded in a six-well plate and infected with various dilutions of the vector in the presence of 4 μg/ml polybrene. The culture medium was replaced 48 h later with fresh medium containing puromycin at a concentration of 1.5 μg/ml. Puromycin-resistant colonies were counted 10 days after transduction. To examine the efficiency of Stat3 downregulation, cells were infected at 5 plaque forming units (pfu)/cell and extracts probed for total Stat3 (Cell Signalling, Danvers, MA).

**Immunofluorescence and apoptosis assays**

Immunofluorescence staining for E-cadherin was performed using a mAb (BD Transduction Labs, Mississauga, ON), biotinylated anti-mouse IgG secondary and Rhodamine-avidin D (Vector Labs, Burlington, Ontario). Fixed cells were assayed for apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Roche, Mississauga, ON) and fluorescence activated cell sorting (FACS) analysis as described (Vultur et al., 2004). Images were captured using a 20x objective (NA 0.45) on a Nikon TE200 epifluorescence microscope equipped with a cooled CCD camera (Roper Scientific, Cool Snap HQ) and Metamorph Software. Fluorescent images were also analyzed on a laser scanning confocal microscope (Leica SP2) with Tsunami 2 photon IR laser using a 100x oil immersion lens (NA 1.4) and LCS software for initial acquisition, as well as Image Pro Plus 6.0 software for further analysis.
**Immunohistochemical staining for E-cadherin and Stat3ptyr705**

For immunohistochemical staining, consecutive tissue sections (6 µm) were cut from a formalin-fixed, paraffin-embedded tissue block of the #4 mammary fatpad from a nulliparous (8-12 wk old) strain 129 mouse bred in the Queen’s University Animal Care Facility. Sections were first deparaffinized in toluene for 4 min, three times and then rehydrated in a graded series of 100%, 85% and 70% ethanol, prior to being submerged in a water bath (10 °C) for 4 min. Antigen retrieval used a 1:10 dilution of 10 mM sodium citrate buffer, pH 6.0 at 95°C for 30 min. Tissues were then cooled to room temperature before washing twice with TBS (tris-buffered saline). Excess buffer was removed and 3% hydrogen peroxide was applied for 5 min. The tissue sections were then permeabilized in 25 µg/ml Digitonin in TBS, and blocked with 5% horse serum in TBS for 30 min. Excess blocking medium was removed and a PAP pen was used to concentrate antibodies over the tissue. Monoclonal anti-E-cadherin antibody (BD Transduction Labs), mouse IgG2α control (Invitrogen Inc., Burlington, ON), or rabbit anti-Stat3ptyr705 antibody (Cell Signalling NEB, Pickering ON) were applied at a predetermined dilution (200 µl) to each tissue section. For Stat3-ptyr705 staining, sections underwent an additional trypsinization step after antigen-retrieval in which sections were incubated in 0.01N hydrochloric acid (HCl) and 0.4% Trypsin for 20 min at 37°C, prior to application of rabbit anti-Stat3ptyr705 antibody. Sections were incubated with antibodies overnight at 4°C in a humidified chamber. The next day, the biotinylated link (Dako North America Inc.) was applied and incubated for 15 min. Streptavidin-peroxidase (Dako North America Inc.) was then applied to the specimens and incubated for another 15 min, according to the manufacturer’s instructions. Finally, 200 µL of DAB (diaminobenzidine) with DAB chromogen substrate (1 drop/mL) (Dako North America Inc.) was added to each section and incubated for 10 min. Tissues were counterstained lightly with hematoxylin, dipped three times in 1% HCl in 70% ethanol and placed in a water bath. The sections were then dehydrated in a graded ethanol series (70%, 85%, 100%)
followed by three toluene baths, and coverslipped using permount. Representative images were acquired under brightfield illumination using a Leitz microscope.

**Western blotting and immunoprecipitation**

Detergent cell extracts were prepared as described (Vultur et al., 2004). Following a careful protein determination (BCA-1 Protein assay kit, Sigma), 30 or 100 μg of clarified cell extract were loaded, as indicated. Blots were cut into strips and probed with antibodies specific for the tyr-705 phosphorylated Stat3 or total Stat3 protein (Cell Signalling NEB), Rac1 or Cdc42 (BD Transduction Labs), the dually phosphorylated form of Erk1/2 (Biosource, Burlington, ON), survivin (Cell Signalling NEB), p21 (Biosource), poly (ADP-ribose) polymerase (PARP, Roche), E-cadherin (BD Transduction Labs), gp130 (Sigma) or Hsp90 (Stressgen, Ann Arbor, Michigan) followed by alkaline phosphatase, or HRP-conjugated secondary antibodies (Jackson Labs, Bar Harbor, Maine). Bands were visualized using enhanced chemiluminescence (ECL, PerkinElmer Life Sciences, Woodbridge, Ontario), or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). Quantitation was achieved by fluorimager analysis using the FluorChem program (AlphaInnotech Corp). In all cases Stat3-ptyr705 band intensities were normalized to Hsp90 levels of the same samples. Jak1 phosphorylation was examined by immunoprecipitation against total Jak1, followed by blotting with a Jak-ptyr1022/1023 antibody (Cell Signalling NEB). Jak kinase assays were performed as described (Vultur et al., 2004).

Rac1/Cdc42 activation assays were performed using GST-PAK-PBD (glutathione-S-transferase – p21-activated kinase binding domain) pull-down assays with the Rac1/Cdc42 activation assay kit (Cytoskeleton, Denver, Colorado, #BK035). Photoshop (Adobe) or Corel Draw software were used for the organization of non-adjusted, original images and blots.
Reverse transcriptase-polymerase chain reaction (RT-PCR) assays

RT-PCR was performed using Superscript III First-Strand Synthesis kit (Invitrogen) in an Eppendorf Personal Mastercycler. cDNAs were synthesized using gene-specific primers and the Superscript III First-Strand Synthesis System kit for RT-PCR (Invitrogen, Cat. #180080-051). PCR was performed in 20μl on an Eppendorf Mastercycler Personal thermo cycler (Mississauga, ON). For the detection of cadherin levels, cDNA was denatured for 2 minutes at 94°C, then the reaction conducted for 30 cycles (94°C for 5 seconds, 55°C for 5 seconds, 72°C for 5 seconds) and the reaction completed at 72°C for 2 minutes. Primer pairs for amplification were, for E-cadherin: forward primer 5’CAT CGC CAC AGA TGA TGG TT and the reverse primer 5’ACC TGC ATG TTT CGA GGT TCT to generate a 111bp fragment; for cadherin-11: forward primer 5’ACC CCC TGA AAT CAT GCA CAA  and the reverse primer 5’ACC GGC ATT CAG GAT GTA G to generate a 251bp fragment.

For the detection of Rac1 and Cdc42 levels, total cDNA from cells grown to different densities was denatured for 2 minutes at 94°C, then the reaction conducted for 30 cycles (94°C for 15 seconds, 55°C for 30 seconds, 72°C for 1 minute) and the reaction completed at 72°C for 6 minutes. The primer pairs for Rac1 amplification were: forward primer 5’GGA CAC AGC TGG ACA AGA AGA and the reverse primer 5’GGA CAG AGA ACC GCT CGG ATA to generate a 368 bp fragment. For Cdc42 the primer pairs were: forward primer 5’CGA CCG CTA AGT TAT CCA CAG and the reverse primer 5’GCA GCT AGG ATA GCC TCA TCA to generate a 325 bp fragment. 18S RNA (153 bp; forward primer 5’AAA CGG CTA CCA CAT CCA AG and reverse primer 5’CCT CCA ATG GAT CCT CGT TA) was used as a control (Sigma).

All quantitative RT-PCR reactions were performed with 1x SYBR Green Master Mix (BioRad, Philadelphia, PA) using the Corbett Rotor-Gene 6000. Serial ten-fold dilutions of 18S RNA were used as a reference for the standard curve calculation. The delta ct (Δct) value was calculated from the given ct value by the formula: Δct = (ct\_sample – ct\_control). The fold change was calculated as the value of $1.94^{-\Delta ct}$, 1.94 being the average PCR efficiency. For the qRT-PCR
cytokine array, we used the PAMM-021A kit (SA Biosciences, Frederick, MD) with an RT-PCR for IL6 run in parallel, according to the manufacturer’s protocol.
Results

E-cadherin engagement can activate Stat3

To explore the nature of the molecule(s) that might be involved in the cell density-mediated Stat3 activation, we first used the normal mouse breast epithelial line HC11, which expresses high amounts of E-cadherin, an extensively studied, calcium-dependent, cell to cell adhesion molecule and member of the classical type I cadherin family [(Merlo et al., 1995); Appendix, Fig. A.1A]. HC11 cells were plated in plastic Petri dishes, and when ~30-80% confluent, and over several days thereafter, detergent cell extracts were probed by western blotting with an antibody specific for the tyrosine-705 phosphorylated form of Stat3 (Stat3-ptyr705, see Materials and Methods). To reduce the variability that might be caused by nutrient depletion in post-confluent cultures, the medium was changed every 24 hours. As a loading control, the same extracts were probed for the abundant heat shock protein, Hsp90. As shown in Fig. 4.1, Stat3-ptyr705 levels were almost undetectable in sparsely growing HC11 cells (A, panel a, and B, lane 1), while density caused a dramatic increase (>50-fold), which peaked at 2-3 days after confluence (A, panel d, and B, lanes 1 vs 6) and eventually decreased slightly at later times. The Stat3-ptyr705 observed in densely growing, normal HC11 cells was nuclear (Appendix, Fig. A.1C) and reached approximately half the levels present in cells transformed by the potent Stat3 activator, Src-527F (Fig. 4.1B, lane 9). Examination of the levels of total Stat3 protein revealed a modest increase with cell density (~2 fold, Fig. 4.1B), possibly due to the fact that the Stat3 promotor itself is one of the Stat3 targets (Narimatsu et al., 2001), although the differences were not as pronounced as the Stat3-tyr705 phosphorylation observed (Appendix Table A.1). This activation was found to be specific to Stat3, since the levels of the extracellular signal regulated kinase (Erk1/2), a signal transducer often coordinately activated with Stat3 by a number of growth factors and oncogenes, remained unaffected by cell confluence (Fig. 4.1B). In addition,
Figure 4.1

Cell density triggers a dramatic activation of Stat3 in HC11 cells.

A: HC11 cells grown to the indicated confluences were photographed under phase-contrast illumination. Bar represents 100 μm.

B: Cell density upregulates Stat3-ptyr705 levels.

Lysates from normal mouse breast epithelial HC11 cells grown to increasing densities were resolved by gel electrophoresis. The blot was cut into strips which were probed for Stat3-ptyr705, phospho-Erk1/2, total Stat3 or Hsp90 as a loading control, as indicated. Numbers under the lanes of the top panels refer to Stat3-ptyr705 and total Stat3 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90 levels, with the peak value of the control, HC11 cells transformed by src-527F (lane 9) taken as 100% (see Materials and Methods and Appendix, table A.1). Numbers at the left refer to molecular weight markers.
cell density increased Stat3 transcription in HC11 cells (Appendix, Fig. A.1D). The above results taken together indicate that cell density causes a specific increase in Stat3-tyr705 phosphorylation and activity in HC11 cells.

To examine whether E-cadherin engagement is directly required for the cell to cell adhesion-mediated Stat3 activation, cadherin adhesive interactions were interrupted through the introduction of a recombinant E-cadherin fragment, encompassing the two distal, extracellular domains of E-cadherin (E/EC12), which was previously shown to retain biological activity (Perret et al., 2002), into the medium of densely growing HC11 cells. In solution, this fragment is expected to compete with the cadherin molecules interacting from opposite cells and dissociate the junctions. As shown in Fig. 4.2A, addition of 40 μM, soluble E/EC12 to the growth medium caused a ~6-fold decrease in Stat3-ptyr705 levels (lane 2 vs 1), while the E/W2A mutant fragment, which is largely defective in cadherin engagement (Perret et al., 2002), had a weaker effect (~2-fold, lanes 3 and 4), strongly suggesting that cadherin engagement is required for the Stat3-ptyr705 increase. To further substantiate this conclusion, we used the peptide Ac-S-HAV-SA-NH2 (SHAVSA), which has been shown to inhibit E-cadherin homophilic interactions in epithelial cells (Makagiansar et al., 2001). As shown in Fig. 4.2A (lanes 6-9), addition of this peptide to the growth medium resulted in a substantial decrease in Stat3-tyr705 phosphorylation (up to 20-fold, lane 9 vs 6). In contrast, the peptide Ac-S-HAV-SS-NH2 (SHAVSS), previously shown to be less effective at inhibiting cadherin engagement had a less pronounced effect (lane 12), while the control, inactive peptide Ac-S-HGV-SA-NH2 (SHGVSA) had no detectable effect (lane 10). On the other hand, addition of the SHAVSA peptide even at 10 mM to HC11 cells expressing the constitutively active form of Stat3, Stat3C (McLemore et al., 2001), was unable to reduce Stat3-ptyr705 levels substantially (Fig. 4.2C). The reduction in Stat3-tyr705 phosphorylation following treatment with the SHAVSA peptide or soluble E/EC12 fragment was accompanied by a specific decrease in Stat3 transcriptional activity as well (Fig. 4.2B). Similar results were obtained with the MDCK, canine kidney epithelial line (not shown). These data
Figure 4.2

Disruption of cadherin engagement by soluble cadherin fragments or synthetic peptides reduces Stat3-tyr705 phosphorylation and activity

**A: Reduction of Stat3, ptyr705 phosphorylation.**
Left: HC11 cells were grown to 1 day post-confluence (lanes 1-4) or 40% confluence (lane 5) and the E/EC12, E-cadherin fragment encompassing the two outermost extracellular domains (lane 2), or the corresponding fragment from the E/W2A mutant (lanes 3 and 4) were added to the medium at concentrations of 40 μM for 48 hours, redosing at 24 hours. Detergent cell extracts were resolved by gel electrophoresis and probed for Stat3-ptyr705 or Hsp90 as a loading control, as indicated. Lanes 1 and 5: Control, untreated cells.
Right: HC11 cells grown to 1 day post-confluence were treated with increasing concentrations of the SHAVSA peptide (lanes 7-9), the inactive SHGVSA (10 mM, lane 10) or the weakly active SHAVSS (10 mM, lane 12) peptides for 48 hours, as indicated, and Stat3-ptyr705 and Hsp90 levels examined as above. Lanes 6 and 11: Control, untreated cells grown to 1 day post-confluence. Lane 13: Untreated HC11 cells grown to 40% confluence.

Numbers under the lanes of the top panels refer to Stat3-ptyr705 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90 levels, with the peak value of the control, untreated cells for each blot (lanes 1, 6 or 11) taken as 100% (see Materials and Methods).
B: Reduction of Stat3 transcriptional activity.

HC11 cells, transfected with the Stat3-dependent pLucTKS3 and the Stat3-independent pRLSRE reporters, were grown to the indicated densities and postconfluent cells treated with the E/EC12 or E/W2A cadherin fragments, the E-cadherin-disrupting SHAVSA, or the control SHGVSA peptides, as indicated. Firefly (■) or Renilla (□) luciferase activities were determined in detergent cell extracts (Vultur et al., 2004). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.

C: Stat3C expression rescues Stat3-ptyr705 levels following inhibition of cadherin engagement.

Stat3C was stably expressed in HC11 cells, which were grown to 1 day post-confluence. Following addition of 5 mM or 10 mM of the cadherin-disrupting, SHAVSA peptide, detergent cell extracts were probed as above for Stat3-ptyr705 or Hsp90 as a loading control, as indicated. Numbers under the lanes refer to Stat3-ptyr705 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90, with levels in the control, untreated HC11-Stat3C cells taken as 100%.

Further reinforce the conclusion that E-cadherin engagement is required for the cell to cell adhesion-mediated, Stat3-tyr705 phosphorylation, in both mammalian species.

To examine the generality of the E-cadherin requirement for the density-mediated, Stat3 activation, we employed an E-cadherin-defective, mouse embryonic stem (ES) cell line (null), and the parental ES line (Ecad+/+) as a control (Larue et al., 1996). As expected, density caused a 10-fold increase in Stat3-ptyr705 phosphorylation in the wild-type (Ecad+/+) cells, which peaked at 2-3 days after confluence (Fig. 4.3A, top, lanes 1 vs 6). In stark contrast however, Stat3-ptyr705 levels were approximately 6-fold lower in null cells, at all densities examined (Fig. 4.3B vs A, top). The low, background Stat3-ptyr705 levels present in null cells (Fig. 4.3B) could conceivably be due to the LIF, a known Stat3 activator, present in the growth medium of ES cells (Appendix Figs. A.2, and A.3). However, the possibility of other cadherins, or alternative pathways of Stat3 activation being present, cannot be excluded. In any event, the dramatic Stat3-ptyr705 increase with cell confluence (Fig. 4.3A, lane 1 vs 6), and decrease with cadherin ablation (Fig. 4.3B vs A and Appendix Fig. A.4A), indicates that the E-cadherin-induced, Stat3 phosphorylation is much stronger than the activation due to LIF. The decrease in Stat3-705
phosphorylation in null cells was accompanied by a similar decrease in Stat3 DNA binding and transcriptional activity (Appendix Fig. A.4C and D). The above data taken together indicate that E-cadherin ablation in ES cells can have a striking and specific effect upon the density-induced increase in Stat3-tyr705 phosphorylation and activity, and further suggest that E-cadherin is required for this activation.

The HC11 line was originally derived from normal mouse breast epithelium (Danielson et al., 1984; Kimball et al., 1984). Therefore, to assess the relevance of the cadherin-Stat3 interactions observed at high cell densities to the in vivo microenvironment, the expression patterns of E-cadherin and Stat3-ptyr705 were examined in normal mouse breast tissues by immunohistochemistry staining. As shown in Fig. 4.4A, there is intense E-cadherin staining at the plasma membrane, while Stat3-ptyr705 staining is concentrated mostly in the nuclei of luminal breast epithelial cells. In contrast, there is little expression of either protein in the surrounding adipose tissue. These findings demonstrate the presence of constitutively activated Stat3 in the corresponding normal breast tissue where E-cadherin is engaged, thus revealing a distinct correlation in the state of these same molecular markers observed in HC11 cells growing at high, but not low densities in culture. This conclusion was reinforced by examination of Stat3-ptyr705 levels in cultured primary mouse breast epithelial cells, which display similar levels of Stat3-ptyr705 as confluent HC11 cells (Fig. 4.4B).

**E-cadherin engagement is sufficient to activate Stat3**

The above results demonstrate that E-cadherin is required for the cell to cell adhesion-mediated Stat3 activation in normal cells. The question still remaining is whether Stat3 activation is a direct consequence of E-cadherin engagement or whether E-cadherin interactions are simply required to bring cell surfaces into proximity, to initiate signals which are not direct effects of
Figure 4.3

E-cadherin ablation inhibits the density-mediated, Stat3 activation.

Ecad+/+ (A) or null (B) mouse ES cells were grown to increasing densities, up to 8 days post-confluence. Detergent cell lysates were resolved by gel electrophoresis and probed for Stat3-ptyr705 (top), total Stat3 (middle) or Hsp90 as a loading control (bottom), as indicated.
Figure 4.4

Expression of Stat3-ptyr705 and E-cadherin in normal mouse breast ductal epithelium

A. Serial tissue sections from a formalin-fixed, paraffin embedded mouse mammary (#4) fatpad from a nulliparous 8 wk old mouse were subjected to immunohistochemical staining for Stat3-ptyr705 (top) or E-cadherin (bottom), as described in Materials and Methods. Representative images taken with a 40x or 100x objective are shown. Scale bar = 100 µm.

B. Lysates from primary mouse breast epithelial cells (lane 3) and normal mouse breast epithelial HC11 cells grown to 40% (lane 1) or 100% (lane 2) confluence were resolved by gel electrophoresis. The blot was cut into strips which were probed for Stat3-ptyr705, E-cadherin or Hsp90 as a loading control, as indicated. Numbers at the left refer to molecular weight markers.
cadherin ligation. To definitively demonstrate whether the Stat3 activation observed at high cell
densities is a direct effect of cadherin engagement *per se*, the E/EC12 fragment was used to
functionalize petri dishes by covalent immobilization (Fig. 4.5A). This fragment has been shown
to retain biological activity when immobilized on surfaces and is recognized specifically by E-
cadherin-expressing cells (Perret et al., 2002).

Plastic petri dishes were functionalized with increasing amounts of purified E/EC12 or
E/W2A fragments and HC11 cells plated on these surfaces (see Materials and Methods). No
change in the morphology of HC11 cells upon growth on these fragments was noted (Fig. 4.5A).
Detergent cell extracts were prepared 48 hours later, when cells were 40% confluent, and probed
for Stat3-ptyr705 as above. As shown in Fig. 4.5B, there was a dramatic and graded increase in
Stat3-ptyr705 levels, in proportion to the amounts of E/EC12 used to decorate these surfaces
(lanes 4-12 and 16). As expected, coating with the E/W2A fragment had only a small effect (~4x
lower than the E/EC12 fragment, Fig. 4.5B, bar graph), consistent with its impaired ability to
engage wild- type E-cadherin (Perret et al., 2002). The increase in Stat3-ptyr705 was most
pronounced when cells were grown for more than 20 hrs on E/EC12-coated dishes (Appendix,
Fig. A.5A), and when the confluence at harvest time was 40% or less (Appendix, Fig. A.5B).
The increase in Stat3-ptyr705 levels was specific to Stat3, since no differences were noted in the
levels of activated Erk1/2 in the same cell extracts (Fig. 4.5B). Similar results were obtained with
MDCK cells (not shown). At the same time, Balb/c 3T3 cells, which are naturally devoid of E-
cadherin (Fig. 4.6A and Appendix, Fig. A.1A) showed no increase in Stat3-ptyr705 upon growth
on surfaces coated with the E/EC12 fragment (Fig. 4.5B, lanes 1-2). The above findings
demonstrate a dramatic increase in Stat3-ptyr705 upon direct E-cadherin engagement in mouse
epithelial cells, which is specific to Stat3 and proportional to the density of E/EC12 present on the
culture surface, indicating that E-cadherin engagement alone is sufficient to activate Stat3, in the
absence of direct cell to cell contact.
Figure 4.5

E-cadherin engagement is sufficient to activate Stat3.

