CHAPTER I

INTRODUCTION

Non-receptor tyrosine kinase Src

The Src family kinases (SFK) are a group of non-receptor tyrosine kinases that are implicated in several aspects of cell signaling. Among the proteins that belong to the SFK, six are usually restricted to hematopoietic tissues (Lyn, Lck, Hck, Blk, Fgr and Yrk), three are ubiquitously expressed in most tissues (Src, Yes and Fyn) and a subgroup is predominantly expressed in epithelial cells (Frk-related kinases). Src kinase is considered the prototypical protein of the Src family kinases (reviewed in Thomas and Brugge, 1997).

Src is one of the oldest oncogenes, and its investigation from the early 1900s resulted in two Nobel Prizes to date (Peyton Rous in 1966, Bishop and Varmus in 1989). Peyton Rous first described a virus that seemed to promote tumor formation in chickens (Rous, 1911, Rous, 1991b). The unsettling concept of a viral agent causing cancer was not well accepted until 1970, when Steven Martin (Martin, 1970) demonstrated that chicken cells transformed with a temperature-sensitive mutant of Src reversed the transformed phenotype when kept at the restrictive temperature. The viral Src gene (*v-Src*) was identified and sequenced in 1976, and great advances have since been made regarding its function. In 1976, the *c-Src* genetic sequence was found in the normal cellular genome, suggesting that v-Src could be derived from normal cells and incorporated into the viral genome by recombination (Stehelin, 1976). Interestingly, v-

Src has a C-terminal deletion not observed in the cellular Src sequence (Takeya, 1983), which turned out to be critical for Src activity regulation (Cooper, 1986; Cooper, 1986b; Cartwright, 1987).

The linear structure of Src kinase is displayed in figure 1. The Src structure contains four *Src homology* (SH) domains: SH1 corresponds to the kinase domain and contains the auto-phosphorylation site (Tyr418 in mouse Src, Tyr416 in human Src); the SH2 domain interacts with the C-terminal auto-inhibitory domain; the SH3 domain interacts with the linker region (Figure 2) and the SH4 domain contains the myristoylation site, important for targeting Src to the membrane. Note that v-Src protein differs from c-Src because of the lack of an autoinhibitory domain in the C-terminus, resulting in constitutive kinase activation of the v-Src protein (Takeya, 1983).

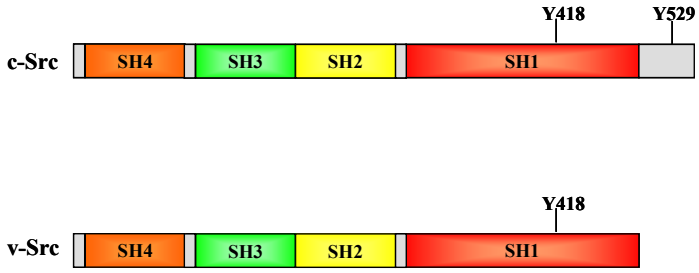


Figure 1: Diagram of Src kinase structure. The structures of c-Src (top) and v-Src (bottom) are essentially conserved, except for the loss of the auto-inhibitory domain (Y529) at the C-terminus. SH1 domain corresponds to the kinase domain. SH – Src homology domain.