A. Schematic drawing of epithelial cells plated at low density on plastic petris coated with an E-cadherin fragment encompassing the two outermost EC domains (E/EC12) (Boggon et al., 2002). Right: Phase-contrast photographs of HC11 cells grown for 48 hours on an E/EC12-coated surface (0, 100 or 1,000 µg/ml). Bar corresponds to 100 µm.

B: 0.3x10^5 HC11 cells were grown on plastic 3 cm dishes coated with increasing amounts of E/EC12, from 0.5 to 1,000 µg/ml. 48 hours later, extracts were probed for Stat3-ptyr705 or total Stat3, as indicated. As a further control (lanes 1 and 2), Balb/c 3T3 cells, which are devoid of E-cadherin (Appendix, Fig. A.1A), were grown on the same surfaces. Lanes 13 and 14: Surfaces were coated with the largely inactive, mutant fragment of E-cadherin, E/W2A.

Bar graph: The average relative Stat3-ptyr705 levels across 3 independent experiments were quantitated by fluorimeter analysis, and graphed as a function of the loading control, Hsp90 (☉), or as a function of total Stat3 (■). The peak value of HC11 cells grown to 2 days postconfluence on an uncoated surface was taken as 100%.

The same extracts were probed for phospho-Erk1/2 or Hsp90 as a loading control, as indicated (lower panels).

Cadherin-11 engagement in Balb/c 3T3 can also activate Stat3

Cadherins were originally identified as cell surface glycoproteins responsible for calcium-dependent, cell to cell adhesion in development. More than 100 family members have been discovered to date with diverse structures, but with the characteristic extracellular, EC repeats [reviewed in (Halbleib and Nelson, 2006; Gumbiner, 2005)]. To examine whether other cadherins might also be able to activate Stat3, we used the mouse fibroblast line Balb/c 3T3 which expresses cadherin-11 (Orlandini and Oliviero, 2001), a prototype type II classical cadherin, but not E-cadherin (Fig. 4.6A). Examination of Stat3-ptyr705 levels at different densities revealed a distinct increase (Fig. 4.6B), similar to HC11 (Fig. 4.1B) or 10T½ fibroblasts [(Vultur et al., 2004; Vultur et al., 2005) and Appendix, Fig. A.11], suggesting that cadherin-11 homophilic interactions may be indispensable for the cell to cell adhesion-mediated, Stat3 activation. The levels of Erk1/2 also remained unaffected by density in this cell line (Fig 4.6B) indicating that cell confluence causes a specific increase in Stat3-ptyr705 levels in mouse Balb/c 3T3 fibroblasts. Stat3 activity levels mirrored the increase in ptyr705 (Fig 4.6C). In addition,
cell density increased the levels of the Stat3 transcriptional target, survivin (Fig. 4.6C). These observations are in keeping with previous data indicating that Stat3 transcriptional activity increases with the density of cultured fibroblasts (Vultur et al., 2004; Vultur et al., 2005), and further point to the possibility that cadherin-11 may be responsible for the Stat3 activation observed at high cell densities in Balb/c 3T3 fibroblasts.

To examine whether cadherin-11 was responsible for the density-mediated increase in Stat3-ptyr705 levels, cadherin-11 was knocked down through shRNA expression (see Materials and Methods). As shown in Fig. 4.6D, downregulation of cadherin-11 resulted in a reduction in Stat3-ptyr705 levels, indicating that cadherin-11 is indeed responsible for the Stat3, tyr705 phosphorylation observed at high densities.

To further demonstrate whether, like E-cadherin, direct cadherin-11 engagement might also be able to lead to Stat3 activation, Balb/c 3T3 cells were plated on petri dishes coated with a cadherin-11 fragment encompassing the two outermost EC domains (11/EC12). The results revealed that this treatment caused a dramatic increase in Stat3-ptyr705 (Fig. 4.7C, lanes 2-5), while there was no increase in Stat3-ptyr705 when Balb/c 3T3 cells were plated on petri dishes coated with the E-cadherin-derived fragment, E/EC12 (Fig. 4.7C, lane 1). As a further control, HC11 cells were plated on surfaces coated with 11/EC12 or on E/EC12, and Balb/c3T3 cells were plated on surfaces coated with the E/EC12 fragment. As expected, there was no increase in Stat3-ptyr705 when HC11 cells, which are naturally devoid of cadherin-11 (Fig. 4.6A), were grown on surfaces coated with 11/EC12 (Fig. 4.7B, lane 8), while there was an increase upon growth of HC11 cells on E/EC12-coated surfaces (lane 9). The increase was specific to Stat3, since no increase in Erk1/2 upon plating of Balb/c 3T3 cells on 11/EC12-coated surfaces was noted (Fig. 4.7C). These results indicate that, apart from E-cadherin, cadherin-11 can also increase Stat3-ptyr705 phosphorylation levels in the appropriate cellular context.
Figure 4.6

Cadherin-11 engagement can trigger Stat3 phosphorylation in Balb/c 3T3 cells

A: Examination of E-cadherin and cadherin-11 mRNA levels in HC11 and Balb/c 3T3 cells.
Extrasts from Balb/c3T3 or HC11 cells were probed by RT-PCR for E-cadherin (lanes 1 and 2) or cadherin-11 (lanes 3 and 4), with 18S RNA as a control (lanes 6 and 7) (see Materials and Methods). Numbers on the left refer to the molecular weight marker lane (M).

B: Cell density upregulates Stat3-ptyr705 levels in Balb/c3T3 cells.
Lysates from Balb/c3T3 fibroblasts grown to increasing densities were resolved by gel electrophoresis and probed for Stat3-ptyr705, total Stat3, phospho-Erk1/2 or Hsp90 as a loading control, as indicated.

C: Cell density upregulates Stat3 transcriptional activity in Balb/c 3T3 cells.
Upper panel: Balb/c 3T3 cells were transfected with the Stat3-dependent pLucTKS3 reporter driving a firefly luciferase gene under control of the C-reactive gene promoter element, and the Stat3-independent pRLSRE reporter driving a Renilla luciferase gene under control of the c-fos serum response element (SRE) promoter, respectively. Cells were grown to the indicated densities with daily media changes and firefly (■) or Renilla (□) luciferase activities determined.
in cytosolic extracts with the peak value of the control, v-src transformed cells taken as 100% (see Materials and Methods). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means±SEM of at least 3 experiments, each performed in triplicate. **Lower panel:** Detergent cell extracts of Balb/c 3T3 cells were probed for survivin or Hsp90 as a loading control, as indicated.

**D: Cadherin-11 knockdown dramatically reduces Stat3 phosphorylation**

Lysates from Balb/c 3T3 fibroblasts or Balb/c 3T3 stably expressing an shcad11 construct (see Materials and Methods) were grown to increasing densities. Lysates were resolved by gel electrophoresis and probed for Stat3-ptyr705, and Hsp90 as a loading control.

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![Figure 4.7](image)

**Figure 4.7**

**Cadherin-11 engagement is sufficient to activate Stat3 in Balb/c 3T3 fibroblasts**

**A:** Schematic drawing of fibroblasts plated at low density in plastic petris coated with a cadherin-11 fragment encompassing the two outermost EC domains (11/EC12) (Boggon et al., 2002).
Balb/c 3T3 cells were grown in plastic 3cm dishes, coated with increasing amounts of the cadherin-11 fragment, 11/EC12, as indicated. 48 hours later, cell lysates were probed for Stat3-ptyr705, phospho-Erk1/2 or Hsp90 as a loading control, as indicated. As controls Balb/c 3T3 cells, which are devoid of E-cadherin (see Fig. 4.6A above), were grown on a surface coated with the corresponding E-cadherin fragment (E/EC12, lane 1) and HC11 cells which are devoid of cadherin-11 were grown on surfaces coated by cadherin-11 (lanes 7 and 8), or by E-cadherin (lane 9).

**Cell to cell adhesion increases protein levels and activity of Rac1 and Cdc42**

Results from a number of laboratories have indicated that E-cadherin-mediated cell to cell contacts can activate the Rac1 and Cdc42, Rho family GTPases (Kovacs et al., 2002; Noren et al., 2001; Nakagawa et al., 2001). Therefore, to assess the potential role of Rac1 in the cadherin-dependent, Stat3 activation, we investigated whether Rac1 activity does, in fact, increase with cell density; cells were plated in petri dishes and when ~30% confluent, and over several days thereafter, Rac1 activity was determined by measuring the binding between Rac1-GTP and its effector, p21-activated kinase (PAK), in cell extracts using pull-down assays (see Materials and Methods). As shown in Fig. 4.8A, left, there was a gradual increase in Rac1 activity with density in HC11 cells, which plateaued at approximately 80-100% confluence, that is ~24 hours before the rise in Stat3-ptyr705. Cdc42 activity displayed a similar increase with cell confluence (Fig. 4.8A, right), in a manner parallel to Rac1. In addition, cell density led to an upregulation of Rac1-GTP and Cdc42-GTP levels in Balb/c 3T3 cells (Fig. 4.10A), which express cadherin-11. The above results demonstrate that cell density causes an increase in Rac1-GTP and Cdc42-GTP (~6-fold), which is detectable before the rise in Stat3 activity, in both HC11 epithelial cells and Balb/c 3T3 fibroblasts.

It was previously demonstrated that epithelial cell scattering brought about by HGF can induce the proteasome-mediated degradation of Rac1, pointing towards a new mechanism of Rac1 regulation involving protein stability (Lynch et al., 2006). Therefore, to explore the potential effect of cell density upon the levels of total Rac1 protein, detergent extracts from cells
Figure 4.8

Cell density increases the activity as well as total protein levels of Rac1 and Cdc42 in HC11 cells.

A: Rac1-GTP, Cdc42-GTP as well as total Rac1 and Cdc42 protein levels are dramatically increased by cell density.

HC11 cells were grown to increasing densities, up to 11 days post-confluence, and total protein extracted. Rac1/Cdc42 activation assays were performed using beads coated with glutathione-S-transferase (GST) fused to the binding domain of PAK (PAK-PBD) in pulldown assays (see Materials and Methods). Detergent lysates were resolved by gel electrophoresis and blots were cut into strips which were probed for Stat3-pty705, active Rac1-GTP and total Rac1 (left) or active Cdc42-GTP and total Cdc42 (right), or Hsp90 as a loading control, as indicated. See Appendix, table A.1 for quantitation.

B: Rac1 and Cdc42 mRNA levels are not affected by density.

Total mRNA from HC11 cells grown to different densities was subjected to RT-PCR analysis, with the 18S RNA as a control (see Materials and Methods). Bottom: Quantitative RT-PCR was performed as described in Materials and Methods. The relative expression of each sample was determined according to 18S RNA expression levels as an internal control.
E-cadherin engagement increases Rac1 protein levels and activity in the absence of direct cell to cell contact

A: Cadherin engagement is sufficient to increase Rac1 activity and protein levels.

**Lanes 1-4:** HC11 cells were grown in plastic petris coated with 1 mg/ml of the E/EC12 (lane 3) or the largely inactive E/W2A (lane 2) fragment. 48 hours later, detergent cell extracts were resolved by gel electrophoresis and blots were cut into strips which were probed for Stat3-ptyr705, total Rac1 or Hsp90 as a loading control, as indicated. **Lanes 5-6:** Cadherin engagement increases Rac1 activity: Sparsely growing HC11 cells were plated on uncoated petris (lane 5) or petris coated with 1 mg/ml of the E/EC12 fragment (lane 6) and Rac1 activity determined (see Materials and Methods).

Numbers under the lanes refer to Stat3-ptyr705, Rac1-GTP and total Rac1 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90 levels, with the peak value of the control untreated cells taken as 100%.

B: Inhibition of E-cadherin engagement prevents Stat3 phosphorylation and the increase in Rac1 activity and protein levels.
Lanes 1-5: HC11 cells were grown to 1 day post-confluence (lanes 1-4) or 40% confluence (lane 5) and treated with 40 µM soluble E/EC12 fragment (lane 2), or the E/W2A mutant (lanes 3 and 4).

Lanes 6-13: HC11 cells were grown to 1 day post-confluence (lanes 6 or 11, untreated) or 40% confluence (lane 13, untreated) and subject to increasing concentrations of the SHAVSA peptide (lanes 7-9), the inactive SHGVSA (lane 10) or the weakly active SHAVSS (lane 12). Rac1 levels were determined in the same extracts and the same blot as in Fig. 4.2A.

Lanes 14-17: Inhibition of cadherin engagement reduces Rac1 activity. HC11 cells were grown to 1 day post-confluence, treated with the indicated amounts of peptide for 48 hours and Rac1 activity determined (see Materials and Methods).

Figure 4.10

Cadherin-11 increases Rac1/Cdc42 activity and protein levels in Balb/c 3T3 cells.

A: Rac1 and Cdc42 activity, as well as expression, and Stat3-ptyr705 levels are dramatically increased by cell density in Balb/c 3T3 cells.
Balb/c 3T3 cells were grown to different densities, up to 5 days post-confluence. Detergent cell lysates were probed for Stat3-ptyr705, active Rac1-GTP, total Rac1, active Cdc42, total Cdc42 or Hsp90 as a loading control, as indicated.

**B: Cadherin-11 engagement is sufficient to increase Rac1 protein levels and activity.**

Balb/c 3T3 cells were grown in plastic petris coated with 1mg/ml of the E/EC12 fragment or the E-cadherin-derived, E/EC12 fragment. 48 hours later, detergent cell extracts were probed for Stat3-ptyr705, Rac1 or Hsp90 as a loading control, as indicated.

**C: Rac1 and Cdc42 mRNA’s are not affected by cell density.**

Balb/c 3T3 cells were grown to different densities as indicated and Rac1 or Cdc42 mRNA levels examined by RT-PCR, using 18S RNA as a control (see Materials and Methods).

grown to different degrees of confluence were probed for Rac1. As shown in Fig. 4.8A, left, and Fig. 4.10A, there was a ~50-fold increase in total Rac1 protein levels with cell density. The increase in total Rac1 protein was sharp, in contrast to the gradual increase in Rac1 activity, and could explain the further increase in active Rac1-GTP at higher densities. Total Cdc42 levels mirrored Rac1 and increased at approximately the same time (Fig. 4.8A, right, and Fig. 4.10A).

At the same time, Rac1 and Cdc42 mRNA levels did not change significantly with cell density, as shown by quantitative RT-PCR (Figs. 4.8B and 4.10B), pointing to a post-mRNA mechanism. The above results indicate that, in addition to Rac1 and Cdc42 activity, cell to cell adhesion also causes a dramatic increase in total Rac1 and Cdc42 protein levels in HC11 and Balb/c 3T3.

To better control the degree of cell to cell contact, irrespective of differences in cell growth patterns, we repeated the experiment by plating different numbers of HC11 cells (ranging from 0.05-3x10^6 per 3cm petri), so that they would reach the same densities as above within 24 hrs. At that time, cells were lysed and Rac1, Cdc42 and Stat3 phosphorylation levels analysed as above. In all cases, very similar results were obtained, indicating that it is the extent of cell to cell contact, regardless of time in culture beyond 24h, that is responsible for the increase in Rac1 or Cdc42 protein levels and Stat3-ptyr705 (Appendix, Fig. A.7). Similar results were obtained with Balb/c 3T3 (not shown).
E-cadherin and cadherin-11 engagement can increase protein levels and activity of Rac1 and Cdc42

To examine whether E-cadherin or cadherin-11 engagement alone is able to increase Rac1 protein levels in the absence of cell to cell contact, HC11 cells or Balb/c 3T3 cells were plated in E/EC12- or 11/EC12-coated dishes, as in Fig. 4.5B or Fig. 4.7B, respectively, and Rac1 levels examined. As shown in Fig. 4.9A, plating HC11 cells on E/EC12-coated surfaces (lanes 1-4), besides leading to an increase in Stat3-ptyr705 (top), also caused a ~50-fold increase in Rac1 protein levels (lanes 1 vs 3) and activity (lanes 5 and 6), while the mutant E/W2A fragment had a less pronounced effect (lane 2). Similarly, plating Balb/c 3T3 cells on surfaces coated with the 11/EC12 fragment led to a striking increase in Rac1 protein levels (Fig. 4.10C, lanes 1 vs 3) and activity (lanes 8 vs 9), while plating them on E/EC12 had no effect on Stat3-ptyr705 or Rac1 levels (Fig. 4.10C, lanes 1 vs 2).

To further examine whether cadherin engagement is required for the increase in Rac1 protein levels, E-cadherin interactions were disrupted through the introduction of the E/EC12 fragment into the medium, as in Fig. 4.2A. As shown in Fig. 4.9B, besides a reduction in Stat3-ptyr705, addition of 40 μM, soluble E/EC12 fragment to the medium of densely growing HC11 cells caused a ~6-fold decrease in Rac1 levels (lane 2), while the mutant E/W2A had a weaker effect (~3-fold, lanes 3 and 4). Inhibition of cadherin engagement with the SHAVSA peptide had a similar effect upon Rac1 activity (Fig. 4.9B, lanes 14-17) as well as total Rac1 and Stat3-ptyr705 levels (Fig. 4.9B, lanes 6-9). Preliminary data also show that Balb/c 3T3 cells stably expressing shcad11 have reduced Rac1 protein levels and activity (not shown). These results taken together further demonstrate that E-cadherin or cadherin-11 engagement is required for the cell to cell adhesion-mediated increase in total Rac1 protein levels and activity in HC11 or Balb/c cells, respectively.
Rac1 and Cdc42 are required for the cell to cell adhesion-mediated, Stat3 activation

To examine whether the increase in cellular Rac1 and Cdc42 we observe in confluent cultures is actually required for the Stat3 increase, their activity was blocked in HC11, Balb/c 3T3 or 10T½ cells using the potent inhibitor of the Rho family GTPases, toxin B of Clostridium difficile (Aktories, 1997). As shown in Fig. 4.11A, besides a reduction in Rac1 and Cdc42 activity (right), toxin B treatment caused a dramatic reduction in Stat3-ptyr705 levels in HC11 at all densities (~5-fold, left). Similar results were obtained with 10T½ fibroblasts, which express cadherin-11 (Appendix, Fig. A.9, A-B) and Balb/c 3T3 fibroblasts (not shown), indicating that these GTPases may be required for the density-induced, Stat3 stimulation in both cell types.

To further examine the relative contribution of each Rho family GTPase individually, we assessed the ability of their dominant-negative mutants (Rac1N17 and Cdc42N17, respectively), or Rac1- and Cdc42-specific shRNAs, to restrain the density-mediated increase in Stat3-ptyr705 levels. These constructs were expressed in HC11, 10T½/2 or Balb/c 3T3 cells and Stat3-ptyr705 examined at different cell densities as above. As shown in Fig. 4.11B and C, Appendix, Figs. A.9D, A.10 and Tables A.1A and B, there was a significant reduction in Stat3-ptyr705 levels (~2-fold), upon expression of Rac1N17 or shRac1 and Cdc42N17, or shCdc42 at all densities examined. The residual Stat3-ptyr705 following Rac1 downregulation (Fig. 4.11B, lanes 5-8 and C, lanes 7-12 and Appendix, Fig. A.9D, top panel, lanes 8-14) could be due to Stat3 phosphorylation mediated by Cdc42, and vice versa, following Cdc42 downregulation. Rac1 downregulation with the Rac1N17 or shRNA caused a similar decrease in Stat3 transcriptional activity (Fig. 4.11B and C). These results were confirmed using the Rac1 specific inhibitor NSC23766 (Appendix, Fig. A.9C). Similar results were obtained in Balb/c 3T3 upon shRac1 expression (not shown).

Conversely, to ensure that Rac1 activation is not caused by Stat3, Stat3 activity was reduced by [1], expressing a Stat3-specific, shRNA with a lentiviral vector, using a scrambled sequence as a control (see Materials and Methods), or [2], treating the cells with the compound

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Figure 4.11

Inhibition of Rac1 or Cdc42 prevents the density-induced, Stat3 activation.

A: Toxin B, a Rho GTPase protein family inhibitor prevents the density-mediated, Stat3-tyr705 phosphorylation.

Left: HC11 cells were grown from 70% to 7 days post-confluence with daily media changes and treated with 50 pg/ml toxin B for 5 hours (lanes 6-10), or the DMSO carrier alone (lanes 1-5), and lysates resolved by gel electrophoresis. Extracts were probed for Stat3-ptyr705 (top) or Hsp90 as a loading control (bottom), as indicated.

Right: HC11 cells were grown to 1 day post-confluence and treated with 50 pg/ml toxin B for 5 hours (lane 2) or the DMSO carrier alone (lane 1) and the activity of Rac1 or Cdc42 determined (see Materials and Methods).

B: The dominant-negative Rac1\textsuperscript{N17} mutant prevents the density-mediated, Stat3-tyr705 phosphorylation and transcriptional activity

Left: Increasing numbers of HC11 cells as indicated, before (lanes 1-4) or after (lanes 5-8) transfection with the dominant-negative mutant Rac1\textsuperscript{N17} were plated in 3 cm dishes. Detergent cell extracts were resolved by gel electrophoresis and blots were probed for Stat3-ptyr705, active Rac1-GTP, total Rac1, the myc-tag or Hsp90, as above, as indicated.

Right: Increasing numbers of HC11 cells as indicated, before or after transfection with Rac1\textsuperscript{N17} and the Stat3-dependent pLucTKS3 and the Stat3-independent pRLSRE reporters, were lysed and firefly (■) or Renilla (□) luciferase activities determined (Vultur et al., 2004). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.

C: Rac1 downregulation through expression of an shRNA prevents the density-mediated, Stat3-tyr705 phosphorylation and transcriptional activity

Top: HC11 cells before (lanes 1-6) or after (lanes 7-12) stable expression of a Rac1-shRNA were grown to different densities and lysates probed, as above, for Stat3-ptyr705, active Rac1-GTP (right), total Rac1 or Hsp90, as indicated.