Regulation of Src kinase activity

Src is tightly regulated in normal cells, and aberrant kinase activity is often reported in several types of cancer (Frame, 2002; Summy and Gallick, 2003). The three-dimensional structure of Src shows that the SH2 and SH3 domains are critical in regulating the kinase activity of Src by interacting with other domains of the protein and keeping it in a 'closed', inactive conformation (Xu, 1997). As indicated in figure 2, Src kinase activity is regulated by intramolecular interactions between the SH2 domain and the auto-inhibitory tyrosine site (Tyr529 in mouse Src). The tyrosine in the auto-inhibitory domain can be phosphorylated by the Carboxy-terminal Src kinase (Csk; Nada, 1991), creating affinity for the SH2 domain and keeping Src in a closed (inactive) conformation. Expression of a dominant negative mutant of Csk promotes invasiveness in colon cancer cell lines, while overexpression of the wild type Csk reduced migration and invasion (Rengifo-Cam, 2004), indicating that the regulation of Src activity is critical for colon cancer progression. The tyrosine in the auto-inhibitory domain can be dephosphorylated by several protein tyrosine phosphatases, including (PTP)α, PTPγ, PTP1B and SHP-2. Another critical intramolecular interaction that negatively regulates Src activity is the binding of the SH3 domain to the poly-proline region present on the linker region, which lies between the kinase and the SH2 domains (see figure 2). The autophosphorylation site (Tyr418 in mouse Src) in the catalytic domain is another mechanism of Src kinase activity regulation, allowing the protein to adopt an open (active) conformation (Smart, 1981). Although this tyrosine site is not required for Src kinase activity (Snyder, 1983), mutation on this site has been shown to reduce the transforming potential of both v-Src and mutant variations of c-Src, indicating that this

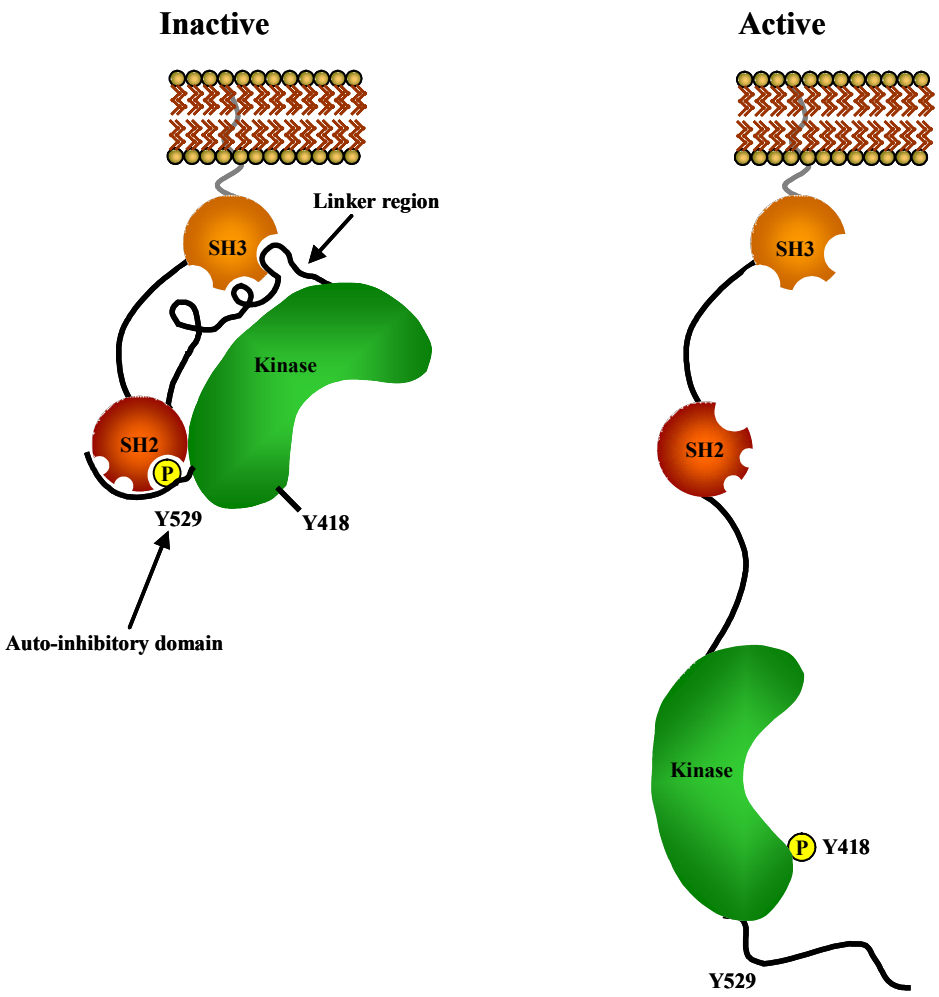


Figure 2: Diagram of Src kinase activation. Diagram represents the inactive (left) and active (right) conformations for comparison. The two intramolecular interactions that regulate Src activation are indicated by arrows. Upon phosphorylation of the auto-inhibitor site (Y529 for mouse Src, see text for more details) the C-terminus of Src binds to the SH2 domain and the linker region binds to the SH3 domain, resulting in a closed molecular structure. This closed (inactive) conformation reduces the access of Src substrates to the kinase domain. On the other hand, dephosphorylation of Y529 results in an open (active) molecular structure, giving Src substrates access to the kinase domain. Full Src activation requires the autophosphorylation of Y418 (in mouse; see text for more details). Binding to other molecules such as CAS (Crk-associated substrate) or FAK (focal adhesion kinase) displaces the intramolecular inhibitory interactions and results in activation of Src (not shown in the figure). The gray line indicates myristoylation sites important for membrane localization. Model adapted from Thomas and Brugge (1997).

phosphorylation site is important *in vivo* (Kmiecik, 1987; Piwnica-Worms, 1987; Snyder, 1984).

Src kinase substrates

Cell-ECM adhesion molecules and Src signaling

Src kinase has several cellular substrates with diverse function (reviewed in Thomas and Brugge, 1997) some of which are implicated in critical roles in several biological processes, including cell proliferation (Irby, 1997; Staley, 1997), disruption of cell-cell adhesions (Frame, 2002), invasion (Pories, 1998), epithelial-to-mesenchymal transition (EMT, Avizienyte, 2004) and metastasis (Boyer, 2002). In particular, several Src substrates, including paxillin, CAS (*Crk*-associated substrate), talin, vinculin, tensin, FAK (focal adhesion kinase) and the β1 integrin subunit have been identified in focal adhesions, sites of cell-ECM (extracellular matrix) adhesion, suggesting that Src might be a regulator of focal adhesion dynamics. The dynamics of focal adhesions can deeply impact the behavior of both normal and transformed cells.

Two of the major Src substrates, FAK (Hanks, 1992; Schaller, 1992) and CAS (Reynolds, 1989; Matsuda, 1990) were shown to interact not only with Src, but also directly with each other, in an intricate mechanism to promote CAS activation (Ruest, 2001). CAS signaling has been implicated in the regulation of the actin cytoskeleton, cell motility, growth and survival (Bounton, 2001). More details regarding CAS signaling are discussed in Chapter V.

FAK is activated upon integrin engagement or growth factor receptor activation

through autophosphorylation of its Tyr397 site, followed by binding of the Src kinase SH2 domain to this phosphorylated site. Phosphorylation of FAK Tyr397 results in increased affinity not only to Src, but also to signaling molecules such as: PI3K (phosphatidylinositol-3-OH kinase), phospholipase C-γ and adaptor proteins Nck2 and Shc (Src homology and collagen protein) and Grb7. Upon Src binding, tyrosine phosphorylation of FAK creates binding sites for other molecules and activates several signaling pathways (reviewed in Brunton, 2004; Parsons, 2008; Siesser and Hanks, 2006). Phosphorylation of the FAK Tyr397 and Tyr925 residues results in association of Grb2 and SOS with FAK, followed by activation of the Ras-MAPK pathway (Schlaepfer, 1994). Proteins such as CAS, GRAF (a Rho-GAP), p190RhoGEF and ASAP1 (an Arf1 and Arf6 GAP) associate with FAK by binding to proline rich sequences (PR1 and PR2) in FAK, while paxillin binds to the C-terminal FAT (focal adhesion targeting) domain. With this remarkable signaling versatility, it is not surprising that FAK has been implicated in several cellular processes such as migration, invasion, survival and proliferation, which aberrant regulation can lead to cancer (reviewed in Frame, 2002; McLean, 2005; Mitra, 2006; Parsons, 2008; Siesser and Hanks, 2006). FAK expression is elevated in invasive and metastatic colon and breast cancer tumors, compared to normal, surrounding tissues. Moreover, evaluation of pre-invasive tumorigenic lesions revealed that FAK levels are enhanced in early stages of tumor progression (Owens, 1995).