Bottom: HC11 cells before or after expression of a Rac1-shRNA as indicated, were transfected with the Stat3-dependent pLucTKS3 and the Stat3-independent pRLSRE reporters, grown to different densities, then lysed and firefly (■) or Renilla (□) luciferase activities determined (Vultur et al., 2004). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.

S3I-201 (Siddiquee et al., 2007), shown to inhibit Stat3 by binding to its SH2 domain (see Materials and Methods). No significant change in Rac1 levels or activity was noted in HC11 cells under either condition (Fig. 4.12B and Table 4.1). Similar results were obtained in 10T\textfract{1}{2} and Balb/c 3T3.
Table 4.1
Stat3 downregulation promotes apoptosis in confluent HC11 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>H11</th>
<th>HC11-Stat3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Confluence (%)</td>
<td>Stat3-ptyr705 (%)</td>
</tr>
<tr>
<td>c-RNA</td>
<td>80</td>
<td>32±11</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>80±15</td>
</tr>
<tr>
<td></td>
<td>+3d</td>
<td>100±12</td>
</tr>
<tr>
<td>shRNA</td>
<td>80</td>
<td>10±3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>30±9</td>
</tr>
<tr>
<td></td>
<td>+3d</td>
<td>39±8</td>
</tr>
<tr>
<td>S3I-201</td>
<td>80</td>
<td>18±6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>29±5</td>
</tr>
<tr>
<td></td>
<td>+3d</td>
<td>51±11</td>
</tr>
</tbody>
</table>

HC11 or HC11-Stat3C cells were grown to different densities and treated with the Stat3-shRNA, a control, scrambled c-RNA or 200μM of the inhibitor S3I-201, as indicated, and Stat3-ptyr705, active Rac1-GTP and apoptosis examined.

* Confluence was estimated visually and quantitated by imaging analysis of live cells under phase contrast (see Materials and Methods).

† Stat3-ptyr705 levels were quantitated by fluorimager analysis and normalized to Hsp90 levels, with the values for HC11 cells at 2 days post-confluence, treated with the control RNA taken as 100%. The results are averages of at least three independent experiments ± s.e.m. In all cases, the EMSA and transcriptional activity values obtained paralleled the Stat3-705 phosphorylation levels indicated.

‡ To quantitate apoptosis, cells were stained with propidium iodide and their sub-G1 profile analysed by FACS sorting. Numbers refer to cells in subG1 as a % of total cells. The results are averages of at least three independent experiments ± s.e.m.

§ Rac1 activity was measured as described in Materials and Methods.
The above data taken together indicate that the Rac1 and Cdc42 Rho family GTPases are essential components of the pathway whereby cadherin engagement triggers the Stat3 phosphorylation and activity increase observed at post-confluence in epithelial cells and fibroblasts.

**JAKs are required for the cadherin-mediated, Stat3 activation**

Previous results indicated that cell density induces an increase in Jak1 kinase activity (Vultur et al., 2004). To further investigate the role of Jak1 in the cadherin-mediated, Stat3 activation, we at first examined whether Jak1 is activated by E-cadherin engagement, by examining Jak1 phosphorylation at tyr1022/1023, shown to be important in the regulation of Jak1 activity (Gauazzi et al., 1996; Leonard and O'Shea, 1998). HC11 cells were grown in plastic petri dishes coated with the E/EC12 fragment and Jak1 phosphorylation at 1022/1023 measured by blotting with a phospho-specific antibody (see Materials and Methods). As shown in Fig. 4.12A (lanes 8 and 9), there was a 5-fold increase in Jak1 1022/1023 phosphorylation upon growth on E/EC12-coated surfaces, which paralleled the phosphorylation of Stat3 (lanes 1 and 2). To examine whether Jak1 is actually required for Stat3 phosphorylation, HC11 cells were plated on the E/EC12 fragment and treated with the pan-JAK inhibitor, JAK inhibitor 1. The results showed a reduction in Stat3-ptyr705 levels, proportional to the concentration of the inhibitor added (Fig. 4.12A, lanes 3-7). Treatment of Balb/c 3T3 with JAK inhibitor I also reduced the cell-cell adhesion dependent Stat3-tyr705 phosphorylation (see Appendix, Fig. A.12B). Similar results were obtained with the AG490 JAK inhibitor ((Zhang et al., 2000); not shown). These data suggest that the JAK kinases may be involved in the cadherin-mediated increase in Stat3-ptyr705 levels.

We next examined the effect of downregulation of the Rho GTPases upon Jak1, 1022/1023 phosphorylation (Fig. 4.12A). HC11 cells were treated with toxin B as in Fig. 4.11A, left and Jak1 ptyr1022/1023 phosphorylation measured as above. As shown in Fig. 4.12A,
Figure 4.12

**JAKs are required for the cadherin-dependent Stat3-tyr705 phosphorylation.**

**A: JAK inhibitor-1 reduces the cadherin-dependent, Stat3-tyr705 phosphorylation.**

**Left:** HC11 cells were grown to a confluence of 40% on 3 cm dishes coated with the E-cadherin fragment, E/EC12, as indicated, and treated with the pan-JAK, JAK inhibitor-1 at the indicated concentrations (lanes 4-7) or not (lanes 1-3) for 48 hours, at which time cell lysates were probed as above, for Stat3-ptyr705, Rac1 or Hsp90.

**Right:** Lysates were immunoprecipitated against total Jak1 and blotted against p-Jak1022/1023, total Jak1, or Hsp90 as a loading control, as indicated.
**Bottom:** HC11 cells were grown to 1 day post-confluence and treated with 50 pg/ml toxin B for 5 hours (lane 2) or the DMSO carrier alone (lane 1) (see Materials and Methods). HC11 (lane 3) and HshRac1 (lane 4) cells were grown to 1 day post confluence. Lysates were immunoprecipitated against total Jak1 and blotted against p-Jak1022/1023, total Jak1, or Hsp90 as a loading control, as indicated.

**B: Stat3 inhibition does not affect Rac1 protein levels.** HC11 cells were grown to different densities as indicated and treated with 100 μM of the S3I-201, Stat3 inhibitor (lanes 2,4,6,8), or the DMSO carrier alone (lanes 1,3,5,7) for 24 hrs, at which time cell lysates were resolved by gel electrophoresis and blots cut into strips which were probed for Stat3-ptyr705, Rac1 or Hsp90.

*bottom*, lane 2, toxin B treatment caused a 5-fold reduction in Jak1 phosphorylation. To further demonstrate the effect of Rac1 upon Jak phosphorylation, we examined Jak1, ptyr1022/1023 levels in HC11 cells expressing the Rac1 shRNA. As shown in Fig. 4.12A, *bottom*, lane 4, Rac1 knockdown reduced Jak1 1022/1023 phosphorylation by ~2-fold. Jak1 *in vitro* kinase assays gave similar results (Vultur et al., 2004). These findings taken together indicate that Rac1 activity is required for the activation of Jak1 observed at high densities.

**Cell to cell adhesion triggers cytokine gene expression**

As shown in Figs. 4.8A and 4.10A, the increase in Stat3-tyr705 phosphorylation occurs ~24 hrs after the surge in Rac1 levels, which points to the possibility of an indirect mechanism. To explore the likelihood that the Stat3 activation by cadherin may be mediated by secreted factors, medium conditioned by HC11 cells grown to high densities was added to sparsely growing HC11 cells for 15 minutes and Stat3-ptyr705 examined. The results revealed a ~20-fold increase in Stat3-ptyr705, indicating the presence of autocrine factors, able to activate Stat3 (Fig. 4.10A, lane 2 vs 1). To examine the nature of the cytokines that may be expressed, we conducted a quantitative RT-PCR array for 86 cytokines, and compared sparsely growing HC11 or Balb/c 3T3 cells to cells grown as dense cultures (see Materials and Methods). The results revealed an increase in mRNA levels of a number of cytokines, including the IL6 family, known to act through the common gp130 subunit, shared by a number of Stat3 activating cytokines, such as
IL6, LIF, CT-1 and IL27 in HC11 [76-fold increase for IL6 mRNA, see Appendix, Table A.2A; (Fischer and Hilfiker-Kleiner, 2007)]. In Balb/c 3T3 cells IL6 and LIF mRNA gave a similar increase in confluent cells (Appendix, Table A.2B). To examine whether these cytokines are required for the Stat3 activation observed in confluent cultures, gp130 levels were reduced through expression of shRNA (see Materials and Methods). As shown in Fig. 4.13B, a 70% gp130 knockdown reduced Stat3-ptyr705 levels in HC11 by ~60%, indicating that gp130 activation is at least partly responsible for the Stat3-ptyr705 increase. Stat3-ptyr705 levels were also reduced in Balb/c 3T3 cells upon gp130 knockdown (not shown).

Results from a number of labs indicated that, besides Stat3, IL6 stimulation results in activation of Erk1/2 (Erk) (Fischer and Hilfiker-Kleiner, 2008). However, as shown in Figs. 4.1B and 4.7B, E-cadherin or cadherin-11 engagement does not activate Erk. To solve this apparent paradox, we examined the ability of IL6, or conditioned medium, to activate Erk in confluent cultures. HC11 cells were grown to 60% or 100% confluence, serum-starved and following IL6 stimulation, cell extracts were probed for Erk or Stat3-ptyr705. As shown in Fig. 4.13C, at a confluence of 60%, IL6 addition caused an increase (~10-fold) in both Erk and Stat3, in agreement with previous results (Fischer and Hilfiker-Kleiner, 2008). As expected, cell density 

Inhibition of E-cadherin engagement at confluence promotes apoptosis

Previous results have shown that Stat3 signalling contributes to the induction of anti-apoptotic genes, such as bcl-xL and mcl-1 (Grandis et al., 2000; Epling-Burnette et al., 2001) while it downregulates the p53 promotor (Niu et al., 2005), thus protecting tumor cells from
Figure 4.13

Cell to cell adhesion triggers cytokine gene expression.

A: Confluence induces autocrine secretion of Stat3-activity factors. Conditioned medium from HC11 cells grown to 100% confluence was added for 15 min to HC11 cells grown to 40% (lane 2) or 100% (lane 4) confluence. At this time detergent cell extracts were resolved by gel electrophoresis and blots were cut into strips which were probed for Stat3-ptyr705, p-Erk1/2 or Hsp90 as indicated. Numbers under the lanes refer to Stat3-ptyr705 or p-Erk1/2 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90 levels, with the highest value taken as 100%.
B: gp130 downregulation reduces the density-mediated Stat3 activation. HC11 cells before (lanes 1-8) or after (lanes 9-16) expression of shRNA for gp130 were grown to different densities and lysates probed for Stat3-ptyr705, gp130 (bottom), Rac1 or Hsp90, as indicated. Numbers under the lanes refer to Stat3-ptyr705 or gp130 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90 levels, with the highest value taken as 100%.

C: IL6 activates Stat3 but not Erk at high densities. IL6 was added at 10 ng/ml for 15 min to HC11 cells grown to 60% (lane 2) or 100% (lane 4) confluence and cell extracts probed for Stat3-ptyr705, p-Erk1/2 or Hsp90 as indicated. Numbers under the lanes refer to Stat3-ptyr705 or p-Erk1/2 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90, with the highest value taken as 100%.

apoptosis. The importance of Stat3 in survival of confluent, HC11 cells was examined by reducing Stat3 activity by shRNA expression or treatment with S3I-201 as above. As a control, the constitutively active mutant, Stat3C, was expressed with a retroviral vector in HC11 cells prior to treatment (McLemore et al., 2001). As shown in Table 4.1, Stat3 downregulation in confluent HC11 epithelial cells using either approach induced apoptosis, in agreement with previous data from mouse NIH3T3 or 10T1/2 fibroblasts (Anagnostopoulou et al., 2006; Vultur et al., 2004), indicating that the increased Stat3 activity may reflect a survival mechanism that is engaged as cells reach confluence. Similar results were obtained with the ES, Ecad+/+ cells, as well as following reduction of Stat3 levels through Rac1 (Fig. 4.11) or Cdc42 (Appendix, Fig. A.10) or gp130 (Fig. 4.13B) downregulation (not shown). At the same time, Stat3C expression rescued HC11 cells from apoptosis induced by Stat3 inhibition, thus reinforcing this conclusion (Table 4.1).

To evaluate the functional consequences of the E-cadherin-mediated, Stat3 activation, we examined the effect of E-cadherin ablation in ES cells. Ecad+/+ and null cells were plated in plastic petri dishes and apoptosis examined by TUNEL and PARP cleavage experiments as well as by FACS analysis, at different densities. As shown in Fig. 4.14, the E-cadherin null cells succumbed to apoptosis (~70%) when confluent (A, panel f, B, bottom, and D, lanes 4-6), while no significant apoptotic death was noted with the Ecad+/+ cells (A, panel b, B, top, and D, lanes
**Figure 4.14**

E-cadherin engagement promotes survival.

**A:** Ecad+/+ (a-d) or null (e-h) cells were grown to densities of two days post-confluence and stained for E-cadherin (a, e), DAPI (c, g) or TUNEL to examine apoptosis (b, f) (see Materials and Methods). Scale bar in overlayed images (d, h) represents 30 μm.

**B:** Ecad+/+ (top) or null (bottom) cells were grown to densities of two days post-confluence, stained with propidium iodide and subject to FACS analysis (see Materials and Methods). Bar indicates cells with a sub-G1 DNA content.

**C:** Inhibition of cadherin engagement with the SHAVSA peptide induces apoptosis, which can be abrogated by Stat3C expression.

b: HC11 cells were grown to a density of two days post confluence, treated with 10 mM of the SHAVSA peptide for 48 hours and apoptosis examined by TUNEL staining. d: Stat3C-expressing, HC11 cells, similarly treated (note the absence of apoptosis). a, c: Same panels as in b and d, phase contrast illumination. Scale bar represents 100 μm.

**D:** Cadherin engagement is required for cellular survival.

**Left:** Ecad+/+ (lanes 1-3) or null (lanes 4-6) cells were grown to different densities and apoptosis examined by PARP cleavage analysis.

**Right:** HC11 (lanes 2 and 3) or HC11-Stat3C cells were treated with the cadherin-inhibitory peptide, SHAVSA (lanes 1 and 3) or not (lane 2) and apoptosis examined by PARP cleavage analysis.

Arrows point to PARP (113 kDa) and cleaved PARP (89 kDa), respectively.

1-3). This observation is in agreement with previous data pointing to a positive role for E-cadherin engagement in cell survival signalling (Liu et al., 2006). To further reinforce this conclusion, we examined the consequences of inhibition of cadherin engagement in HC11 cells using the cadherin-antagonistic, SHAVSA, or the inactive SHGVSVA peptides. Following treatment for 48 hours, confluent cells were fixed and apoptotic death assessed. As shown in Fig. 4.14C, b (and Appendix, Table A.3), concomitant with a Stat3 activity reduction, addition of the cadherin-antagonizing peptide induced apoptosis (~65%), which was more pronounced in confluent cultures. Expression of the constitutively active Stat3C mutant rendered HC11 cells (line HC11-Stat3C, Fig. 4.2C) resistant to apoptosis triggered by inhibition of cadherin
engagement (Fig. 4.14C, c and d, and D, right, and Appendix, Table A.3) indicating that Stat3 activation can in fact restore survival.

In the context of cancer, a more aggressive role has been assigned to cadherin-11, which was shown to be aberrantly expressed in tumor cells of epithelial lineage with a more invasive phenotype. Our preliminary results indicate that genetic knockdown of cadherin-11 through shRNA in Balb/c 3T3 leads to an increase in apoptotic death as observed by TUNEL staining. These data further emphasize the central role of cadherin/Stat3 signalling in promoting cell survival, a mechanism which could be exploited even in the later stages of cancer.
Discussion

Cellular interactions with neighboring cells profoundly influence a variety of signalling events including those involved in mitogenesis, survival and differentiation. Unlike cells cultured in two dimensions, cells in a tumor have extensive opportunities for adhesion to their neighbors in a three-dimensional structure. For this reason, in the study of these cellular processes it is important to take into account the effect of surrounding cells. In the present communication we demonstrate that homophilic interactions by E-cadherin or cadherin-11 can dramatically activate Stat3. Furthermore, Stat3 activation is preceded by a striking increase in the total levels of Rac1 and Cdc42 Rho family GTPases, as well as their activity, and is followed by potent survival signalling. Our finding that downregulation of Rac1 and Cdc42 through expression of dominant-negative mutants, shRNA or pharmacological inhibitors causes a dramatic reduction in Stat3-ptyr705 levels, demonstrates that their activation is part of the pathway(s) whereby cadherin engagement leads to Stat3 activation and survival.

These findings raise important questions: [1] How does cadherin engagement and Rac1/Cdc42 activate Stat3? [2] What is the functional significance of the cadherin-mediated Stat3 activation? These are crucial questions in the context of understanding the molecular mechanisms involving cadherin-mediated adhesion in cellular survival.

Cadherin engagement increases Rac1 and Cdc42 total protein levels and activity

Earlier reports indicated that cell to cell adhesion results in the rapid activation of the Rac1 (Noren et al., 2001; Nakagawa et al., 2001) and Cdc42 (Kim et al., 2000) Rho family GTPases (Fukuyama et al., 2006). Our findings, revealing an increase in Rac1 and Cdc42 activity as cells approach confluence, are in agreement with the above data. Still, the activity of these enzymes at confluences greater than 100%, when the opportunity for cell to cell adhesion is
maximized is unknown. Our results reveal a continuing increase in Rac1-GTP and Cdc42-GTP at higher densities. Unexpectedly, we also found that in addition to this cadherin-induced activation of Rac1, there is a dramatic increase in total Rac1 protein levels with confluence, leading to a further increase in activity. This could be due, at least in part, to inhibition of proteasome-mediated degradation of Rac1 following cadherin engagement (Lynch et al., 2006). A similar mechanism could hold true for Cdc42, which mirrored Rac1 activity and protein levels at all densities examined. Moreover, the absence of a significant increase in Rac1 or Cdc42 mRNA levels with cell density further points to a post-mRNA mechanism.

In transmitting a proliferative signal, cadherins can act directly as receptors propagating an intracellular signal, or they may function primarily to bring cells into contact with each other, to signal via other, juxtacrine receptors. Plating epithelial cells on surfaces coated with the E/EC12 fragment, or fibroblasts on 11/EC12, demonstrated that cadherin engagement alone is sufficient to increase Rac1 and Cdc42 activity and protein levels, pointing to a direct mechanism in the HC11, MDCK and Balb/c 3T3 cell systems. The above findings add to the growing body of evidence indicating that cadherins can provide direct, functionally relevant signalling beyond their structural role.

**Rac1 and Cdc42 transduce the cadherin signal to Stat3**

Data from a number of labs have demonstrated a functional link between the Stat3 and Rho family pathways (Simon et al., 2000; Faruqi et al., 2001; Debidda et al., 2005). Our results showing that both pharmacological and genetic Rac1/Cdc42 inhibitors blocked the cadherin-mediated Stat3 activation, clearly demonstrate that Rac1/Cdc42 are the mediators of the cadherin signal to Stat3. Given the complexity of the immediate effector networks controlled by cadherins (Jaffer and Chernoff, 2004), it is likely that these Rho GTPases may engage multiple effectors, which could indirectly lead to Stat3 activation. Our findings further indicate that both tyr-705
and ser-727 are phosphorylated and JAKs are required for Stat3 activation following cadherin engagement [(Figs. 4.12 and (Vultur et al., 2004)], expanding on the above studies.

Extensive previous studies using both genetic ablation and pharmacological inhibition approaches indicated that the cell confluence-mediated, Stat3 activation is resistant to inhibition of the Src, Fyn, Yes, EGFR, Insulin-like Growth Factor-1 Receptor and Fer kinases, individually (Vultur et al., 2004). Our present data demonstrate that cell density causes the expression of a number of cytokines, most importantly the IL6 family. The role of Rac1/Cdc42 activation in increasing mRNAs for IL6 family cytokines will be discussed in Chapter 5. Although it is certainly possible that other receptors may also be involved, the fact that downregulation of the common subunit, gp130, did reduce Stat3 activity indicates that this family is at least in part responsible for the Stat3 activity increase observed at high cell densities. It is especially noteworthy that IL6 cannot activate Erk in densely growing cultures, which explains the observation that cadherin engagement activates Stat3 exclusively, while Erk remains unaffected in our system. The reasons for the inability of IL6 to activate Erk in confluent cultures is presently under investigation. In any event, these results further reveal that, despite the fact that these two pathways are often both activated by growth factors or oncogenes, they are not coordinately regulated by cadherin engagement.

**Cadherin-mediated Stat3 activation leads to survival signalling**

The role of E-cadherin in cell proliferation and survival is complex. It is thought to be a tumor suppressor molecule largely because it is frequently down-regulated in carcinomas (Gottardi et al., 2001), while in cultured, human colon carcinoma and mammary carcinoma cell lines, E-cadherin plays a negative role in cell proliferation (Perrais et al., 2007). However, there is also evidence that E-cadherin is associated with increased cell proliferation. Ovarian cancers up-regulate E-cadherin, the suppression of which inhibits their proliferation (Sundfeldt, 2003; Reddy et al., 2005). More recently, E-cadherin engagement in cultured normal rat kidney or
mammary epithelial cells was shown to stimulate cell proliferation (Liu et al., 2006). The results presented here indicate that in our cellular systems, E-cadherin engagement promotes cell survival. Indeed, we observed a dramatic induction of apoptosis in ES cells lacking E-cadherin when grown to high densities, or in epithelial cells such as HC11 or MDCK following inhibition of cadherin engagement.