Similar to the effects seen in CAS and Src knockout; FAK knockout results in embryonic lethality and fibroblasts derived from these mice display defects in motility (Ilic, 1995). The migration defects of FAK-/- fibroblasts are rescued by overexpression

of v-Src (Sieg, 1998). v-Src is capable of transforming FAK-/- fibroblasts, however, Src-transformed FAK-/- cells are not capable of activating MMP2 (matrix metalloproteinase) or invading matrigel, indicating that although FAK is not required for Src transformation, FAK does have a critical role in the invasion of Src-transformed fibroblasts (Hsia, 2003). Evidence that FAK can promote tumorigenesis *in vivo* comes from mouse models wherein the FAK gene was conditionally deleted. On mice with FAK conditionally depleted from keratinocytes, chemically induced skin tumor formation was abrogated, and interestingly, when FAK was conditionally depleted from keratinocytes of already existing tumors, the transition from benign to malignant tumors was impaired (McLean, 2004). Because of its multiple roles in cancer and its interaction with other critical proteins, including Src kinase, FAK is being targeted for drug development for cancer therapeutics (McLean, 2004; Parsons, 2008).

Cell-cell adhesion molecules and Src signaling

Epithelial cells are characterized by the presence of apical and basolateral domains, separated by the apical junctional complex (AJC) (Farquhar and Palade, 1963). The AJC comprises the desmosomes, tight junctions and adherens junctions. Src kinase has been observed at cell-cell junctions and contributes to their normal functioning; however, elevated Src kinase activity results in weakening and disruption of epithelial cell adhesions (Behrens, 1993; Takeda, 1995).

Elevated Src activity has profound effects in epithelial cell morphology and function and it is associated with enhanced phosphorylation of Src substrates, including E-cadherin, β-catenin and p120 catenin, components of the adherens junctions (reviewed

in Frame, 2002; Frame, 2002b). Phosphorylated E-cadherin fails to localize to the membrane and cell-cell adhesions, abrogating the recruitment of other cell-cell adhesion proteins at these locations (Avizienyte, 2002). Phosphorylation of E-cadherin has also been shown to downregulate both protein levels and gene expression, leading to EMT in carcinoma cells (Menke, 2001). Another Src substrate implicated in the regulation of cell-cell adhesions, p120 catenin (Reynolds, 1989) is able to bind and stabilize E-cadherin (Reynolds, 1994). Src phosphorylation of p 120 catenin increases its affinity for E-cadherin (Roura, 1999), yet the significance of this property is still unclear. Tyrosine sites phosphorylated by Src have been identified in β-catenin (Tyr-86 and Tyr-654) and phosphorylation of these sites results in a decreased affinity for E-cadherin and displacement from cell-cell adhesions (Roura, 1999). The free pool of β-catenin in the cytoplasm can translocate to the nucleus and act as a transcription factor in association with TCF (T-cell factor) (reviewed in Fodde, 2001). More details regarding β-catenin signaling are discussed in Chapter III.

Ras and Src signaling

Ras is a small GTPase which cycles between inactive (bound to GDP) and active (bound to GTP) states, and three isoforms have been described: K-Ras, H-Ras and N-Ras. Ras is activated by Ras-GEFs (guanine exchange factor; Wolfman, 1990) and inactivated by Ras-GAPs (GTPase-activating protein; Gibbs, 1988). Ras-GEFs function by increasing the dissociation rates of GDP and Ras, and since the concentration of GTP in the cell is much higher than GDP, dissociation results in Ras binding to GTP. The spontaneous exchange of GDP for GTP bound to Ras can be inhibited by the guanine

nucleotide dissociation inhibitors (GDIs), keeping Ras inactive. Conversely, Ras-GAPs increase the hydrolysis of GTP, resulting in Ras bound to GDP and release of the phosphate group. Therefore, Ras-GAP is critical for the inactivation of Ras and disruption of the Ras-GAP/RasGTPase complex correlates with an increase in the oncogenic potential of Ras (Scheffzek, 1997). Another critical step in the regulation of Ras activity is post-translational modifications that target the protein to the plasma membrane, where Ras will be activated and in turn, activate its downstream effectors. The major Ras post-translational modifications are farnesylation (all isoforms), palmitoylation (N-Ras and H-Ras) (Hancock, 1989, 1990) and geranylgeranylation (N-Ras and K-Ras) (McCubrey, 2006).