Preliminary data indicate that cadherin-11 inhibition also results in apoptosis of Balb/c 3T3 fibroblasts. Cadherin-11 is aberrantly expressed in cancer cells of epithelial lineage with a more invasive phenotype (Pishvaian et al., 1999; Feltes et al., 2002). It was also recently shown that although cadherin-11 is not expressed in normal human prostate epithelial cells, it is detected in prostate cancer, with its expression increasing from primary to metastatic disease to the bone, a tissue where cadherin-11 is abundantly expressed. In the same report it was shown that cadherin-11 knockdown with shRNA in PC3, prostate carcinoma cells, which are derived from a bone metastasis, decreased their metastatic ability to the bone following intracardiac injection in mice. These findings suggest that cadherin-11 promotes metastasis of prostate cancer cells specifically to the bone, and it has been proposed that cadherin-11 inhibition may inhibit bone metastasis (Chu et al., 2008). Our observations, combined with the fact that cadherin-11 has been shown to promote metastasis, may point to Stat3 as a central survival, rather than metastasis, factor. Most importantly, cadherin-11 inhibition could induce apoptosis (through Stat3 downregulation) in metastatic cells specifically, while normal cells expressing E-cadherin would be spared.

Our findings further demonstrate that this cell survival signal may be mediated by an increase in Rac1 and Cdc42 activity, leading to Stat3 stimulation. Such a schema could offer an explanation for our previous data showing that Stat3 inhibition under conditions of extensive cell to cell adhesion can induce apoptosis, even in normal cells (Anagnostopoulou et al., 2006). Apoptosis could be mediated by p53 downregulation, through direct binding of Stat3 to the p53 promotor (Niu et al., 2005). The above findings illustrate the fact that cadherin function is modulated by the cellular context.
The relevance of cell interactions observed in densely growing cultured epithelial cells to that of normal breast is clearly demonstrated by our findings. We observed high levels of Stat3 activity in the luminal epithelial cells of normal mouse breast duct lobule, where E-cadherin is engaged, thus revealing a distinct association of the signalling of these molecular markers with that of HC11 cells growing at high but not low densities. Furthermore, the relevance of cell interactions observed in densely growing, cultured cells to human cancer is emphasized by recent findings demonstrating a close correspondence of genes expressed specifically in lymph node carcinoma of the prostate (LNCaP) cells cultured to high densities, with genes associated with prostate cancer in vivo (Chen et al., 2006). The fact that these genes were different from genes identified in LNCaP cells grown under log-phase conditions further underscores the importance of the examination of signalling pathways in densely growing cells. The dramatic activation of Stat3 by cell density may also explain previous discrepancies regarding activation of Stat3 by the Rho GTPases in cultured cells (Faruqi et al., 2001; Simon et al., 2000; Debidda et al., 2005). In any event, disruption of cadherin engagement inhibits Stat3 activity and induces apoptosis, which may indicate that the role of Stat3 in densely growing cells may be to counteract apoptotic death. At least two different cadherins can perform this function by activating two distinct Rho family GTPases in an apparently redundant fashion in at least two mammalian species, a finding which could have important therapeutic implications.
References


Chapter 5

Activated Rac1 requires gp130 for Stat3 activation, cell proliferation and migration

These data are included in the following manuscripts:


Author contributions:

RA generated all the cell lines and performed the bulk of the experiments, MG conducted some of the migration assays and growth curve analysis, HF provided insightful advice and was involved in discussing results and revising the manuscript. Special thanks to Dr. Graham Côté and Emilia Furmaniak-Kazmierczak for the Rac1 and Cdc42 vectors and Dr. Harvey Ozer and Douglas Gray for the ts20 cell line.
Abstract

Rac1 (Rac) is a member of the Rho family of small GTPases which controls cell migration by regulating the organisation of actin filaments. Previous results suggested that mutationally activated forms of the Rho GTPases can activate Stat3, but the exact mechanism is a matter of controversy. We recently demonstrated that Stat3 activity of cultured cells increases dramatically following E-cadherin or cadherin-11 engagement in epithelial cells or fibroblasts, respectively. To better understand this pathway, we now compared Stat3 activity levels in mouse HC11, 10T1/2 or Balb/c 3T3 cells before and after expression of the mutationally activated Rac1 (RacV12), at different cell densities. The results revealed for the first time a dramatic increase in protein levels and activity of both the endogenous Rac and RacV12 with cell density, which was due to inhibition of proteasomal degradation. In addition, RacV12-expressing cells had higher Stat3, tyrosine-705 phosphorylation and activity levels at all densities, indicating that RacV12 is able to activate Stat3. Further examination of the mechanism of Stat3 activation showed that RacV12 expression caused a surge in mRNA of IL6 family cytokines, known potent Stat3 activators. Knockdown of gp130, the common subunit of this family reduced Stat3 activity, indicating that these cytokines may be responsible for the Stat3 activation by RacV12. The upregulation of IL6 family cytokines was required for cell migration and proliferation induced by RacV12, as shown by gp130 knockdown experiments, thus demonstrating that the gp130/Stat3 axis represents an essential effector of activated Rac for the regulation of key cellular functions.
Introduction

Rho family GTPases are intracellular molecular switches cycling between the active, GTP-bound state and the inactive, GDP-bound form (Etienne-Manneville and Hall, 2002). Upon activation, the Rho GTPases can activate a distinct panel of effectors to regulate cellular functions. In fact, the Rho family members RhoA, Rac1 and Cdc42 are best known as master regulators of the actin cytoskeleton, promoting the formation of stress fibers, lamellipodia or filopodia, respectively. In addition, Rho GTPases are known growth stimulators that act by modulating key cell cycle regulators, such as cyclin D1 and the transcription factor NFκB, at the transcriptional level (Joyce et al., 1999; Perona et al., 1997).

STATs were discovered as latent cytoplasmic transcription factors that are activated by a number of cytokines and growth factors. Among seven mammalian STAT genes identified, Stat3 is found to be overexpressed in a number of tumor cell lines and carcinomas (Frank, 2007). The fact that a constitutively active form of Stat3 alone is sufficient to transform cultured fibroblasts to anchorage-independence and tumorigenicity points to an etiological role for Stat3 in neoplasia (Bromberg et al., 1999). Like other STAT proteins, Stat3 is latent in the cytoplasm in an unstimulated cell. Following ligand engagement and receptor phosphorylation, Stat3 binds the activated receptor through its Src homology 2 (SH2) domain and is activated through phosphorylation by the receptor itself or by the associated JAKs or Src family kinases. Phosphorylation at a critical tyr-705 activates Stat3 by stabilizing the association of two monomers through reciprocal SH2-phosphotyrosine interactions. The Stat3 dimer then migrates to the nucleus where it binds to target sequences, leading to the transcriptional activation of specific genes, such as myc, bcl-xL, cyclin D, survivin, hgf (Hung and Elliott, 2001) and to the downregulation of the p53 antioncogene (Niu et al., 2005; Yu and Jove, 2004).
Previous results suggested a functional link between the Rho GTPases and Stat3 but the mechanism is still unclear. Indeed, while in one study it was reported that mutationally activated Rac1 can directly interact with Stat3 in co-immunoprecipitation assays (Simon et al., 2000), other data showed that Rac1 indirectly activates Stat3 through autocrine induction of IL6 (Faruqi et al., 2001), while another group reported that the Rho GTPases can activate Stat3 independent from IL6 action (Debidda et al., 2005). We and others recently demonstrated a dramatic increase in the activity of Stat3 triggered by cell to cell contact in a variety of cell lines (Kreis et al., 2007; Onishi et al., 2008; Steinman et al., 2003; Su et al., 2007; Vultur et al., 2004). For this reason, the modulation of Stat3-ptyr705 levels by cell density must be taken into account in experiments assessing the effect of proto-oncogenes such as the Rho GTPases upon Stat3 function.

In this communication, we revisited the question of the mechanism of Stat3 activation by Rac1 and Cdc42, in light of the above findings. The results indicate that cell density alone causes a dramatic increase in protein levels and activity of both the endogenous cRac1 (Rac) and Cdc42, and the mutationally activated Rac1 (RacV12) and Cdc42V12 in mouse epithelial cells and fibroblasts, through inhibition of proteasomal degradation. Furthermore, lines expressing activated RacV12 had higher Stat3 activity levels at all cell densities examined, indicating that RacV12 is, in fact, able to activate Stat3. The Stat3 increase was mediated through the expression of IL6 family cytokines, as shown through knockdown of gp130, common subunit of the family. Gp130 function and Stat3 activation were required for cell migration and increase in proliferation induced by mutationally activated Rac and Cdc42, as shown by genetic knockdown experiments, thus demonstrating that the gp130/Stat3 axis represents an essential effector of activated Rac in the regulation of both of these essential cellular functions.
Materials and Methods

Cell lines, culture techniques and gene expression

The normal mouse mammary epithelial line HC11 is a prolactin-responsive cell clone originally isolated from the COMMA-1D mouse mammary epithelial cell line derived from a female Balb/c mouse in mid-gestation (Ball et al., 1988). Normal mouse Balb/c 3T3 and mouse 10T1/2 fibroblasts have been previously described (Vultur et al., 2004). The ts20, Balb/c3T3 A31-derived line was grown as described (Chowdary et al., 1994). Cell confluence was estimated visually and quantitated by imaging analysis of live cells under phase contrast using a Leitz Diaplan microscope and the MCID-elite software (Imaging Research, St. Catharine’s Ont.).

For NFκB inhibition, cells were treated with 20 µM IkB kinase (IKK)-inhibitorIII (BMS-345541) or 20 µg/ml caffeic acid phenethyl ester (CAPE, EMD Biosciences). JAK-inhibitor-1 (EMD Biosciences) or MG132 (Sigma) were added at the indicated concentrations. Treatment with the JAK-inhibitor-1 was for 24 hrs and with MG132 for 8 hrs. The CPA7, platinum Stat3 inhibitor was prepared as described (Littlefield et al., 2008) and used at a 50µM concentration. Cell viability was assessed by trypan blue exclusion and by replating cells in medium lacking the inhibitors. IL6 was purchased from Invitrogen.

For gp130 knockdown, a mouse pSM2 retroviral target gene shRNA set (Cat#. RMM4530-NM_010560, Appendix, Table A.4) was purchased from Open Biosystems. V2MM-70734 was the most efficient. Infected cells were selected for puromycin resistance. RacV12 was expressed with a retroviral vector (a gift of Dr John Collard (Sander et al., 1998)). RacL61, Cdc42V12 and wtRac1 were expressed through plasmid transfection under control of the cytomegalovirus (CMV) promotor (plasmids were a gift of Dr. Graham Côté, Queen’s University). Transfections for wtRac1 and RacL61 expression were performed with Lipofectamine Plus (Invitrogen). To effectively compare the consequences of wtRac1 vs RacL61 expression upon
Stat3 activity, HC11 cells were transfected with each of their respective plasmids and the next day plated at 3x10^6 cells/3 cm petri, equivalent to 2 days post confluence, as before (Arulanandam et al., 2009). 24 hrs later, cell extracts were prepared and Western blots probed with the indicated antibodies.

For neutralisation of IL6 the #ab6672 antibody (Abcam) was used, while for neutralisation of LIF we used the antibody #L9152 (Sigma), at concentrations of 0.425 μg/ml and 1 ng/ml, respectively, according to the manufacturers’ protocols.

**Western blotting and immunoprecipitation**

Detergent cell extracts were prepared as described (Vultur et al., 2004). Following a careful protein determination (BCA-1 Protein assay kit, Sigma), 30 or 100 μg of clarified cell extract were submitted to SDS-PAGE, as indicated. Blots were cut into strips and probed with antibodies specific for the tyr-705 phosphorylated Stat3 (Cell Signalling), total Stat3 (Cell Signalling), the dually phosphorylated form of Erk1/2 (Biosource), survivin (Cell Signalling), p21 (Biosource), gp130 (Sigma) or Hsp90 (Stressgen) followed by alkaline phosphatase, or HRP-conjugated secondary antibodies (Jackson Labs). Rac1 and Cdc42 antibodies (BD Transduction Labs) recognised both the endogenous and mutant Rac or Cdc42, respectively. To examine the degree of Rac ubiquitination, extracts were immunoprecipitated with anti-ubiquitin antibodies (Biomol) and blotted against Rac1 or myc-tag (for Rac^{L61}, antibody 9E10, Sigma). In all cases, bands were visualized using enhanced chemiluminescence (PerkinElmer Life Sciences), or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Quantitation was achieved by fluorimager analysis using the FluorChem program (AlphaInnotech Corp). In all cases Stat3-ptyr705 band intensities were normalized to Hsp90 levels of the same samples. Jak1 phosphorylation was examined by immunoprecipitation against total Jak1, followed by blotting with a Jak-ptyr1022/1023 antibody (Cell Signalling).
Rac/Cdc42 activation assays were performed using GST-PAK-PBD pull-down assays with the Rac/Cdc42 activation assay kit (Cytoskeleton, #BK035). Briefly, the beads were coated with GST fused to the binding domain of PAK (PAK-PBD) in pulldown assays. Adding twice the amount of PBD-coated beads did not increase the signal, indicating that the amount of binding partner used in the detection was not limiting.

Photoshop (Adobe) or Corel Draw software were used for the organization of non adjusted, original images and blots.

qRT-PCR assays

For quantitative RT-PCR, the delta ct (Δct) value was calculated from the given ct value by the formula: Δct = (ct\text{sample} – ct\text{control}). For the qRT-PCR cytokine array, we used the PAMM-021A kit (SA Biosciences) with an RT-PCR for IL6 run in parallel, according to the manufacturer’s protocol.


Results

Cell density inhibits cRac1 proteasomal degradation

We have demonstrated that cell density causes a dramatic increase in the cellular Rac1 (Rac) and Cdc42 protein levels in HC11 normal mouse breast epithelial cells, or Balb/c 3T3 and 10T1/2 normal mouse fibroblasts, through homophilic engagement of E-cadherin or cadherin-11, respectively (Arulanandam et al., 2009; Arulanandam et al., in preparation). To explore the possibility that the increase in Rac/Cdc42 with cell density observed in HC11 cells (Figs. 5.1A and B, lanes 1-7) might be due to inhibition of proteasome-mediated degradation, we at first made use of the MG132 proteasome inhibitor (Tsubuki et al., 1996). As shown in Fig. 5.2A, MG132 treatment of sparsely growing HC11 cells caused a substantial increase in Rac protein levels, as well as the p21CIP/WAF, p53 substrate serving as a positive control (Zhu et al., 2007). This observation points to a role for the proteasome in Rac degradation. At the same time, the levels of phosphorylated, i.e. activated Erk1/2 remained unchanged following MG132 treatment, suggesting that Rac may be selectively degraded by the proteasome. As shown in Fig. 4.10 and Appendix, Fig. A.10, cell density caused a similar increase in Rac/Cdc42 protein levels in Balb/c 3T3 and 10T1/2 cells, while MG132 treatment of sparsely growing Balb/c 3T3 resulted in high Rac levels compared to untreated cells (Fig. 5.3A).

To further demonstrate the involvement of ubiquitination, we took advantage of the ts20, Balb/c 3T3 A31-derived line. Due to a mutation in the gene for the ubiquitin activating enzyme E1, which makes it susceptible to accelerated destruction, this line is defective in protein ubiquitination at high temperatures (39°C), while ubiquitination is normal at 34°C (Chowdary et al., 1994). To examine the effect of E1 inactivation upon Rac levels, ts20 cells were grown to different densities at 34°C or 39°C, along with the parental Balb/c 3T3 A31, and Rac levels examined. The results revealed that in cells grown to low densities (0.5x10^5 cells/3 cm petri)
Figure 5.1

**Rac\textsuperscript{V12} and Cdc42\textsuperscript{V12} activate Stat3 in HC11 cells at all densities**

**A:** Both endogenous and mutant Rac\textsuperscript{V12} protein levels and activity are dramatically increased by cell density, while Rac\textsuperscript{V12} expression increases Stat3-ptyr705 levels at all cell densities

HC11 cells (lanes 1-7) or their counterparts stably expressing activated Rac\textsuperscript{V12} (lanes 8-14) were grown to increasing densities, up to 11 days post-confluence. Detergent cell lysates were probed for total Rac, active Rac-GTP, Stat3-ptyr705, total Stat3 or Hsp90 as a loading control, as indicated (see Materials and Methods). Numbers at the left refer to molecular weight markers.

**B:** Both endogenous and mutant Cdc42\textsuperscript{V12} protein levels and activity are dramatically increased by cell density, while Cdc42\textsuperscript{V12} expression increases Stat3-ptyr705 levels at all cell densities

HC11 cells (lanes 1-7) or their counterparts stably expressing activated Cdc42\textsuperscript{V12} (lanes 8-14) were grown to increasing densities, up to 11 days post-confluence. Detergent cell lysates...
were probed for total Cdc42, active Cdc42-GTP, Stat3-ptyr705, total Stat3 or Hsp90 as a loading control, as indicated.

C: RacV12 and Cdc42V12-expression increases Stat3 transcriptional activity
HC11 and RacV12 (top panel)- and Cdc42V12 (bottom panel)-expressing cells were transfected with the Stat3-dependent pLucTKS3 and the Stat3-independent pRLSRE reporters and grown to the indicated densities. Firefly (■) or Renilla (□) luciferase activities were determined in detergent cell extracts (Vultur et al., 2004). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.

D: RacL61 is more effective than wtRac1 at increasing Stat3-705 phosphorylation.
HC11 cells (lane 1), or their counterparts transfected with wtRac1 (lane 2) or RacL61 (lane 3) were plated at 3 x 10⁶ cells/3cm plate (see Materials and Methods) and detergent cell lysates probed for total Rac, active Rac-GTP, Stat3-ptyr705, total Stat3 or Hsp90 as a loading control, as indicated. Numbers at the left refer to molecular weight markers.

under permissive conditions, that is in ts20 cells grown at 34°C (Fig. 5.3B, lane 1) or in Balb/c 3T3 cells at either temperature (lanes 7 and 9), levels of Rac were low. In contrast, ts20 cells grown to the same low densities at 39°C had substantially higher Rac levels (Fig. 5.3B, lanes 1 vs 4), pointing to the possibility of a ubiquitination-inhibition effect. The requirement for a continuous inhibition of ubiquitination for the maintenance of high Rac levels was examined next (Fig. 5.3B, lanes 11-14). ts20 cells were grown at the permissive temperature, to 100% confluence at first, at which time Rac levels were high, as expected (lane 13). Trypsinizing and replating them at a low density caused a dramatic decrease in Rac (lane 14). However, replating ts20 cells at 39°C did not cause a decrease in Rac levels, possibly due to low ubiquitination activity because of the destruction of the temperature-sensitive, E1 ligase at this temperature (lane 12). This treatment did not affect Rac levels in the parental Balb/c 3T3 cells (not shown). The above data taken together demonstrate that inhibition of ubiquitin ligase E1 may play an important role in the dramatic increase in Rac levels observed at high cell densities.

We next examined the effect of ubiquitin overexpression upon Rac protein levels, by transfecting HC11 cells with a plasmid consisting of a CMV promotor driving the transcription of an mRNA encoding a multimeric precursor molecule composed of eight, N-terminal hexa
Figure 5.2

**Cell density inhibits the proteasomal degradation of Rac in HC11 cells**

**A: The proteasome inhibitor, MG132 increases total Rac and Stat3-ptyr705 levels.**

Sparsely growing HC11 cells were treated with 10μM of the proteasome inhibitor, MG132 (lane 2), or buffer alone (lane 1) for 8 hrs. Detergent cell extracts were probed for total Rac, Stat3-ptyr705, total Stat3, p21^CIP/WAF, pErk1/2 or Hsp90 as a loading control, as indicated.

**B: Ubiquitin overexpression leads to Rac degradation.**

HC11 cells were transfected with a plasmid consisting of a CMV promotor driving the transcription of an mRNA encoding eight ubiquitin units with a his6 tag (His-Ubq, lanes 1 and 3) and grown to 30% (lanes 1 and 2) or 100% (lanes 3 and 4) confluence. Extracts were probed for Rac, Stat3-ptyr705, total Stat3, pErk1/2 or Hsp90, as indicated. Numbers under the lanes refer to total Rac or Stat3-ptyr705 band intensities obtained through quantitation by fluorimager analysis.
and normalized to Hsp90 levels, with the peak value of the control, HC11 cells grown to confluence (lane 4) taken as 100% (see Materials and Methods).

C-E: Rac is ubiquitinated in vivo, at low cell densities. HC11 or H-RacV12 cells were grown to 30% or 100% confluence and anti-ubiquitin immunoprecipitates of detergent cell extracts (lanes 5-9) blotted against Rac1. As a control, extracts from cells grown to 30% confluence were immunoprecipitated with normal rabbit serum (lanes 3-4) or buffer alone (lanes 1-2). Bracket points to the ubiquitinated Rac.

D: Extracts from HC11 or H-RacV12 cells grown to 30% or 100% confluence were blotted against Rac1.

E: HC11 cells before (lane 1) or after (lanes 2 and 3) expression of a myc-tagged, L61 mutant were grown to 30% or 100% confluence and anti-ubiquitin immunoprecipitates of detergent cell extracts blotted against the myc-tag. H.C., L.C.: IgG heavy and light chains, respectively. Bracket points to the ubiquitinated Rac.

-histidine tagged ubiquitin units (Treier et al., 1994). The results revealed that ubiquitin overexpression caused a reduction in Rac total protein levels which was more pronounced at lower cell densities (Fig. 5.2B).

To further investigate whether Rac itself might be a substrate of the proteasome in sparsely growing cells, we examined whether Rac is modified by ubiquitin tagging. To this effect, we searched for the presence of Rac in the pool of ubiquitinated proteins, by probing anti-ubiquitin immunoprecipitates from HC11 cells for Rac by Western blotting. As shown in Fig. 5.2C (left panel), a diffuse band of ubiquitin-tagged Rac, consistent with short chain polyubiquitination (Lynch et al., 2006), was detected in immunoprecipitates from HC11 cells (Fig. 5.2, lane 5) grown to 30% confluence (lane 5). This band was very weak at 100% confluence (Fig, 5.2C, lane 6), possibly due to inhibition of ubiquitination at high cell densities. When the anti-ubiquitin antibody was omitted (Fig. 5.2C, lane 1) or replaced with normal mouse IgG (Fig, 5.2C, lane 3) or an unrelated, control mouse monoclonal antibody (not shown), no Rac was found in the immunoprecipitates from 30% confluent cultures. Similar results were obtained in Balb/c 3T3 (Fig. 5.3C) and 10T1/2 cells (not shown). The above data taken together indicate that Rac itself is, in fact, a substrate of the proteasome in sparsely growing cultures, and that cell confluence inhibits Rac ubiquitination and proteasomal degradation.
Figure 5.3

Cell density inhibits the proteasomal degradation of Rac in Balb/c 3T3 fibroblasts

A: The proteasome inhibitor, MG132 increases total Rac and Stat3-ptyr705 levels.  
A: Sparsely growing Balb/c 3T3 cells were treated with 10μM of the proteasome inhibitor, MG132 (lane 2), or not (lane 1) for 8 hrs. Detergent cell extracts were probed for Stat3-ptyr705, total Rac, or α-tubulin as a loading control, as indicated.