The highest levels of mutations in *Ras* genes have been reported in pancreas (90%), colon (50%), lung (30%), thyroid tumors (50%), and myeloid leukemia (30%), and most mutations were detected in K-Ras. A comprehensive review of the incidence of Ras mutations in other tumor types is also available (Bos, 1989). Evaluation of the prevalence of *K-Ras* mutations in a large cohort (Netherlands Cohort Study; Brink, 2003) did not find a significant difference between patients with and without a family history of colon cancer, suggesting that K-Ras mutations might be more important for sporadic rather than familial cases of colon cancer (Brink, 2003).

It has been reported that Src kinase activity activates the small GTPase Ras (Satoh, 1990) by activating the Ras-GEF (GTPase exchange factor; Li, 1993b). Spurred by the first evidence that Src kinase regulates Ras activity; work from several groups elucidated the link between Src and Ras (Buday, 1993; Egan, 1993; Gale, 1993; Li, 1993; Oliver, 1993; Rozakis-Adocock, 1993; Simon, 1993). Src regulates Ras activity by

recruiting the adaptor proteins Shc (Src homology containing-protein) and Grb2 (growth-factor receptor bound protein), which in turn recruits Ras-GEF SOS to the plasma membrane. SOS promotes the dissociation of GDP bound to Ras, resulting in binding of GTP and activation of Ras. A summary of these findings are shown in figure 3. The best characterized signaling pathway downstream of Ras is the MAPK (mitogen-activated protein kinase) pathway, nevertheless, there are several reports of other Ras-regulated pathways, including PI3K, TIAM1 (tumor invasion and metastasis inducing protein) and phospholipase C (reviewed in Roberts, 2007; Schubbert, 2007).

Activated Ras recruits Raf to the membrane, where Raf is phosphorylated, mainly on serine and threonine residues, although tyrosine residues are also phosphorylated in the presence of oncogenic Src (Morrison, 1988). Once Raf is activated, the following step on the MAPK pathway is the phosphorylation of MEK (mitogen-activated and extracellular-signal regulated kinase kinase), a dual kinase that phosphorylates ERK both on tyrosine and threonine (Crews, 1991; Crews, 1992). Activation of ERK leads to the activation of several substrates, mainly nuclear proteins, including transcription factors, phosphatases, kinases and cytoskeletal proteins (reviewed in Yoon and Seger, 2006).

Development and clinical trials with small molecules targeting most, if not all, components of the Src-Ras-MAPK pathway are underway (Roberts, 2007) and seem to be promising as cancer therapeutics.