B: Inhibition of the E1 ubiquitin activating enzyme increases Rac protein levels.  
Left panel: ts20 (lanes 1-6) or Balb/c 3T3 (lanes 7-10) cells were grown to different densities, at 34°C (lanes 1-3 and 7-8) or 39°C (lanes 4-6 and 9-10), as indicated. Detergent cell extracts were
probed for Stat3-ptyr705, Rac or Hsp90 as a loading control. Note the high levels of Stat3-ptyr705 at a low confluence, at 39°C (lane 4 vs lane 1).

**Right panel:** ts20 cells were grown to a confluence of 100% at 39°C (lane 11) or 34°C (lane 13). Cells were subsequently trypsinized and replated at a low confluence at 39°C (lane 12) or 34°C (lane 14). Detergent cell extracts were probed for Stat3-ptyr705, Rac or Hsp90 as a loading control.

Note the dramatic reduction of both Stat3-ptyr705 and Rac levels upon replating sparsely at 34°C (lane 14), which was abrogated when the E1 enzyme was inhibited by replating at 39°C (lane 12).

C: Rac1 is ubiquitinated in vivo. Balb/c 3T3 cells were grown to 30% or 100% confluence and anti-ubiquitin immunoprecipitates of detergent cell extracts (lanes 3 and 4) blotted against Rac (bracket). Lanes 5 and 6: As a control, extracts were immunoprecipitated with normal rabbit serum and probed for Rac in a similar manner. H.C.: Heavy chain.

**Cell density upregulates activated RacV12 levels through inhibition of proteasomal degradation**

It was previously shown that the active, GTP-bound form of Rac is preferentially degraded by the proteasome (Pop et al., 2004). Therefore, to examine the impact of cell density upon the levels of mutational activation of RacV12 or RacL61, the latter labeled with a myc-tag, these genes were stably expressed in HC11 cells (termed H-RacV12 or H-RacL61, see Materials and Methods). A number of colonies were picked and, since cell density affects Rac protein levels, screening for total Rac or myc-Rac by Western blotting was performed at 40% confluence. Cells were plated in 3 cm dishes and when 30% confluent and at different times thereafter, Rac protein levels were evaluated by Western blotting. As shown in Fig. 5.1A (lanes 8-14), the levels of RacV12 increased dramatically with cell density. Interestingly, H-RacV12 cells had ~3x higher total Rac levels than the parental HC11, at all densities examined. Two other RacV12 and three RacL61-expressing clones gave the same results (not shown). Similar results were found in 10T1/2 lines stably expressing RacV12 (see Appendix, Fig. A.11, lanes 7-12). The above data, taken together, indicate that, similar to cRac1, activated RacV12 and RacL61 are also subject to density-dependent upregulation.
The effect of cell density upon RacV12 activity was examined next. Cells were plated in Petri dishes and when ~30% confluent, and over several days thereafter, Rac activity was measured by assessing the binding between Rac-GTP and its effector p21-activated kinase (PAK) in cell extracts using pull-down assays (see Materials and Methods). As shown in Fig. 5.1, there was a dramatic increase in Rac activity with cell density, in both the parental HC11 (Fig. 5.1, lanes 1-7), and in RacV12-expressing cells (Fig. 5.1, lanes 8-14), at all densities examined, in parallel with Rac protein levels. 10T-RacV12 (Appendix, Fig. A.11) and clones expressing the mutationally activated, Cdc42V12 gave similar results (Fig. 5.1B and Appendix, Fig. A.11). The above findings taken together indicate that cell density, besides an increase in total Rac/Cdc42 protein levels, it also causes a dramatic increase in both Rac/Cdc42 and RacV12/Cdc42V12 activity. It follows that, cell density has to be taken into account when the effect of RacV12 upon Stat3 is examined.

To better control the degree of cell-cell contact, irrespective of differences in cell growth patterns, we repeated the experiments by plating different numbers of HC11 and or H-RacV12 cells (0.4x10^6, 0.7x10^6, 1.0x10^6, 1.3x10^6, 1.8x10^6 or 2.5x10^6 per 3cm petri, respectively), so that they would reach the same densities as above within 24 hours (Vultur et al., 2004). At that time, cells were lysed and Rac protein levels and activity analyzed as above. In all cases, very similar results were obtained, indicating that it is the extent of cell-cell contact, regardless of time in culture beyond 24 hrs that is responsible for the increase in Rac protein and activity (Appendix, Fig. A.7 and not shown).

The potential role of ubiquitination upon the levels of the mutationally activated, RacV12 was examined next. As for Rac in HC11 cells (Fig 5.2C, lanes 5 and 6), anti-ubiquitin immunoprecipitates of H-RacV12 cells grown to different densities were probed with an anti-Rac1 antibody. As shown in Fig. 5.2C (lane 8), a diffuse band of ubiquitin-tagged Rac (Lynch et al., 2006), was detected in immunoprecipitates from cells grown to 30% confluence. Cells grown to 100% confluence on the other hand, had very low levels of ubiquitin-tagged Rac (lane 9),
consistent with inhibition of ubiquitination at high cell densities. When the anti-ubiquitin antibody was omitted (lane 2) or replaced with normal mouse IgG (lane 4), no RacV12 was detected in the immunoprecipitates. At the same time, total Rac protein levels were much higher at high densities, both in the HC11 and H-RacV12 cell lines (Fig. 5.2D). Taken together, these data strongly suggest that RacV12 itself is also a substrate of the proteasome, in sparsely growing cells exclusively. To ensure that it is the mutationally activated Rac which is ubiquitinated, we made use of the myc-tagged, RacL61. As shown in Fig. 5.2E, blotting anti-ubiquitin immunoprecipitates from H-RacL61 cells, expressing a myc-tagged RacL61, grown to 30% confluence, with an anti-myc-tag antibody, yielded a strong band (lane 2), while at 100% confluence no myc-tagged band was detected (lane 3). Similar results were obtained with 10T½ and 10T-RacV12 (not shown). The above data taken together indicate that cell density can cause a dramatic increase in both Rac and RacV12 protein levels, through inhibition of proteasomal degradation.

**Mutationally activated RacV12 and Cdc42V12 can activate Stat3**

Previous results demonstrated that RacV12 expression leads to the activation of Stat3. However, the effect of cell density was not taken into account in any previous report (Debidda et al., 2005; Faruqi et al., 2001; Simon et al., 2000), and this could be a source of apparently conflicting results. To definitively demonstrate the effect of RacV12 upon Stat3 activity, H-RacV12 cells were plated in 3 cm dishes and at different times thereafter Stat3-ptyr705 levels measured by western blotting and compared to the parental HC11 cells, respectively. As shown in Fig. 5.1A (lanes 8-14), H-RacV12 cells had higher Stat3-ptyr705 levels at all cell densities examined. Similar results were obtained with H-RacL61 and 10T- RacV12 (Appendix, Fig. A.11), while overexpression of the cellular Rac1 in HC11 cells (wtRac1, Fig. 5.1D) had a substantially reduced effect upon Stat3-ptyr705 levels, compared to RacV12 or RacL61. Cdc42V12 mirrored the effect of RacV12 regarding Stat3 activation when expressed in both HC11 and 10T½ cell lines.
Examination of the levels of total Stat3 protein revealed a modest increase with cell density, as previously reported (Vultur et al., 2004), and with Rac\textsuperscript{V12}, Rac\textsuperscript{L61} or Cdc42\textsuperscript{V12} expression compared to parental HC11 (Fig. 5.1, A, B, D) or 10T½ lines (not shown), possibly due to the fact that the Stat3 promotor itself is one of the Stat3 targets (Narimatsu et al., 2001), although the differences were not as pronounced as the Stat3-tyr705 phosphorylation observed. Stat3 transcriptional activity also increased upon Rac\textsuperscript{V12} or Cdc42\textsuperscript{V12} expression (Fig. 5.1C), following the pattern of Stat3-tyr705 phosphorylation.

The effect of Rac ubiquitination upon Stat3-ptyr705 levels was examined next. As shown in Fig. 5.2A and Fig. 5.3A, treatment with MG132, which increased Rac levels, also increased the levels of Stat3-ptyr705, while expression of his-ubiquitin, which reduced Rac levels, had a proportional effect upon Stat3-ptyr705 (Fig. 5.2B). Stat3-ptyr705 levels were also increased in subconfluent ts20 cells at the higher, nonpermissive temperature (Fig. 5.3B, lanes 1 vs 4 or lane 14 vs 12).

The above results taken together indicate that mutationally activated forms of both Rac1 and Cdc42 Rho family GTPases are indeed able to activate Stat3 in epithelial cells and fibroblasts, above and beyond the activation due to cell density.

**NFκB and JAKs are required for the Rac\textsuperscript{V12}-mediated, Stat3 activation**

Early data showed that Rac1 can activate NFκB (Sulciner et al., 1996). To examine whether NFκB may be actually required for the Rac\textsuperscript{V12}-mediated Stat3 activation, sparsely growing HC11 cells were trypsinized and plated to a high density (2.5x10\textsuperscript{6} cells/3 cm petri). Following attachment, cells were treated with the IKK-inhibitor-III (BMS-345541) or the DMSO carrier alone, for 48 hours. As shown in Fig. 5.4A, treatment with the inhibitor caused a dramatic reduction in Stat3-ptyr705 levels, in both the parental HC11 (lanes 3 vs 2), or H-Rac\textsuperscript{V12} (lanes 5 vs 4). These data indicate that both the cell density-mediated, and Rac\textsuperscript{V12}-mediated increase in Stat3-tyr705 phosphorylation requires NFκB. On the other hand, Rac levels were unaffected by
Figure 5.4

Rac\textsuperscript{V12}-dependent Stat3-ptyr705 phosphorylation requires JAK and NF\textsubscript{κ}B

**A:** The IKK inhibitor III inhibits the density-mediated, Stat3, tyr705 phosphorylation in both HC11 and H-Rac\textsuperscript{V12} cells. HC11 (lanes 1-3) or Rac\textsuperscript{V12} (lanes 4 and 5) cells were trypsinised and plated at a high density (2.5\times10^6 cells/3 cm plate). Following attachment 30 min later, cells were treated with the IKK inhibitor III (lanes 3 and 5), or the DMSO carrier (lanes 2 and 4) for 48 hrs. Cell extracts were probed for Stat3-ptyr705, total Stat3, Rac or Hsp90, as a loading control. Lane 1: HC11 cells immediately after attachment to the plastic.

**B:** Rac\textsuperscript{V12}-expression increases Jak1-1022/1023 phosphorylation.

Lysates from HC11 (lanes 1 and 3) or H-Rac\textsuperscript{V12} (lanes 2 and 4) cells were grown to 30% confluence (lanes 1 and 2), or one day after confluence (lanes 3 and 4) and probed for p-Jak1022/1023, total Jak1 or Hsp90 as a loading control, as indicated.

**C:** JAK inhibitor 1 reduces the Rac\textsuperscript{V12}-dependent, Stat3-tyr705 phosphorylation.

HC11 (lanes 1-3) or Rac\textsuperscript{V12} (lanes 4-6) cells were grown to a high density and treated with 0 (lanes 1 and 4), 10 (lanes 2 and 5) or 2 (lanes 3 and 6) µg/ml JAK inhibitor 1. Cell lysates were probed for Stat3-ptyr705, total Stat3, Rac or Hsp90 as a loading control, as indicated.
NFκB inhibition (Fig. 5.4A), which further reinforces the conclusion that NFκB is downstream of Rac, ie it is required for the Rac-mediated, Stat3 activation exclusively. Similar results were obtained with caffeic acid phenethyl ester (CAPE), another known inhibitor of NFκB activity (not shown) and in parental Balb/c 3T3 (Appendix, Fig. A.12A).

To explore the role of Jak1 in the RacV12-mediated, Stat3 activation, we at first investigated whether Jak1 is activated by RacV12, by examining Jak1 phosphorylation at tyr1022/1023, shown to be important in the regulation of Jak1 activity (Gauzzi et al., 1996; Leonard and O'Shea, 1998). Jak1 phosphorylation at 1022/1023 was measured by blotting lysates from H-RacV12 and HC11 cells with a phospho-specific antibody (see Materials and Methods). As shown in Fig. 5.4B, there was a ~2 fold increase in Jak1, 1022/1023 phosphorylation upon RacV12 expression at one day after 100% confluence (lanes 3 vs 4), which paralleled the phosphorylation of Stat3 at this density (Fig. 5.4A, lanes 2 vs 4), while at 30% confluence the difference was more pronounced (Fig. 5.4B, lane 1 vs 2). To examine whether Jak1 is actually required for the RacV12-mediated increase in Stat3 activity, HC11 cells grown to densities of 1 day post confluence were treated with the pan-JAK inhibitor, JAK inhibitor 1. The results showed a dramatic reduction in Stat3-ptyr705 levels, which essentially plateaued at 2μM of inhibitor, consistent with previous findings (Arulanandam et al., 2009), while Rac levels remained unaffected (Fig. 5.4C, lanes 1-6). Similar results were obtained with the AG490 JAK inhibitor and in parental Balb/c 3T3 cells [(Zhang et al., 2000), and Appendix, Fig. A.12B]. These findings suggest that the JAK kinases are required for the RacV12-mediated increase in Stat3-ptyr705 levels.

RacV12 triggers cytokine gene expression and requires gp130 for Stat3 activation

To explore the possibility that the RacV12-induced, Stat3 activation may be mediated by autocrine factors, medium conditioned by sparsely growing, H-RacV12 cells was added to the parental HC11 cells growing to a low density and Stat3-ptyr705 examined. The results revealed a
3-fold increase in Stat3-ptyr705, indicating the presence of autocrine factors (not shown). To explore the nature of the cytokines responsible, we conducted a quantitative RT-PCR array experiment for 86 cytokines, comparing mRNA levels in H-Rac^{V12} cells with levels in the parental HC11 line (see Materials and Methods). Given the effect of density upon Stat3 levels, to examine the effect of Rac^{V12} per se, we compared cells expressing Rac^{V12} with the parental HC11, while both were grown to densities of 40%. The results revealed an increase in mRNA levels of two cytokines of the IL6 family, IL6 (18-fold) and LIF (~10-fold), known to act through the common gp130 subunit, shared by a number of Stat3 activating cytokines [(Hilfiker-Kleiner et al., 2004), Appendix, Table A.5]. In fact, addition of neutralizing antibodies against two members of the family, IL6 and LIF, separately reduced Stat3-ptyr705 levels by approximately 30%, while a combination of the two caused a reduction of ~50% (Fig. 5.5A, top panel). To further demonstrate the requirement for IL6 family cytokines for the Rac^{V12}-mediated, Stat3 activation, the levels of gp130 were reduced through stable expression of a specific shRNA, using a retroviral vector (see Materials and Methods). As shown in Fig. 5.5A, gp130 knockdown reduced Stat3-ptyr705 levels in H-Rac^{V12} cells (line H-Rac^{V12}-shgp130) at all densities examined (lanes 1-3 vs 4-6), pointing to the possibility that the two IL6 family cytokines play an important role in the activation of Stat3 by Rac^{V12}. Stat3-tyr705 phosphorylation was also reduced in 10T-Rac^{V12} cells following shgp130 expression (not shown).

**Rac^{V12}-induced IL6 upregulation is unable to activate Erk1/2 in confluent cultures**

Results from a number of labs indicated that, in addition to Stat3, IL6 is able to activate the Erk1/2 kinase [Erk (Fischer and Hilfiker-Kleiner, 2008)]. Since Rac^{V12} expression leads to IL6 upregulation, we investigated whether Rac^{V12} can activate Erk. H-Rac^{V12} and HC11 cells were grown to 30% confluence, serum-starved for 24 hrs and levels of the dually phosphorylated, i.e. activated Erk1/2 (pErk) examined by Western immunoblotting. As shown in Fig. 5.5B, pErk levels were higher in H-Rac^{V12} cells when grown to 30% confluence than the parental HC11 (lane
Figure 5.5

gp130 is required for the RacV12-mediated, Stat3 activation

A: Top: Reduction in Stat3-ptyr705 levels by neutralising antibodies to IL6 and LIF. H-RacV12 cells were grown to 30% confluence (lane 1) or one day post confluence and an antibody to IL6 (lane 3) or to LIF (lane 4), or a combination of the two (lane 6) added to the growth medium for 6 hrs. Detergent cell extracts were blotted for Stat3-ptyr705, total Stat3 or Hsp90 as a loading control. Lanes 2 and 5: Control lanes, cells treated with buffer alone.

Bottom panel: Lysates from H-RacV12 cells before (lanes 1-3) or after (lanes 4-6) expression of a gp130-specific, shRNA were probed for Stat3-ptyr705, total Stat3 or Hsp90 as a loading control, as indicated.

B

HC11 H-RacV12 H-C11 H-RacV12 H-C11-shgp130 H-RacV12-shgp130 H-C11-shgp130 H-RacV12-shgp130

30 100+1day 30 100+1day

p44 p42 pErk1/2 Hsp90

35

Hsp90

p44 p42 pErk1/2

IL6 + - + -

60 100

1 2 3 4 5 6 7 8
Right panel: Lysates from H-Rac\textsuperscript{V12} cells, before (lane 1) or after (lane 2) knockdown of gp130 were probed for gp130 or Hsp90 as a loading control, as indicated.

**B: Rac\textsuperscript{V12} does not activate Erk at high cell densities.**

Left panel: Extracts from the indicated cell lines, before (lanes 1-4) or after (lanes 5-8) expression of the gp130-specific, shRNA were probed for pErk1/2 or Hsp90 as a loading control, as indicated.

Right panel: IL6 was added at 10 ng/ml for 15 min to HC11 cells grown to 60\% (lane 2) or 100\% (lane 4) confluence and cell extracts probed for Erk1/2 or Hsp90 as a loading control, as indicated.

At high density however, there was no Erk activity increase upon Rac\textsuperscript{V12} expression (lanes 4 vs 3), although Stat3 phosphorylation was increased (Fig. 5.1A). To investigate whether the Erk activation by Rac\textsuperscript{V12} might be due to the IL6 family cytokines produced, pErk levels were examined in the gp130 knockdown cells. As shown in Fig. 5.5B (lanes 5-8), shgp130-expressing cells had low pErk levels, even after Rac\textsuperscript{V12} expression (lane 6 vs 2), indicating that IL6 activity is required for pErk upregulation by Rac\textsuperscript{V12}. Similar results were obtained in 10T\frac{1}{2} and 10T-Rac\textsuperscript{V12}, with or without shgp130. To further verify the effect of IL6 upon Erk, we directly examined the role of density upon the activation of Erk by purified IL6. HC11 cells were grown to densities of 60\% or 2 days after confluence, stimulated with IL6 for 15 min and Erk activity examined by probing for pErk (Fig. 5.5B, right panel). The results demonstrated that although IL6 could activate Erk in subconfluent cultures (lane 2 vs 1), as previously reported (Fischer and Hilfiker-Kleiner, 2008), there was no Erk activity increase upon IL6 addition in cells grown to high confluence (lane 4). The above findings clearly indicate that, although Rac\textsuperscript{V12} induces the expression of IL6, a known Erk activator, IL6 is unable to activate Erk in cells grown to high densities. This observation explains earlier data indicating that pErk levels are unaffected by confluence in a number of cellular systems (Vultur et al., 2004), and reveals a profound effect of cell to cell adhesion upon the response of HC11 cells to IL6.
**Rac\(^{V12}\)-induced cell proliferation and migration require IL6 family cytokines**

The Rho family GTPases are known to play a role in the control of cell proliferation (Guo and Zheng, 2004). To examine the effect of IL6 family cytokines upon the Rac\(^{V12}\)-induced cell growth, HC11, H-shgp130, H-Rac\(^{V12}\) and H-Rac\(^{V12}\)-shgp130 cells were plated in 3 cm dishes and their growth rate determined. As shown in Fig. 5.6A, shgp130 expression caused a ~2.2 fold reduction in the growth rate of both H-Rac\(^{V12}\) cells and the parental HC11, indicating that gp130 is an essential component of a pathway leading to Rac-induced cell proliferation. Similar results were observed when comparing 10T1/2, 10T-shgp130, 10T-Rac\(^{V12}\) and 10T-Rac\(^{V12}\)-shgp130 cell lines (not shown).

It is well established that increased Rac activity can increase cell motility through the formation of lamellipodia at the leading edge of cells in a wound healing assay (Nobes and Hall, 1999). To examine the role of the gp130 subunit in the Rac-induced cell migration, HC11, H-Rac\(^{V12}\), H-shgp130 and H-Rac\(^{V12}\)-shgp130 cells were plated in plastic petri dishes. At two days post-confluence, a scratch-wound was introduced to the monolayer using a plastic pipette tip, and the cells allowed to migrate into the gap. As shown in Figs. 5.6B and C, Rac\(^{V12}\)-expressing cells were able to move faster to the open wound than the parental HC11 (panel f vs b). However, gp130 downregulation abolished this effect (panel h), indicating that gp130 is an integral component of the Rac\(^{V12}\) pathway leading to the increase in cell migration. gp130 shRNA expression in the parental HC11 line also decreased cell migration (panel d). Very similar observations were noted when comparing 10T1/2, 10T-shgp130, 10T-Rac\(^{V12}\) and 10T-Rac\(^{V12}\)-shgp130 cell lines (not shown).