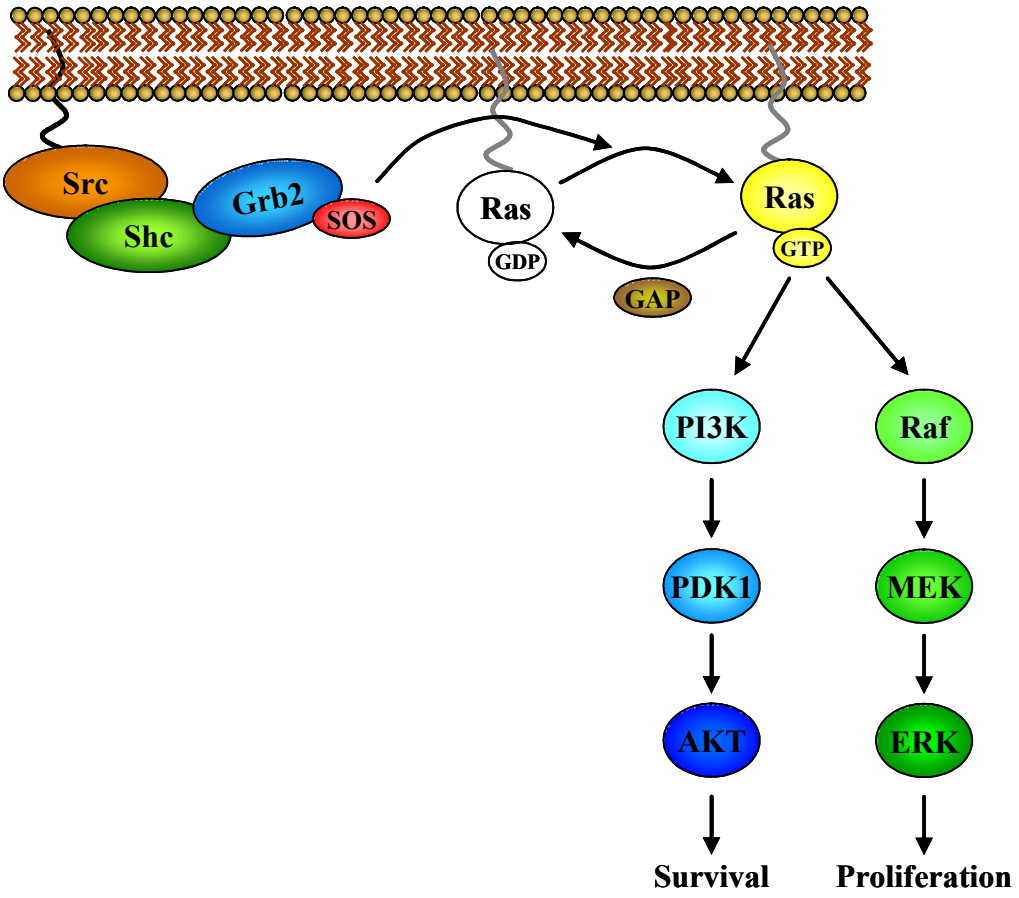


Figure 3: Diagram of Src-mediated activation of Ras. Src recruits the adaptor proteins Shc and Grb2, which recruits the Ras-GEF SOS to activate Ras. Conversely, Ras-GAP inactivates Ras by promoting hydrolysis of GTP into GDP. Two major pathways, implicated in survival and proliferation, are depicted here. Shc – Src homology containing protein; Grb - growth-factor-receptor bound protein; GEF - guanine exchange factor; GAP - GTPase-activating protein; GDP - guanosine diphosphate; GTP - guanosine triphosphate; MEK - mitogen-activated and extracellular-signal regulated kinase kinase; ERK - extracellular signal-regulated kinase; PI3K - phosphatidylinositol 3-kinase; PDK - PI3K-dependent protein kinase.

PI3K and Src signaling

In an effort to define a mechanism for Src transformation, several research groups have revealed that Src kinase activity results in activation of multiple pathways. For example, Sugimoto and collaborators (Sugimoto, 1984) reported that v-Src transformation is associated with elevated phosphatidylinositol activity. Several years later, a protein with a molecular weight of approximately 81 kDa was identified in a complex with Src and the middle T antigen in polyoma-virus transformed cells (Courtneidge, 1987; Kaplan, 1987). The protein turned out to be the regulatory subunit of PI3K, indicating that Src may regulate lipid signaling (Otsu, 1991).

Elevated PI3K signaling has been reported in several types of cancer, including prostate, breast and lung cancer, as well as melanomas and leukemia (reviewed in McCubrey, 2006). PI3K is a heterodimeric protein and contains two subunits, an 85 kDa regulatory unit and a 110 kDa catalytic subunit, named p85 and p110, respectively. The activation of PI3K can be mediated by Ras or by Src. In the first mechanism, Ras binds directly to the catalytic p110 subunit to activate PI3K. Moreover, experiments *in vivo* have shown that expression of a *Ras* dominant negative mutant correlates with reduced growth factor-induced PI3K activity, and overexpression of Ras increases PI3K levels (Rodriguez-Viciana, 1994). Direct activation of PI3K by Src was first identified in v-Src transformed fibroblasts, which contain high levels of PI3K activity. The regulatory p85 subunit of PI3K was shown to associate with the SH3 domain of Src (Liu, 1993), resulting in recruitment of the catalytic p110 subunit and activation of PI3K (Pleiman, 1994). Src can also activate PI3K via Ras, by recruiting and activating the adaptor

protein Shc (Gu, 2000). Phosphorylation of Shc creates a binding site for the SH2 domain of the adaptor protein Grb2, which recruits SOS (a Ras GEF) to activate Ras.