Although Rac\(^{V12}\) cells grow faster than cells expressing both Rac\(^{V12}\) and shgp130, the accelerated growth rate cannot account for the increase in rate of migration and gap closure. Downregulation of Stat3 through shRNA knockdown (Geletu et al., 2009), or treatment with the CPA7 inhibitor (Littlefield et al., 2008) caused a similar decrease in cell migration of H-Rac\(^{V12}\) cells (not shown), as previously reported in other systems (Debida et al., 2005). These findings
Figure 5.6

Rac-induced cell migration and proliferation require gp130

A: Cell proliferation: HC11, H-shgp130, H-RacV12, or H-RacV12-shgp130 cells were grown in Petri dishes in 10% serum and cell numbers obtained over several days, as indicated. Values represent averages of 3 independent experiments.

B: Cell migration: HC11, HC11-shgp130, H-RacV12 or H-RacV12-shgp130 cells were cultured to confluence before a scratch was made through the monolayer using a plastic pipette tip. Cells were photographed at 0 (panels a, c, e, g) or 24 hrs (panels b, d, f, h) after 12 hrs of culturing in 0.5% fetal calf serum.

C: Quantitation of the time (hrs) required by the different cell lines for gap junction closure.

HC11, HC11-shgp130, H-RacV12 or H-RacV12-shgp130 cells were cultured to confluence before a scratch was made through the monolayer using a plastic pipette tip. The time taken for gap junction closure was determined by microscopic observation. Numbers represent averages of 3 independent experiments.
indicate that gp130 is required for cell migration triggered by the cellular Rac, and that expression
of the mutationally activated RacV12 cannot overcome the gp130 knockdown. Taken together,
these results demonstrate that gp130 represents an essential effector of activated Rac in the
regulation of both cell proliferation and migration.
Discussion

We previously demonstrated that cell to cell adhesion induces a dramatic increase in the levels of Stat3 activity in a number of cell lines [(Arulanandam et al., 2009; Vultur et al., 2004; Vultur et al., 2005); reviewed in (Raptis et al., 2009)]. An important piece of information that came out of these studies is that cell density must be taken into account in experiments to assess the effect of an oncogene upon Stat3 activity. Therefore, to investigate the effect of activated Rac upon Stat3, Stat3 activity levels were examined at different cell densities, following expression of two mutationally activated Rac constructs, Rac\textsuperscript{V12} and Rac\textsuperscript{L61}. The results revealed that Stat3 activity was increased with activated Rac expression at all cell densities examined, indicating that activated Rac can, in fact, activate Stat3, above and beyond the activation due to cell density.

These observations raise important questions: 1. What is the mechanism of increase in Rac and Rac\textsuperscript{V12} levels with cell density? 2. What is the mechanism of Stat3 activation by Rac\textsuperscript{V12}? 3. What is the functional significance of the Stat3 activation in Rac\textsuperscript{V12}-expressing cells?

**Cell density inhibits the proteasomal degradation of Rac.**

We have demonstrated that cell density also increases Rac levels in both HC11 epithelial cells and Balb/c 3T3 or 10T1/2 fibroblasts (Arulanandam et al., 2009; Arulanandam et al., in preparation). Our data indicate that this increase could be due to inhibition of Rac proteasomal degradation with confluence. In fact, previous results indicated that Rac can be degraded through the proteasome pathway (Pop et al., 2004). A mutational analysis further indicated that constitutive activation of Rac, as well as binding of effectors, which might be acting as ubiquitin E3 ligases, are necessary for Rac degradation (Pop et al., 2004). Our results, demonstrating that, although activated, Rac\textsuperscript{V12} and Rac\textsuperscript{L61} protein levels are increased dramatically with cell density, indicate that cell density can overcome the degradative effect of activation. These findings
extend and reinforce previous data indicating that epithelial cell scattering brought about by HGF can induce the proteasome-mediated degradation of Rac1 (Lynch et al., 2006). A similar mechanism could hold true for Cdc42, which mirrored Rac levels and stability increases with cell density.

**IL6 family cytokines transmit the signal from activated Rac\textsuperscript{V12} to Stat3.**

Although earlier reports (Simon et al., 2000) indicated that Rac can directly interact with Stat3 in co-expression/co-immunoprecipitation assays, later findings unequivocally demonstrated that Stat3 activation by the Rho GTPases can occur without the formation of a stable complex [(Debidda et al., 2005); and data not shown]. In addition, Faruqi et al. have demonstrated that Rac\textsuperscript{V12} could indirectly activate Stat3 through autocrine induction of IL6 (Faruqi et al., 2001). However, through the use of specific antibodies added to the medium it was later demonstrated that Stat3 activation can also occur independently of IL6 stimulation (Debidda et al., 2005). To resolve this apparent controversy, we examined mRNA levels for 86 cytokines in an array analysis. The results revealed an increase in two cytokines of the IL6 family (IL6 and LIF), indicating that this family may be involved in Stat3 activation by mutationally activated Rac. Furthermore, downregulation of gp130, the common subunit of the family, abolished the Rac\textsuperscript{V12}-mediated, Stat3 activation indicating that this subunit is actually required for Stat3 activation. The fact that the LIF also, rather than IL6 alone, is upregulated by Rac\textsuperscript{V12} explains earlier data indicating that IL6-specific antibodies did not reduce Stat3 activity (Debidda et al., 2005), since these antibodies, directed against IL6 would inhibit IL6 exclusively, while LIF would still be free to activate Stat3. In any event, these results demonstrate that the total Stat3 activity in the cell is the sum of effects of both cell to cell adhesion, plus the Stat3 activating, Rac\textsuperscript{V12} or Cdc42\textsuperscript{V12} oncogene present.

Previous results indicated that IL6 activates Erk, as well as Stat3. Since Rac\textsuperscript{V12} causes the upregulation of IL6 family cytokines, it is expected that Rac\textsuperscript{V12} expression would activate Erk.
However, although Rac$^{V12}$-expressing cells had higher pErk levels than the parental HC11, when sparsely grown, there was no pErk increase in H-Rac$^{V12}$ cells grown to high densities. To solve this apparent paradox, HC11 cells were grown to different densities and stimulated with IL6. Indeed IL6 itself, although able to activate Erk in subconfluent cultures, was unable to do so once cells reached confluence (Fig. 5.5B), although Stat3 was still activated (Fig. 5.1). It is possible that Erk-specific phosphatases such as Cdc25A are activated at high cell densities (Lazo et al., 2002), or that other adaptors required for Erk activation by IL6, or phosphorylation of IL6-R sites, are downregulated following cadherin engagement and establishment of cell to cell contacts. In any event, these results further demonstrate that, despite the fact that these two pathways are often both activated by oncogenes, cytokines or growth factors, they are not coordinately regulated by IL6 in densely growing cells.

**Gp130 is required for Rac-mediated cell proliferation and migration.**

It is well established that Rac activation can increase cell motility, in part due to regulation of the actin cytoskeleton. However, the role of the IL6/Stat3 axis in this effect has not been examined. Our results showed that, as observed before (Nobes and Hall, 1999), Rac$^{V12}$-expressing cells were able to move faster into an open wound introduced with a plastic pipette tip into a cell monolayer than the parental line (Etienne-Manneville and Hall, 2002). Furthermore, the Rac-mediated motility required the activity of IL6 family cytokines, since downregulation of the gp130, common subunit of the family, reduced the rate of migration. The fact that activated Rac$^{V12}$ upregulates IL6 and Stat3, and that this may occur, at least, in both epithelial cells and fibroblasts, gives further credence to the model of Stat3 activation by E-cadherin or cadherin-11 engagement, respectively, via Rac and IL6 (Arulanandam et al., 2009).

**Conclusions:** Our results definitively demonstrate that activated Rac/Cdc42 expression leads to Stat3 activation, by a mechanism requiring NFκB, gp130 and JAK. This pathway is required for
cell proliferation and migration, that is, the gp130/Stat3 axis represents an essential effector of activated Rac for the regulation of key cellular functions (Fig. 5.7).

**Proposed model for Rac/Cdc42-mediated, Stat3 activation**

Constitutively active Rac/Cdc42 activates Stat3 through induction of IL6 family cytokines, such as IL6 and LIF which possess the common subunit, gp130. This leads to activation of their respective receptors, Jak activation and phosphorylation of Stat3, resulting in cell proliferation and migration.
References


transcription 3 is required for myocardial capillary growth, control of interstitial matrix deposition, and heart protection from ischemic injury. Circ. Res. 95, 187-195.


Chapter 6

Discussion
Malignant cancer progression is associated with mutational events that may lead to persistent mitogenic signalling or loss of negative control, resulting in activation of cell division even in the absence of stimuli. While Stat3 has recently emerged as an ideal target in anticancer therapy due to its ability to coordinate proliferative, anti-apoptotic, immunosuppressive and angiogenic responses triggered by a number of different aberrant molecular signalling events, its mechanism of action is incompletely understood. The current literature suggests that constitutive signalling to Stat3 can occur from its persistent phosphorylation by growth factor receptors, cytokine receptors and cytoplasmic tyrosine kinases. The loss of key negative regulators of Stat3 has also been observed in a number of tumors and tumor-derived cell lines. Our lab has done pioneering research in demonstrating a novel role for cell-cell adhesion in the activation of Stat3. Indeed, these findings indicate that the Stat3 activation observed in confluent cultures may constitute a potent survival signal which could be exploited by cancer cells. The work presented here, revealing that homophilic cadherin ligation and increased protein stability of Rac1 and Cdc42 GTPases can increase ptyr705-Stat3 phosphorylation and activity levels, broadens our understanding of Stat3 signalling while promoting the validation of this pathway for anticancer drug design.

**Direct cadherin engagement activates Stat3**

Cells in normal tissues or in tumors have extensive opportunities for adhesion to their neighbors in a three-dimensional organization, and it recently became apparent that in the study of fundamental cellular processes, such as mitogenesis, survival or differentiation, it is important to take into account the effect of surrounding cells. Dense cell cultures, albeit only two dimensional, may in part mimic some of the physiological stress signals that occur in fast-growing tumors. Indeed, many of the cells in tumor nests lack direct interaction with surrounding connective tissue and they may depend on survival signalling generated by cell-cell contacts. Not
surprisingly, the world of cells growing at high densities in 2-dimensions can be dramatically different from the one of cells dividing peacefully at subconfluence.

While both structural and signalling functions have been extensively characterized for the integrin family of cell-matrix adhesion receptors, which are known to stimulate the activity of the focal adhesion tyrosine kinase (FAK), our understanding of the signals generated at adherens junctions is more recent and less well understood. Early data have demonstrated an increase in total cellular tyrosine phosphorylation in confluent MCF-10A human mammary epithelial cultures, and more specifically upon E-cadherin engagement, which was reduced following calcium chelation or treatment with E-cadherin blocking antibodies (Kinch et al., 1997; Batt and Roberts, 1998). In line with these findings, my work has identified Stat3 as a tyrosine-phosphorylated substrate downstream of signals brought about by homophilic ligation of E-cadherin in both murine and canine epithelial cells (Arulanandam et al., 2009b). In fact, our approach of employing E/EC12 fragments to functionalize plastic petri dishes offered the possibility for a definitive demonstration that the Stat3 activity increase observed at high densities is a direct effect of cadherin engagement, rather than a secondary effect of cell to cell proximity.

Interestingly, examination of cell lines which are known to lack E-cadherin, such as the chinese hamster ovary (CHO) and the cervical carcinoma A431D, also revealed the same increase in Stat3 activity with density (Appendix, Fig. A.13). Hence, these data led us to investigate whether other cadherins may also be activating Stat3. In the context of metastasis, loss of E-cadherin is associated with a concomitant gain in expression of the mesenchymal N-cadherin and cadherin-11. This prompted the examination of the role of cadherins associated with the metastatic phenotype in activating Stat3. Accordingly, our data reveal that cadherin-11, a well characterized type II classical cadherin, is also a potent stimulator of Stat3 activity in Balb/c 3T3 and 10T½ fibroblasts (Arulanandam et al., in preparation). My data so far also attribute a role for N-cadherin in Stat3 activation (Appendix, Fig. A.14). Indeed, comparison of ptyr-705 levels in ES, E-cad null cells, which express very low levels of known cadherins, including N-cadherin, to
null cells where the N-cadherin gene was added back [Ncad-addback cells (Larue et al., 1996)], reveals a dramatic increase in Stat3 upon N-cadherin expression. Interestingly, independent studies have identified the short type PB-cadherin, a novel adhesion receptor in the rat testes, as a direct activator of Jak2 and Stat3 (Wu et al., 2005). Hence, the fact that a number of different cadherins activate Stat3 in tissues originating from at least three mammalian species emphasizes the potentially universal nature and significance of this pathway. In addition, our findings add to the growing body of evidence indicating that cadherins can provide direct, functionally relevant signalling beyond their structural role.

**Cadherin engagement upregulates Rac1 and Cdc42 protein levels through inhibition of proteasomal degradation**

Further examination of the mechanism of Stat3 activation by cadherin engagement in HC11 cells revealed a role for the Rac1 and Cdc42, Rho family GTPases. While our results confirm an increase in GTP-bound Rac1 and Cdc42 as cells approach confluence (Noren et al., 2001; Nakagawa et al., 2001), we further expose an unanticipated rise in Rac1 and Cdc42 total protein levels, brought about by cadherin ligation. Our results reveal for the first time that the dramatic rise in the activity of these GTPases is due to an increase in the stability of Rac1/Cdc42, brought about by an increase in their protein levels, at high confluences. Indeed, Rac1 was shown to be modified by ubiquitin tagging in the absence of cell to cell contact, thereby leading to its degradation by the proteasome. Interestingly, the degradation of GTP-bound Rac1 and its subsequent ubiquitination was also shown in the context of epithelial cell scattering brought about by HGF (Lynch et al., 2006).

*Mutationally activated Rac and Cdc42 are also targeted to the proteasome, in the absence of cell-cell contact*

In addition, our results demonstrate that the levels of mutationally activated forms of Rac1 (RacV12 or RacL61) and Cdc42 (Cdc42V12) also increase with cell density, and can be subject to ubiquitination, which would occur in the absence of cell to cell contact. The ubiquitination of
Rac1 has been demonstrated following its deamidation at Gln61, which causes its constitutive activation by the bacterial toxin, cytotoxic necrotizing factor (CNF) found in *Escherichia coli* (Lerm et al., 2002; Pop et al., 2004). Others have reported the proteasomal degradation of mutationally activated Rac\textsuperscript{V12} following treatment of endothelial cells with reactive oxygen species (ROS), and have identified a destruction box domain, originally found in mitotic cyclins, in the primary structure of Rac, which may be a signal for its modification by ubiquitin (Kovacic et al., 2001; Jacobs et al., 2001). Hence, the present literature implies that ubiquitin tagging of Rac requires its modification and activation, which leads to effector binding. Studies have shown that point mutations in the switch I domain, involved in mediating effector interactions, may prevent the degradation of Rac following CNF treatment (Pop et al., 2004). One promising candidate is the Rac1 effector, POSH, which, in addition to serving as a scaffolding protein linking together activated Rac1 with downstream modulators of the JNK cascade, was shown to also target proteins for degradation via the ubiquitin proteasome pathway (Xu et al., 2003). Examination of the POSH sequence has revealed a zinc ring finger domain near the N-terminus with characteristic E3 ligase activity (Xu et al., 2003). POSH was first shown to direct its own proteasomal degradation, resulting in its autoregulation (Xu et al., 2003). Further studies have revealed POSH to be involved in the proteasomal targeting of a number of other substrates, including the HGF-regulated tyrosine kinase substrate, Hrs, an early endosomal resident sorting factor (Tuvia et al., 2007; Kim et al., 2006; Alroy et al., 2005; Tsuda et al., 2005). Most interestingly, overexpression of the Rac1-binding domain of POSH in HEK 293 cells following CNF treatment, or in MDCK epithelial cells after HGF stimulation could prevent Rac1 degradation (Pop et al., 2004; Lynch et al., 2006). However, these results are at variance with findings by Visvikis et al., who have observed the continuous degradation of Rac\textsuperscript{V12}, but not endogenous Rac1, in HEK 293 cells, but still claim that POSH expression did not promote Rac\textsuperscript{V12} ubiquitination (Visvikis et al., 2008). However, cell density was not taken into account in these experiments and might explain this apparent discrepancy. Given the above findings, the
examination of the role of POSH in the ubiquitin modification of Rac1, or activated Rac1 mutants, in the absence of cell-cell adhesion, would be essential to the further characterization of this GTPase as a binding partner and substrate of this unique effector with E3 ligase activity.

The regulation of other Rho GTPase family members by ubiquitin-mediated proteasomal degradation is less well understood. However, an E3 ligase responsible for the ubiquitination of dominant negative, or GDP-bound, RhoA in HEK 293 cells has been identified as the Smad-ubiquitin regulatory factor (Smurf) (Wang et al., 2006; Zhang et al., 2004). On the other hand, while the ubiquitin targeting of Cdc42 has not yet been validated, the degradation of Cdc42 was demonstrated following its cleavage by caspases during Fas ligand-induced apoptosis (Tu and Cerione, 2001). However, it is interesting to note that activated Cdc42 can form complexes with the E3 ligases, Cbl and Hakai (Wu et al., 2003; Shen et al., 2008). While Cdc42 was shown to function in sequestering Cbl, thereby preventing its binding to its substrate, the EGF receptor, activated Cdc42 could promote the ubiquitination of E-cadherin by Hakai, following calcium chelation (Wu et al., 2003; Shen et al., 2008). Indeed, E-cadherin harbours a PEST sequence, a motif associated with ubiquitin targeting, which overlaps the site for β-catenin binding (Huber et al., 2001). Hence, cadherins that are uncoupled from β-catenin may be endocytosed and degraded in order to reduce the population of cadherins at the cell surface following calcium chelation and even in the context of sparsely growing cells (Le et al., 1999; Shen et al., 2008; Fujita et al., 2002). These findings taken together, further point towards the possibility that Cdc42, or Rac1, could also be targeted for destruction in a complex with adherens junction components in the absence of cell-cell contact. In line with these findings, my unpublished data indicate that E-cadherin, along with Rac1 and Cdc42 total protein levels, are reduced in the absence of cell-cell contacts (Appendix, Fig. A.15). However, whether Rac1/Cdc42 associates with inactive E-cadherin to form a complex which may be destroyed by the proteasome remains to be seen. Nevertheless, our results, demonstrating that, although Cdc42 and Rac1, as well as
Cdc42V12, RacV12, RacL61, protein levels are increased dramatically with cell density, strongly suggest that cell density can overcome the degradative effect of activation.

**IL6 family cytokines transmit the signal from activated Rac to Stat3.**

Data from a number of labs have demonstrated a functional link between the Stat3 and Rho family pathways in a variety of cell systems (Faruqi et al., 2001; Debidda et al., 2005; Turkson et al., 1999; Simon et al., 2000). However, the exact mechanism whereby these GTPases may affect Stat3 is a topic of some controversy. While a direct association between Rac1V12 and Stat3 has been proposed (Simon et al., 2000), later findings unequivocally demonstrated that Stat3 activation by the Rho GTPases can occur without the formation of a stable complex (Debidda et al., 2005). My unpublished data, demonstrating that Rac1 and Stat3 do not co-immunoprecipitate in confluent cultured cells are in agreement with the latter observations.

In addition to their role in the regulation of the actin cytoskeleton, Rho GTPases have been implicated in the modulation of gene expression, mainly due to their ability to activate the NFκB transcription factor (Perona et al., 1997; Aznar and Lacal, 2001). Further data by Faruqi *et al.* demonstrated that RacV12, and Cdc42V12, could indirectly activate Stat3 through NF-κB-mediated autocrine induction of IL6 (Faruqi et al., 2001). However, through the use of blocking antibodies added to the medium, Debidda et al. demonstrated that Stat3 activation by RacV12 can also occur independently of IL6 stimulation (Debidda et al., 2005). To resolve this apparent controversy, we examined H-RacV12 mRNA levels for 86 cytokines in an array analysis. The results revealed an increase in two cytokines of the IL6 family (IL6 and LIF), indicating that this family may be involved in Stat3 activation by mutationally activated Rac. In addition, we demonstrate that inhibition of NF-κB in H-RacV12 cells may prevent Stat3 activation, suggesting that Rac-NFκB activation may be inducing the expression of IL6 family cytokines. Furthermore, downregulation of gp130, the common subunit of the family, abolished the RacV12-mediated,
Stat3 activation indicating that this subunit is actually required for Stat3 activation. The fact that the LIF also, rather than IL6 alone, is upregulated by Rac\textsuperscript{V12} explains earlier data indicating that IL6-specific antibodies did not reduce Stat3 activity (Debidda et al., 2005), since these antibodies, directed against IL6 would inhibit IL6 exclusively, while LIF would still be free to activate Stat3. In any event, these results demonstrate that the total Stat3 activity in the cell is the sum of the effects of both cell to cell adhesion, plus the Stat3 activating, Rac\textsuperscript{V12} or Cdc42\textsuperscript{V12} oncogene present.

**Rac1 and Cdc42 transduce the cadherin signal to Stat3**

Our results reveal that in addition to being activated by Rac\textsuperscript{V12} and Cdc42\textsuperscript{V12}, Stat3 activation can also result from the dramatic upregulation of endogenous levels and activity of these GTPases following direct cadherin ligation. In contrast to Rac and Cdc42, expression of a dominant-negative RhoA construct did not seem to affect Stat3 activity levels (not shown). Interestingly, Kinch et al. have observed increased tyrosine phosphorylation of Ras-GAP in confluent cells (Kinch et al., 1997), while Ras-GAP was shown to associate with Rho-GAP, which leads to a corresponding decline in Rho activity (Settleman et al., 1992). This could, in part, explain the reduced Rho-GTP levels previously observed upon E-cadherin engagement (Noren et al., 2001; Fukata and Kaibuchi, 2001) and the fact that RhoA was found to not play a role in the density-mediated Stat3 activation in our system.

**Cell-cell adhesion induces the autocrine production of IL6 family cytokines**

The mechanism whereby Rac1 and Cdc42 may activate Stat3 with cell density is likely autocrine, since conditioned medium from densely growing cells did increase Stat3 activity in cells grown to low density. Our data further demonstrate that cell confluence causes the expression of a number of cytokines in both E-cadherin and cadherin-11 expressing cells, most importantly members of the IL6 family. Indeed, knockdown of the common gp130 subunit by
stable transfection could significantly inhibit the density-mediated Stat3 activation indicating that this family of cytokines is at least in part responsible for the Stat3 activity increase observed at high cell densities.

In addition to the IL6 family, our qRT-PCR array analysis in HC11 cells pointed to other cytokines which employ different pathways from gp130/Stat3. Prominent among them is a tumor necrosis factor (TNF) family member, the receptor activator of NFκB (RANK) ligand, which was found to be upregulated ~50-fold in densely growing, compared to sparse HC11 cells. RANK-L was shown to play a critical role in mammary gland development in pregnancy, by promoting the proliferation and survival of mammary gland epithelial cells (Fata et al., 2000). The role of RANK-L in the activation of Stat3 by E-cadherin is the subject of further study.

In both HC11 and Balb/c 3T3, the most significant increase was noted in IL6 mRNA in confluent vs. sparse cells (76 and 32 fold, respectively). IL6 is the most potent activator of Stat3 and relies heavily on receptor-associated JAK kinases to fulfill this role. Interestingly, recent findings have revealed that phosphorylation of Stat3 on tyr-705 by JAKs may also depend on its association with the male germ cell Rac1-GAP (MgcRacGAP). Previous data identified a crucial role for MgcRacGAP in cytokinesis through its binding to tubulin and Rho GTPases (Hirose et al., 2001). Conditional knockout of MgcRacGAP can also result in apoptosis even before cytokinesis in B220+ T cells, indicating that it may also function in promoting cell survival (Yamada et al., 2008). More recently, MgcRacGAP was revealed to directly associate with a region in the DNA binding domain of STATs (Kawashima et al., 2006). A classical NLS was identified in MgcRacGAP, which enables it to act, together with GTP-bound Rac1, as a nuclear chaperone for phosphorylated STATs by enabling the association of this complex with importin-α (Kawashima et al., 2006). In addition, downregulation of MgcRacGAP was also shown to prevent the tyrosine phosphorylation and activation of Stat3 following its stimulation by IL6 in a chicken B cell line (Kawashima et al., 2009). The authors indicate that MgcRacGAP can bind Jak2 and hence, along with Rac1-GTP, may mediate Stat3 phosphorylation by serving as a
scaffold for the interaction between Jak2 and Stat3 (Kawashima et al., 2009). Hence, it would be interesting to study the role of MgcRacGAP in the activation of Rac1 and Stat3 observed in our cell systems, in the context of cell-cell adhesion.

The autocrine model of Stat3 activation leaves a question unanswered: The IL6 receptor family invariably activates other pathways, such as Erk1/2 (Fischer and Hilfiker-Kleiner, 2008). To solve this apparent paradox, HC11 cells were grown to different densities and stimulated with IL6. Indeed IL6, although able to activate Erk in subconfluent cultures, it was found to be unable to do so once cells reach confluence, although Stat3 was still activated. This could not have been due to a general inability of the cells to activate Erk in this setting, since my data indicate that confluent cells did respond to EGF with Erk activation (not shown). It is possible that IL6-associated phosphatases that are specific for Erk are activated at high cell densities, or that other adaptors required for Erk activation by IL6, or phosphorylation of certain IL6 receptor sites, are downregulated following cadherin engagement. Our preliminary results indicate a role for the phosphatase Cdc25A, known for its role as an essential regulator of cell cycle transition in mammalian cells, by dephosphorylating the Raf kinase (Xia et al., 1999) as well as the cyclin-dependent kinases, Cdk2 and Cdk4 (Draetta and Eckstein, 1997). Cdc25A was also shown to bind to, dephosphorylate and inactivate Erk as well, both in purified preparations, and in cultured Hep3B hepatoma cells (Wang et al., 2005). Indeed, treatment of densely growing, HC11 cells with the potent, Cdc25A-selective inhibitor, NSC-67212 (Nemoto et al., 2004) caused a dramatic increase in Erk levels in densely growing HC11 cells (Geletu et al., unpublished data). The fact that in confluent cells, Stat3 could be immune to inactivation by Cdc25A, and possibly other phosphatases (not shown), at a time when Erk activity is drastically curtailed, further emphasizes the signalling specificity of this pathway triggered by cadherin ligation and points towards a primordial significance. These data reveal that, despite the fact that the Erk and Stat3 pathways are often both activated by growth factors or oncogenes, they are not coordinately regulated by cadherin engagement.
Calcium phosphate and lipofectamine transfection increase Stat3 activity

The fact that transient transfection regiments alone could activate Stat3 prompted us to generate stable cell lines for all studies on the effect of Stat3 modulators. While the specific mechanism of tyr-705 phosphorylation upon plasmid DNA transfection using calcium phosphate precipitation or certain cationic lipid-based methods has not been fully elucidated, it has been demonstrated that the gene transfer of nucleic acids, including unmethylated plasmid DNA, or siRNA, can trigger proinflammatory responses in vitro or in vivo through IL6 production (Yew et al., 1999; Yoo et al., 2006). Indeed, one of the problems facing non-viral gene therapy for the correction of genetic deficiencies is the activation of a strong innate immune response. While inflammatory cytokine production was generally found to be triggered by unmethylated CpG residues found in plasmid DNA (Yonenaga et al., 2007; Scheule, 2000), a study by Yoo et al. has demonstrated that certain cationic lipid based transfection reagents alone may increase the production of IL6, IL8, IL18 and IL1A (Yoo et al., 2006). Inhibition of endosomal maturation by chloroquine treatment was found to significantly lower IL8, and potentially IL6 expression, brought about by transfection of siRNA or lipid reagent alone (Yoo et al., 2006). Cytokine expression following transient transfection was suggested to occur as a result of nucleic acid binding Toll-like receptor signaling, which could lead to NFκB activation. Interestingly, studies have revealed that Stat3 can also be targeted to signalling or sequestering endosomes, thereby adding a new twist to the original paradigm of Stat3 activation by cytokine receptors (Xu et al., 2007; Sehgal, 2008). Hence, it is possible that the increased Stat3 activity observed following lipofectamine transfection may occur through endosomal interactions.

We have shown that in addition to cationic lipid-based methods, calcium phosphate transfection can also trigger Stat3 activation. An additional explanation for our findings could be that the physical presence of a precipitate in the Petri dish or the increase in calcium concentration may increase the opportunities for cell-cell adhesion. While my unpublished results suggest that adding higher amounts of calcium in the presence or absence of DNA did not
increase Stat3 tyr-705 phosphorylation, it remains to be seen whether these transfection techniques may encourage cadherin engagement.

**Role of the cadherin/Rac1/gp130/JAK/Stat3 axis in cell survival and cancer**

The biological consequences of the cell-cell adhesion mediated Stat3 activation were first examined in our lab by downregulating Stat3 activity in normal mouse epithelial or fibroblast cultures using a variety of methods, such as electroporation of peptides or peptidomimetics to block the Stat3, SH2 domain, or through treatment with two platinum compound inhibitors (Turkson et al., 2004a; Turkson et al., 2004b). The results demonstrated a profound difference in the effect of Stat3 inhibition in sparsely growing vs. confluent cells (Vultur et al., 2004; Anagnostopoulou et al., 2006; Vultur et al., 2005). Although Stat3 levels are very low in normal mouse NIH 3T3 fibroblasts or human breast epithelial MCF-10A cells grown to a low confluence, Stat3 inhibition does cause a growth retardation, indicating a role for Stat3 in cell proliferation in this setting. In sharp contrast, in densely growing, normal cells, Stat3 inhibition induced apoptosis. In fact, both growth retardation and apoptosis following Stat3 inhibition appear to be mediated by a rise in p53 levels. Treatment with the CPA-7 inhibitor (Turkson et al., 2004a; Turkson et al., 2004b) resulted in a dramatic increase in p53 at all cell densities, consistent with previous findings that Stat3 dowregulates p53 by binding directly to its promotor (Niu et al., 2005). Interestingly, p21, the substrate responsible for p53-mediated growth retardation, was elevated at lower densities exclusively (Anagnostopoulou, 2009, thesis). Whether Stat3 inhibition at high densities induces alternate p53 targets associated with apoptosis, such as the PUMA and NOXA members of the Bel-2 family (Pietsch et al., 2008), is currently under investigation.

The results presented demonstrate that direct cadherin engagement can promote cell survival through Stat3 activation. Indeed, we observed a dramatic induction of apoptosis in cells lacking E-cadherin when grown to high densities, or in HC11 cells following inhibition of
cadherin engagement. Contrary to E-cadherin, the mesenchymal cadherins, cadherin-11 and N-cadherin, both have key functions in promoting the metastatic spread of cancer cells, therefore, the demonstration that they also activate Stat3 may point to Stat3 as a central survival, rather than metastasis, factor. Most importantly, cadherin-11 or N-cadherin inhibition could induce apoptosis (through Stat3 downregulation) in metastatic cells specifically, while normal cells expressing E-cadherin would be spared.

Our findings demonstrate the presence of constitutively activated Stat3 in normal breast tissue where cadherin is engaged, thus revealing a distinct correlation of the state of these molecular markers between cells growing to high, but not low densities with the same type of cell in vivo. The expression of Stat3 in normal tissues surrounding human breast tumors has been examined by Diaz et al. (Diaz et al., 2006). Using tissues fixed within less than 15 min after surgery, to preserve the integrity of Stat3-ptyr705, which is known to be rapidly dephosphorylated after surgery (Diaz et al., 2006; Mora et al., 2002), they definitively demonstrated that Stat3-ptyr705 was present in luminal cells, so that overall levels in the non-neoplastic tissues were only two-fold lower than the levels in the tumor itself, as determined by immunohistochemistry and computer-assisted quantitation (Diaz et al., 2006). Remarkably, Her2/neu levels were very high in the tumors but were undetectable in the normal tissues surrounding the breast tumor. Furthermore survivin levels were undetectable in normal tissues but were increased in the tumors pointing towards a marked difference in Stat3 target gene expression profiles in normal vs tumor tissues. The presence of phosphorylated Stat3 staining in normal luminal cells by this group is consistent with our data from the established, mouse HC11 cells, cultured primary breast epithelial cells and breast tissues. These findings by Diaz et al. also correlate with our observations that Stat3-ptyr705 levels in normal cells are generally two-fold lower than cells transformed by oncogenes such as vSrc or activated Rac.

Our demonstration of the central role of gp130 in the density-dependent Stat3 activation explains earlier findings indicating that inhibition of certain tyrosine kinases commonly activated
in cancer such as EGFR, IGFR, Fer or the Src family in mouse epithelial cells or fibroblasts do not play a role (Vultur et al., 2004; Steinman et al., 2003). It is interesting to note that the EGFR independence could perhaps explain why inhibition of members of this family of receptors (e.g. ErbB2) has been ineffective in a large number of ErbB2-positive cancers (Hynes and Lane, 2005; Vogel et al., 2002; Harries and Smith, 2002; Cobleigh et al., 1999). Indeed, emerging evidence has identified a critical role for IL6 signalling in activating Stat3 and promoting malignancy in a number of cancers (Catlett-Falcone et al., 1999a; Lou et al., 2000; Berishaj et al., 2007). A study by Gao et al. has shown that while the presence of nuclear, tyrosine phosphorylated Stat3 was found in 50% of lung adenocarcinomas, its activation was not directly affected by the EGFR, but likely triggered by transcriptional induction of IL6 and the activation of its receptor (Gao et al., 2007). Similar findings were noted in breast cancer cell lines where inhibition of EGFR or Src had minimal effect on Stat3 compared to pJAK or gp130 downregulation (Berishaj et al., 2007). These results indicate that targeting the gp130/JAK/Stat3 axis may present a more effective strategy compared to EGFR or Src inhibition in these cancers.

In addition to autocrine stimulatory loops, paracrine feedforward mechanisms of cytokine signalling to Stat3 have been demonstrated in certain human cancers and can play a role in inducing and maintaining a cancer-promoting, inflammatory environment. Hyperactive Stat3 in tumor cells can lead to the production of cytokines and growth factors that in turn may stimulate Stat3 signalling in stromal cells (Yu et al., 2009). Hence a feedforward stimulation of Stat3 can result from the tumor microenvironment. This is exemplified in multiple myeloma, where bone marrow stromal cells contribute to the production of IL6, thereby fueling cancer progression through the persistent activation of the IL6/JAK/Stat3 axis in tumor cells and the induction of antiapoptotic genes (Shain et al., 2009; Catlett-Falcone et al., 1999b). Our demonstration of a novel pathway of Stat3 signalling through cadherin ligation further suggests that in addition to the production of cytokines such as IL6, stromal cells may also contribute to Stat3 signalling through direct cell-cell adhesion, thereby emphasizing the role of cell communication with the
extracellular milieu in cancer progression. Indeed, this promotion of Stat3 activation by the microenvironment could also be crucial to tumor cell survival following the administration of chemotherapeutic agents, thereby indicating that the cadherin-mediated Stat3 activation could be a key contributing factor in multidrug resistance (Shain and Dalton, 2001).

Conclusion

Over the past decade, Stat3 has received much attention because its constitutive activation has shown to promote the growth and survival of tumor cells. The work presented here furthers our understanding of this transcription factor by identifying three novel pathways leading to its activation (Fig. 6.1A). The fact that Stat3 tyr-705 phosphorylation, DNA-binding and transcriptional activation, as well as Rac1 and Cdc42 activity and levels, may increase through simple cell-cell contact by cadherins, or that ptyr705-Stat3 can result from certain common transfection techniques further indicates that caution is required upon the interpretation of data involving these extensively studied signalling proteins.

Pioneering work from our lab revealed that the world of densely growing cells is vastly different from that of cells growing peacefully at subconfluence. Our data has greatly contributed to the growing list of proteins which are affected by cell to cell contact; these now include pJak1 (Vultur et al., 2004; Arulanandam et al., 2009b), Rac1/Cdc42 (Arulanandam et al., 2009b), activated Rac1V12/Rac1L61/Cdc42V12 (Arulanandam et al., 2009a), caveolin-1 (Mohan et al., unpublished), cdk2 (Steinman et al., 2003), connexin-43 (Geletu et al., 2009), key housekeeping proteins such as αtubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Greer et al., 2009), as well as Stat3 targets such as p21 and p27 (Steinman et al., 2003), VEGF-D (Orlandini and Oliviero, 2001), survivin (Arulanandam et al., 2009b; Arulanandam et al., 2010), and p53 (Anagnostopoulou, Thesis). The significance of our work is brought to light by findings indicating that confluent cell systems may be more representative to the in vivo situation, as revealed by a close correspondence of genes expressed specifically in the prostate carcinoma line,
LNCaP, cultured to high densities, with genes associated with prostate cancer (Chen et al., 2006). The fact that these genes were different from genes identified in LNCaP cells grown under log-phase conditions further stresses the importance of the examination of signalling pathways in densely growing cells. In line with these data, our observations that cultured breast epithelial cells, known to express RANK-L \textit{in vivo}, have high RANK-L levels when grown to high densities, and the absence of RANK-L in fibroblasts at all densities, that is in a cell type that does not express it \textit{in vivo}, further confirm the relevance of data from cells cultured to high densities to the \textit{in vivo} situation. Hence, dense cultures offer a more relevant view of the internal mechanisms of cellular communication which is crucial to the identification and in depth understanding of the signalling pathways exploited in cancer. It is interesting to note that this novel mechanism of Stat3 activation by cadherin-mediated cell-cell interactions not only promotes cell survival, but may also enhance resistance to chemotherapeutic agents (Nakamura et al., 2003; Shain and Dalton, 2001), a finding which could have significant therapeutic implications.

While significant advances have been made in cancer diagnosis, conventional approaches in cancer therapy, including surgery, radiation and chemotherapy, appear to be reaching a plateau in terms of their efficacy. The complexity of cancer as a disease has now become apparent and rather than generally interfering with cell division, which was the goal of traditional chemotherapy, the use of targeted therapies will assist in the identification and specific inhibition of signaling pathways unique to each tumor type. This application of personalized medicine to the field of oncology is presently seeing some promising results. However, while many current FDA-approved targeted therapies have focused on tyrosine kinases, our identification of a novel pathway of Stat3 activation that is independent of a number of kinases would be a promising target in the treatment of cancers where such inhibitors, e.g. Herceptin, are ineffective. Indeed, the data included in this thesis clearly reveal that the gp130/Stat3 axis is highly activated upon the engagement of a number of cadherins, including ones activated during the metastatic switch, and also lies downstream of Rac1 and Cdc42, which are often overexpressed in cancer. Our findings
further expose Stat3 at a central determinant of the balance between cell proliferation and apoptosis (Fig. 6.1B). Inhibition of Stat3 in tumors can promote apoptosis, through p53-dependant or p53-independent mechanisms. My unpublished work has shown that CPA-7 treatment of SV40 Large T expressing cells or tumors in vivo could lead to cell death and tumor regression possibly through E2F-mediated apoptosis, which is normally inhibited by Stat3. From our findings presented here, we anticipate that Stat3 inhibitors will be effective in patients with tumors expressing strong, nuclear ptyrStat3 levels and high serum IL6, indicative of autocrine gp130 signaling. As Stat3 inhibitors are currently undergoing clinical trials across the United States, this work will further our understanding of how these inhibitors may exert their anti-tumor action and also contributes to the growing body of evidence in support of the development of targeted cancer therapies against Stat3.
Figure 6.1

A: Stat3 activation by cadherin engagement

1. Ligation of E-cadherin or cadherin-11 results in the upregulation of Rac1 and Cdc42 protein levels and activity. This may lead to the autocrine induction of IL6 family cytokines via NFκB. Stat3 activation by cadherin engagement generates a potent survival signal, possibly via upregulation of survivin, and others such as bcl-xL or mcl-1.
2. Mutationally activated Rac and Cdc42 may activate Stat3 by increasing the expression of IL6 family cytokines leading to Stat3 signalling via the gp130 receptor. Activation of Stat3 is required for Rac\textsuperscript{V12}-mediated migration and cell proliferation.

3. Lipofectamine or calcium phosphate transfection can activate Stat3, even in the absence of DNA. This may occur through endosomal trafficking or Toll-like receptor activation of NF\kappa B, which may trigger the cytokine mediated-Stat3 activation. Alternatively, the physical presence of a precipitate may encourage cell-cell contact and cadherin engagement.

B: Stat3 inhibition can promote apoptosis

The E2F transcription factor lies downstream of a number of oncogenes, such as Ras, Src, and Myc as well as the SV40 Large T antigen (TAg), which may inhibit the binding between the retinoblastoma-susceptibility gene product (Rb) and E2F, leading to transcriptional activation of genes involved in cell division and apoptosis. Stat3 controls cell fate by inhibiting E2F-mediated apoptosis and promoting cell survival. Stat3 inhibition may lead to apoptosis through E2F-dependant mechanisms.

The following publications were the result of this work:


Submitted, or manuscripts in preparation


I also contributed to the following publications which do not constitute part of this thesis:


*equal contribution.

**Manuscripts in preparation**

References


Anagnostopoulou, A. The role of Stat3 in cell division and apoptosis. 2009. Ref Type: Thesis/Dissertation


Appendix
Figure A.1

The effect of cell density on Stat3 activity levels.

A: Examination of E-cadherin levels in HC11 cells.

Detergent cell lysates from HC11 (lane 1) or Balb/c 3T3 (lane 2) cells were resolved by gel electrophoresis. The blot was cut into strips which were probed for E-cadherin (top) or Hsp90 (bottom). Arrows at the right point to the positions of E-cadherin or Hsp90, as indicated. Numbers on the left refer to molecular weight markers.

B: HC11 cells are growth arrested at high densities.

0.3x10^5 HC11 cells were plated in 3 cm petris and cell numbers obtained at different times, as indicated. Cell numbers plateaued at 2 days post-confluence, indicating the absence of overt cell division during this time.

C: Cell density upregulates nuclear Stat3-ptyr705.

HC11 cells were grown to a confluence of 30% (lanes 1-3) or 2 days post confluence (lanes 4-6). Cell fractionation experiments were conducted by lysing cells in Hypotonic buffer (40 mM Hepes pH 6.0, 2 mM EGTA, 2 mM EDTA) and protease and phosphatase inhibitors, followed by Dounce homogenisation. Nuclei were collected by low speed centrifugation and proteins extracted with Triton X-100 from the nuclear pellet (Vultur et al., 2004). Nuclear and cytoplasmic cell fractions were subsequently resolved by gel electrophoresis and blots were cut into strips which were probed for Stat3-ptyr705 or Hsp90 as a loading control.

D: Cell density upregulates Stat3 transcriptional activity

HC11 cells were transfected with the Stat3-dependent pLucTKS3 reporter driving a firefly luciferase gene under control of the C-reactive gene promoter element, and the Stat3-independent pRLSRE reporter driving a Renilla luciferase gene under control of the c-fos SRE promotor, respectively. Cells were grown to the indicated densities with daily media changes and firefly (■) or Renilla (□) luciferase activities determined in detergent cell extracts (see Chapter 3, Materials and Methods). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.
Figure A.2

Weak Stat3 activation in ES cells by the LIF present in the growth medium.