Upon recruitment to the membrane and activation of PI3K, the catalytic subunit p110 phosphorylates phosphoinositides, converting phosphatidylinositol 4,5 phosphate (PIP2) into phosphatidylinositol 3,4,5 phosphate (PIP3) (Auger, 1989). This phosphorylation event recruits phosphoinositol-dependent kinase 1 (PDK1) and AKT to the membrane via its pleckstrin homology (PH) domains, resulting in PDK1-mediated phosphorylation of AKT (reviewed in McCubrey, 2006). AKT has been implicated in several processes that are critical for cancer development, including cell proliferation/cell cycle progression (by targeting GSK3, mTOR, IRS-1, p21 and p27) and apoptosis (by inactivating proapoptotic factors Bad, procaspase-9 and Forkhead family transcription factors). No AKT mutations have been reported to date; however, gene amplifications have been reported in ovarian, pancreas, breast and stomach tumors (reviewed in Vara, 2004).

AKT can activate mTOR (mammalian target of rapamycin) kinase (Gingras, 1998; vonManteuffel, 1996). Activation of mTOR leads to the phosphorylation of $p70^{s6k}$, 4E-binding protein 1 (4E-BP1) and eukaryotic elongation factor 2, all implicated in ribosomal biosynthesis and mRNA translation of proteins necessary for cell cycle progression (reviewed in Gridelli, 2008). AKT and mTOR, as well as many other effectors of Src transformation are being targeted for drug development (Faivre, 2006; Vara, 2004).

There is compelling evidence that PI3K-mTOR and Ras-MAPK may collaborate to promote fibroblast transformation by v-Src. Pharmacological inhibition of each

pathway reduced, but did not abolish, cell transformation, as defined by morphological changes, anchorage-independent growth, hexose uptake and loss of contact inhibition. However, simultaneous inhibition of both pathways, using different dominant negative construct expression or addition of pharmacological inhibitors, reversed the phenotype of v-Src transformed fibroblasts to a normal cell phenotype (Penuel and Martin, 1999).

STAT3 and Src signaling

STATs (signal transducers and activators of transcription) were first described as downstream targets of cytokine signaling, including growth hormone, prolactin, interferon and erythropoietin. Upon binding of the ligand, the receptor-associated kinase JAK (Janus kinase) is activated and recruits STAT to the receptor, resulting in phosphorylation, dimerization, translocation to the nucleus and activation of transcription. Seven STATS have been identified, and while STATs 2, 4 and 6 are only activated by cytokines, STATs 1, 3, 5a and 5b can be activated by growth factor receptors, G-protein agonists and SFK members (reviewed in Martin, 2001; Silva, 2004).

STAT3 was first thought to be a potential mediator of Src transformation when Yu and collaborators (Yu, 1995) showed that the phosphorylation and DNA-binding capacity of STAT3 is elevated in Src-transformed fibroblasts compared to normal fibroblasts. Moreover, increased STAT3 phosphorylation levels and DNA-binding activity correlate well with the potential of Src to promote oncogenesis. Subsequent studies showed that v-Src can directly bind and activate STAT3, enhancing DNA binding and transcriptional activation. While Src was capable of activating STATs in the absence

of JAK, Src and JAK seem to cooperate to achieve maximal activation levels of STAT3 (Zhang, 2000; Garcia, 2001).