To examine the contribution of the LIF present in the ES cell medium, to Stat3 activation, the E-cadherin defective null cells were grown to 30% or 100% confluence in complete ES medium, containing 15% fetal calf serum and 1x10³ units LIF/ml, then grown in medium lacking LIF for 24 hours (lanes 1, 4), followed by complete ES medium containing LIF (lanes 3, 6). Starved null cells were also grown in medium conditioned by growing 100% confluent, Ecad+/+ cells in 10 cm petris with 10 ml serum-free DMEM (lanes 2, 5). Detergent cell extracts were probed for Stat3-ptyr705 or Hsp90, as indicated. Note the small but detectable increase in Stat3-ptyr705 levels upon addition of ES medium (lanes 3, 6 vs 1, 4), but not by conditioned medium (lanes 2, 5). Note that 100 μg protein were loaded to better visualize the differences.
Figure A.3

ES cells do not differentiate when cultured to high densities, in the presence of LIF

Ecad+/+ cells were grown to 30% confluence in the absence of LIF for 5 days (a, b), or to 5 days post-confluence, in the presence of LIF (c, d), both using a particular lot of fetal calf serum which did not allow differentiation in the presence of LIF. Cells were subsequently fixed and stained for the epithelial cell marker, cytokeratin-8 with the TROMA-1 rat monoclonal antibody (Larue et al., 1996). Note the absence of stained, differentiated cells in d and their presence in a and b (arrow).
Figure A.4

**E-cadherin ablation inhibits the density-mediated, Stat3 activation.**

**A:** Proteins in detergent cell extracts (30 μg) from cell lines established from wild-type (Ecad+/+), E-cadherin-/- (null) and null cells where E-cadherin was reexpressed through transfection (addback) were grown to 2 days postconfluence, resolved by gel electrophoresis, and Western immunoblots cut into strips which were probed for E-cadherin, ptyr-705 Stat3, phospho-Erk1/2, or Hsp90 as a loading control, as indicated.

**B:** Stat3 ptyr-705 phosphorylation: The addback cells were grown to increasing densities, up to 8 days post-confluence. Detergent cell lysates were resolved by gel electrophoresis and the blot was probed for Stat3-ptyr705 (top), total Stat3 (middle) or Hsp90 as a loading control (bottom), as indicated.
C: Stat3 DNA binding activity:

Autoradiogram of Stat3:sis inducible element (hSIE) complexes, in nuclear extracts from Ecad+/+ (lanes 1-4) or null (lanes 5-8) cells prepared at the indicated densities, in EMSA assays (Vultur et al., 2005). Arrow points to the position of the Stat3:hSIE complex. In lanes 4, 8, 12 and 15, the same samples as in lanes 3, 7, 11 and 13, respectively, were incubated with an anti-Stat3 antibody. Lane 14: The same sample as in lane 13 was incubated with an anti-Stat1 antibody. The position of the supershifted Stat3-hSIE-antibody complex (lanes 4, 8, 12, 15) is indicated with an asterisk. Note the absence of supershifting with the Stat1 antibody.

D: Stat3 transcriptional activity:

Ecad+/+ and null cells were transfected with the Stat3-dependent pLucTKS3 reporter driving a firefly luciferase gene under control of the C-reactive gene promoter element, and the Stat3-independent pRLSRE reporter driving a Renilla luciferase gene under control of the c-fos SRE promotor, respectively. Cells were grown to the indicated densities and firefly ( nodo) or Renilla ( nodo) luciferase activities determined in detergent cell extracts with the peak value of the control, src-527F transformed cells taken as 100% (Vultur et al., 2004). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.
Figure A.5

Plating HC11 cells on cadherin-coated surfaces for more than 10 hours or at high cell densities does not activate Stat3.

A: Plating HC11 cells on E/EC12-coated surfaces for 10 hrs is not sufficient to activate Stat3.

0.3x10⁵ HC11 cells were plated in 3 cm plastic petris coated with the indicated amounts of the E/EC12, E-cadherin fragment. 10 hours after plating, when confluence was ~40%, 30 μg of detergent cell lysates were resolved by gel electrophoresis. The blot was cut and probed for Stat3-ptyr705 (top) or Hsp90 (bottom). Lane 5: HC11 cells grown on control, uncoated plastic petris and harvested at 2 days post-confluence, at which time Stat3-ptyr705 levels were examined as above (see Materials and Methods).

B: Stat3 activation upon plating HC11 cells on E/EC12-coated surfaces at 70% confluence is less pronounced than at lower cell densities.

1x10⁵ Balb/c 3T3 (lanes 1 and 2) or HC11 (lanes 3-11) cells were plated in 3 cm plastic petris coated with the indicated amounts of the E/EC12, E-cadherin fragment (lanes 2 and 4-8) or the E/W2A mutant (lanes 9-10), as indicated. 48 hours after plating, when confluence was ~70%, 30μg of detergent cell lysates were probed, as above, for Stat3-ptyr705 (top) or Hsp90 (bottom). Lane 11: HC11 cells grown on control, uncoated plastic petris and harvested at 1 day post-confluence, at which time Stat3-ptyr705 levels were examined as above (see Materials and Methods). Arrows point to the positions of Stat3-ptyr705 or Hsp90, respectively. Numbers at the left refer to molecular weight markers.
E-cadherin engagement is sufficient to activate Stat3 in ES cells.

Ecad+/+ (lanes 5-8), null (lanes 1 and 2) and addback (lanes 3 and 4) cells were grown in plastic petris coated with 100 µg/ml E/EC12 E-cadherin fragment or the E/W2A mutant (lane 7), as indicated. To adjust for differences in growth rates among the different cell lines, 0.3x10^5, 0.4x10^5 or 0.5x10^5 cells per 3 cm plate were seeded for the Ecad+/+, addback (i.e. null cells where E-cadherin was re-expressed through transfection) and null cells, respectively. Detergent cell extracts were prepared 48 hours thereafter, when confluences were ~40% for all three lines, and resolved by gel electrophoresis. The blot was cut and probed for Stat3-ptyr705 (top panel) or Hsp90 (bottom panel). Lane 8: Ecad +/- cells were grown on control, uncoated plastic petris and harvested at 2 days post-confluence, at which time Stat3-ptyr705 levels were examined as above. Numbers under the lanes of the top panel refer to Stat3-ptyr705 band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90 levels, with the peak value of the control, Ecad+/+ cells at 2 days after confluence taken as 100% (see Materials and Methods). Arrows point to the positions of Stat3-ptyr705 or Hsp90, respectively. Numbers at the left refer to molecular weight markers.
Figure A.7

**Stat3 activation after plating different numbers of cells for 24 hours.**

Different numbers of HC11 cells (0.05x10^6, 0.1x10^6, 0.5x10^6, 1x10^6, 1.8x10^6, 2x10^6 or 3x10^6 per 3cm petri, respectively) were plated in plastic petri dishes. 24 hours later, detergent cell lysates were resolved by gel electrophoresis and Western blots cut into strips which were probed for Stat3-ptyr705, Rac1, Cdc42 or Hsp90 as a loading control, as indicated.
Figure A.8

Toxin B can inhibit the induction of the density-mediated, Stat3 tyr705 phosphorylation

Sparsely growing, HC11 cells (lane 1) were re-plated to a high density (3 x 10^6 cells / 3 cm plate, lanes 2-4). Following attachment for one hour (lane 2), cells were treated with the DMSO carrier (lane 3) or 50 pg/ml toxin B (lane 4) for 5 hours and cell extracts resolved by gel electrophoresis. The blot was cut into strips and probed for Stat3-ptyr705 or Hsp90 as a loading control, as indicated.
Figure A.9

Reduction of Rac1 or Cdc42 levels or activity prevents Stat3-tyr705 phosphorylation in mouse 10T1/2 fibroblasts

A: Examination of cadherin-11 mRNA levels in 10T1/2, HC11 and Balb/c 3T3 cells. Extracts from 10T1/2, HC11 or Balb/c 3T3 cells were probed by RT-PCR for cadherin-11 (lanes 4-6), with 18S RNA as a control (lanes 1-3, see Materials and Methods). Numbers on the left refer to the molecular weight marker lanes (M). 10T1/2 cells also show some E-cadherin expression by RT-PCR, although not nearly as much as HC11 cells (not shown).

B: The Toxin B, Rho GTPase protein family inhibitor prevents the density-mediated, Stat3-ptyr705 phosphorylation in fibroblasts.

Left: 10T1/2 cells were grown to different densities with daily media changes and treated with 50 pg/ml toxin B for 5 hrs (lanes 6-10), or not (lanes 1-5) and lysates probed for Stat3-ptyr705 (top panel), total Rac1 (middle panel) or Hsp90 as a loading control (bottom panel), as indicated. Right: 10T1/2 cells were grown to 1 day post-confluence and treated with 50 pg/ml toxin B for 5 hours (lane 2) or the DMSO carrier alone (lane 1) and the activity of Rac1 or Cdc42 determined (see Materials and Methods).

C: The NSC23766 Rac1 inhibitor prevents the density-mediated, Stat3-ptyr705 phosphorylation.

Left: For treatment with the NSC23766 Rac inhibitor (Calbiochem), 10T1/2 cells were allowed to grow to various densities and were incubated with 100 μM inhibitor for 12 hours (lanes 9-16), or not (lanes 1-8), as described (Gao et al., 2004), and lysates probed for Stat3-ptyr705 (top panel), or Hsp90 as a loading control (bottom panel), as indicated. Right: 10T1/2 cells were grown to 1 day post-confluence and treated with 100 μM NSC23766 for 12 hours (lane 2) or the DMSO carrier alone (lane 1) and the activity of Rac1 determined (see Materials and Methods).

D: The dominant-negative Rac1^{N17} and Cdc42^{N17} mutants prevent the density-mediated, Stat3-ptyr705 phosphorylation in 10T1/2 fibroblasts.

Left: 10T1/2 or 10T1/2 stably expressing Rac1^{N17} (top) or Cdc42^{N17} (bottom) were grown to different densities, as indicated, and lysates were probed for Stat3-ptyr705 or Hsp90. Right: 10T1/2 and 10T1/2 stably expressing Rac1^{N17} or Cdc42^{N17} were grown to 1 day postconfluence and probed with an antibody specific for the myc-tag. Hsp90 was used as a loading control.
Figure A.10

Reduction of Cdc42 levels or activity prevents Stat3-tyr705 phosphorylation.

A: The dominant-negative Cdc42$^{N17}$ mutant prevents the density-mediated, Stat3-ptyr705 phosphorylation.

Left: Increasing numbers of HC11 cells as indicated, before (lanes 1-4) or after (lanes 5-8) transfection with the dominant-negative mutant Cdc42$^{N17}$ (a gift from Dr Graham Côté) were plated in 3 cm dishes and lysates probed for Stat3-ptyr705, active Cdc42-GTP, total Cdc42, myc or Hsp90, as indicated.
**Right:** Increasing numbers of HC11 cells as indicated, before or after transfection with Cdc42$^{N17}$ and the Stat3-dependent pLucTKS3 and the Stat3-independent pRLSRE reporters, were lysed and firefly (◼) or Renilla (□) luciferase activities determined (Vultur et al., 2004). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.

**B: Cdc42 downregulation through expression of an shRNA prevents the density-mediated, Stat3-tyr705 phosphorylation.**

**Top:** For Cdc42 shRNA expression, cells were transfected with two iLenti-EGFPmCdc42 constructs (Applied Biological Materials), and selected for G418 resistance (see Supplementary Table S3.4). HC11 cells before (lanes 1-4) or after (lanes 5-8) stable expression of a Cdc42-shRNA were grown to different densities and lysates probed for Stat3-ptyr705, active Cdc42-GTP (right), total Cdc42 or Hsp90 as a loading control, as indicated.

**Bottom:** HC11 cells before or after stable expression of a Cdc42-shRNA as indicated, were transfected with the Stat3-dependent pLucTKS3 and the Stat3-independent pRLSRE reporters, grown to different densities, then lysed and firefly (◼) or Renilla (□) luciferase activities determined (Vultur et al., 2004). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means ± s.e.m. of at least 3 experiments, each performed in triplicate.
Figure A.11

Rac$^{V12}$ and Cdc42$^{V12}$ activate Stat3 in 10T1/2 cells at all densities

**A:** Both endogenous and mutant Rac$^{V12}$ protein levels and activity are dramatically increased by cell density, while Rac$^{V12}$ expression increases Stat3-ptyr705 levels at all cell densities

10T1/2 fibroblasts (lanes 1-6) or their counterparts stably expressing activated Rac$^{V12}$ (lanes 7-12) were grown to increasing densities, up to 4 days post-confluence. Detergent cell lysates were probed for total Rac, active Rac-GTP, Stat3-ptyr705, or Hsp90 as a loading control, as indicated (see Materials and Methods). Numbers at the left refer to molecular weight markers.

**B:** Both endogenous and mutant Cdc42$^{V12}$ protein levels and activity are dramatically increased by cell density, while Cdc42$^{V12}$ expression increases Stat3-ptyr705 levels at all cell densities

10T1/2 fibroblasts (lanes 1-7) or their counterparts stably expressing activated Cdc42$^{V12}$ (lanes 8-14) were grown to increasing densities, up to 4 days post-confluence. Detergent cell lysates were probed for total Cdc42, active Cdc42-GTP, Stat3-ptyr705, or Hsp90 as a loading control, as indicated.
Figure A.12

Rac-dependent Stat3-ptyr705 phosphorylation requires JAK and NFκB in Balb/c 3T3 cells

A: The IKK inhibitor III inhibits the density-mediated, Stat3, tyr705 phosphorylation in Balb/c 3T3 cells. Balb/c cells were trypsinised and plated at a high density (2.5x10⁶ cells/3 cm² plate). Following attachment 30 min later, cells were treated for 48 hrs with the IKK inhibitor III (lane 3), or the DMSO carrier (lane 2). Cell extracts were probed for Stat3-ptyr705, Rac or Hsp90, as a loading control.

Lane 1: Balb/c cells immediately after attachment to the plastic.

B: JAK inhibitor 1 reduces the Rac-dependent, Stat3-tyr705 phosphorylation in Balb/c 3T3 cells.

Balb/c 3T3 cells were grown to a high density and treated with 0 (lane 1), or 10 (lane 2) μg/ml JAK inhibitor 1. Cell lysates were probed for Stat3-ptyr705, Rac or Hsp90 as a loading control, as indicated.
Figure A.13

Stat3 can be phosphorylated at critical tyrosine 705 in E-cadherin deficient cell lines

A, top panel: Normal mammary epithelial HC11 cells were grown to different densities and lysates blotted and probed for E-cadherin (lanes 1-3). Chinese Hamster Ovary cells, while deficient in E-cadherin (lanes 4-5), and others, do not undergo contact inhibition when they reach confluence, as a consequence many cells detach from the substratum and remain floating with apoptotic morphology [(Fiore and Degrassi, 1999); not shown]. Mouse L fibroblasts do not express E-cadherin (lanes 6-8), and appear to lack cadherin-based cell adhesion activity (Fukata and Kaibuchi, 2001). The A431D cell line is a derivative of A431, originating from human epidermoid carcinoma of the vulva, that has ceased to express E-cadherin (lanes 9-12), as well as N- or P-cadherins (not shown) due to chronic treatment with dexamethasone (Lewis et al., 1997).

A, bottom panel, and B: E-cadherin expressing HC11 cells, as well as CHO-B1, L cells and A431D were grown to difference densities and lysates blotted and probed for ptyr-705 Stat3 (A, bottom, and B), with Hsp90 as a loading control.

Note the dramatic increase in ptyr-705 Stat3 with cell density in L cells, A431D and CHO-B1, which lack E-cadherin expression. Our results suggest that these cell lines may contain other, possibly unidentified, cadherins that may mediate the cell confluence dependent Stat3 activation, or that additional mechanisms exist to increase ptyr705-Stat3 by cell density. Full
time course experiments indicate that L cells, A431D and CHO-B1 reach their peak of tyr705 phosphorylation much later than HC11, possibly due to their slower growth rate. Interestingly, total Rac1 levels were also found to increase with confluence in all cell lines (not shown).

A431D cells and L fibroblasts were a gift from Dr. Juliet Daniel, McMaster University. Chinese Hamster Ovary cells expressing the B1 isoform of murine Fc receptor II (CHO-B1) were from Dr. Bruce Elliott.

Figure A.14

**N-cadherin expression increases Stat3 tyr705 phosphorylation in ES cells**

Wild type, ES null cells and null cells where the N-cadherin gene was added back [Ncad-addback cells (Larue et al., 1996)] were grown to different densities and total cell lysates blotted against the tyr705, phosphorylated Stat3, and αtubulin as a loading control.
**Figure A.15**

**E-cadherin levels increase with cell density**

HC11 cells were grown to different densities, as indicated, and lysates blotted and probed for E-cadherin (top) or Hsp90 (bottom), as a loading control. Note the increased E-cadherin levels in confluent cells, suggesting the possibility that E-cadherin may be degraded at low cell densities, or that cell density could be increasing transcription from the E-cadherin promoter.
### Table A.1

#### A. Quantitation of Rac1, Cdc42 and Stat3 levels in HC11 cells and their derivatives with reduced Rac1 or Cdc42 protein levels.

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<thead>
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<th>confluence (%)</th>
<th>Rac1-GTP (%)</th>
<th>total Rac1 (%)</th>
<th>Rac1-GTP/total Rac1 (%)</th>
<th>Cdc42-GTP (%)</th>
<th>total Cdc42 (%)</th>
<th>Cdc42-GTP/total Cdc42 (%)</th>
<th>Stat3-705 (%)</th>
<th>total Stat3 (%)</th>
<th>Stat3-705/total Stat3 (%)</th>
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#### B. Quantitation of Rac1, Cdc42 and Stat3 levels in HC11 cells and their derivatives expressing dominant-negative mutants.

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* Confluence was estimated visually and carefully quantitated by imaging analysis of live cells under phase contrast as well as fixed cells stained with Coomasie blue, using a Leitz Diaplan microscope (Rockleigh, NJ).

† Active Rac1-GTP was quantitated by measuring the amount of Rac1 bound to its effector PAK, with the levels for HC11 cells at 7 days post confluence taken as 100% (see Materials and Methods). The results are averages of at least three independent experiments ± s.e.m.

‡ Total Rac1 or Rac1 N17 were measured by Western blotting. The results are averages of at least three independent experiments ± s.e.m.

§ Active Cdc42-GTP was quantitated by measuring the amount of Cdc42 bound to its effector PAK with the levels for HC11 cells at 7 days post confluence taken as 100% (see Materials and Methods). The results are averages of at least three independent experiments ± s.e.m.

|| Total Cdc42 or Cdc42 N17 were measured by Western blotting. The results are averages of at least three independent experiments ± s.e.m.

** Stat3-pyr705 levels were quantitated by fluorimager analysis and normalized to Hsp90 levels, with the values for HC11 cells at 3 days post-confluence taken as 100%. The results are averages of at least three independent experiments ± s.e.m. In all cases, the EMSA and transcriptional activity values obtained paralleled the Stat3-705 phosphorylation levels indicated.

†† Total Stat3 levels were measured by Western blotting. The results are averages of at least three independent experiments ± s.e.m.

‡‡ Cells were counted 48 hours after plating on 3cm dishes.
### Table A.2

#### A. qRT-PCR array for cytokines secreted by densely-growing HC11 cells

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<th>Gene</th>
<th>Fold up- or downregulation (confluent/sparse)</th>
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### B. qRT-PCR array for cytokines secreted by densely-growing Balb/c 3T3 cells

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Table A.3
Stat3 tyr-705 phosphorylation and apoptosis following cadherin ablation or inhibition with peptides*

| Cell line  | E-cadherin levels (%)† | Peptide‡ | Confluence (%)§ | Stat3-ptyr705 (%)|| | Cells in subG1 (%)** |
|------------|------------------------|----------|-----------------|------------------|-------------------|----------------------|
| Ecad+/+    | 100±7                  | -        | 90              | 66±6             | 2±1               |
|            |                        |          | 100             | 73±5             | 5±2               |
|            |                        |          | 100+2 days      | 100±9            | 6±3               |
| addback    | 25±8                   | -        | 90              | 27±3             | 3±2               |
|            |                        |          | 100             | 35±4             | 22±2              |
|            |                        |          | 100+2 days      | 63±3             | 35±9              |
| null       | 0                      | -        | 90              | 8±5              | 5±1               |
|            |                        |          | 100             | 10±7             | 45±9              |
|            |                        |          | 100+2 days      | 20±5             | 71±5              |
| HC11       | 100±5                  | -        | 100+1 day       | 100±7            | 5±2               |
|            |                        |          | 9±3             | 65±5             |
|            |                        |          | 72±4            | 10±4             |
|            |                        |          | 97±4            | 7±8              |
| HC11-Stat3C|                        | SHAVSA   | 100+1 day       | 263±33           | 3±1               |
| HC11-Stat3C|                        | SHAVSS   | 100+1 day       | 239±29           | 4±1               |
| HC11-Stat3C|                        | SHGVSA   | 100+1 day       | 270±24           | 4±1               |

* Lysates from the indicated lines grown to different degrees of confluence and up to 2 days post confluence as indicated, with or without treatment with the peptide inhibitors for 24 hours, were resolved by gel electrophoresis. Western immunoblots were probed for the tyr705 phosphorylated form of Stat3 or for E-cadherin. Averages of at least three experiments ± s.e.m. are shown (see Materials and Methods).
† E-cadherin levels were quantitated by fluorimager analysis and normalized to Hsp90 levels. The results are averages of at least three independent experiments ± s.e.m. with the value for Ecad+/+ or untreated HC11 cells taken as 100%.

‡ HC11 cells were treated with 10 mM of the indicated peptides for 24 hours (see Materials and Methods). The results are averages of at least three independent experiments ± s.e.m.

§ Confluence was estimated visually and carefully quantitated by imaging analysis of live cells under phase contrast as well as fixed cells stained with Coomasie blue, using a Leitz Diaplan microscope (Rockleigh, NJ).

|| Stat3-ptyr705 levels were quantitated by fluorimager analysis and normalized to Hsp90 levels, with the values for Ecad+/+ cells or HC11 cells at 1 day post-confluence taken as 100%. The results are averages of at least three independent experiments ± s.e.m. In all cases, the EMSA and transcriptional activity values obtained paralleled the Stat3-705 phosphorylation levels indicated.

** To quantitate apoptosis, cells were stained with propidium iodide and their sub-G1 profile analysed by FACS analysis. Numbers refer to cells in sub-G1 as a % of total cells. The results are averages of at least three independent experiments ± s.e.m.
## Table A.4

**shRNA sequences**

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Table A.5
qRT-PCR array for cytokines secreted by H-Rac\textsuperscript{v12} cells

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**References**