In vitro and *in vivo* studies suggest that Src-mediated activation of STAT3 is relevant for Src tumorigenesis. The ability of Src-transformed fibroblasts to form colonies in soft-agar was affected by STAT3 levels; while overexpression of STAT3 enhanced the capacity of v-Src-transformed fibroblasts to form colonies, three different STAT3 dominant negative mutations impaired colony formation (Bromberg, 1998). Studies with STAT3 -/- fibroblasts showed that although STAT3 is not required for normal cell proliferation, it has profound effects on the capacity of v-Src to promote soft-agar colony formation. Expression of wild type STAT3 in STAT3 -/- fibroblasts was necessary for v-Src transformation, but expression of a STAT3 with a defective DNA binding site had no effects (Schlessinger, 2005). Recently, the role of STAT3 on Src-transformation was demonstrated *in vivo* (Oneyama, 2008). The transforming capacity of Csk -/- fibroblasts expressing either c-Src, c-SrcYF (constitutively active variant) or v-Src was compared. While c-SrcYF and v-Src were similarly capable of forming large tumors in nude mice, c-Src cells, even in the absence of Csk, had a longer latency and angiogenesis defects. Characterization of the signaling pathways affected by Src expression revealed that STAT3 is phosphorylated in c-SrcYF and v-Src, but not c-Src cells. Introduction of a constitutively active form of STAT3 into c-Src cells increased the tumor volume and reduced latency. This paper suggests that Src is necessary, but not sufficient, to promote tumor progression and metastasis, which requires Src kinase activity. Elevated Src activity, which was associated with STAT3 phosphorylation, correlated with the formation of large tumors with adequate angiogenesis in nude mice.

Thus, activation of STAT3 signaling pathway seems to be required for Src to promote tumor progression and metastasis in this model.

Src kinase and cancer

Aberrant signaling of the protooncogene-encoded Src has been reported in several types of cancer, and its kinase activity is thought to have a causative effect in the progression of colon, breast, lung and skin cancer. In tumors, activation of Src kinase depends on several factors and is usually a critical event for tumor development, as it appears to regulate multiple aspects of tumor progression including: proliferation, disruption of cell-cell contacts, migration, invasiveness, apoptosis resistance and angiogenesis (reviewed in Summy and Gallick, 2003; Summy and Gallick, 2006).

As shown in figure 4, elevated Src activity can contribute to malignancy by impacting multiple receptor signaling centers including cell-cell adhesion, cell-ECM adhesion and receptor tyrosine kinases (reviewed in Avizienyte and Frame, 2005; Thomas and Brugge, 1997; Summy and Gallick, 2003; Summy and Gallick, 2006; Yeatman, 2004). In particular, the effects of elevated Src activity on cell-cell adhesions in epithelial cells result in a transition from an epithelial-to-mesenchymal phenotype. Src activity in epithelial cells weakens or disrupts E-cadherin-based cell-cell adhesion. This weakening results in a more motile and invasive phenotype (Avizienyte, 2004) and induces morphological changes by regulating the actin cytoskeleton (Boyer, 1997). Src kinase activity has been reported to contribute to the metastatic spread of carcinoma (Boyer, 2002), and it is likely that Src-induced EMT has a critical role in this process.

Enhanced Src expression and activity have been observed in ~80% of colon

adenocarcinomas (Bolen, 1987; Rosen, 1986) in stages as early as colonic polyps, where it correlated with high malignant potential (Cartwright, 1990). Interestingly, the enhanced levels of Src activity were not due to the increased levels of Src expression, but instead, it appeared to result from post-translational modifications (Cartwright, 1989; DeSeau, 1987). Further enhancement of Src expression and activity is observed with progression toward a metastatic phenotype (Talamonti, 1993; Termuhlen, 1993). Remarkably, while enhanced Src activity in liver metastases originating from colon carcinomas was observed, no changes in Src activity have been observed in metastatic tissue derived from non-colon tumors. Moreover, enhanced Src activity in metastases originating from colon carcinomas were not restricted to the liver, but has also been observed in extra-hepatic sites (Termuhlen, 1993). Furthermore, high Src activity has been described as an indicator of poor clinical prognosis in colon carcinomas (Allgayer, 2002). Studies on the effects of Src overexpression and Src kinase inhibition on colon epithelial cell lines support a critical role for Src activity in colon cancer progression. Overexpression of c-Src in a poorly metastatic human colon cell line resulted in no proliferative changes *in vitro* or metastatic capacity *in vivo*, but the growth of primary tumors was significantly higher than in control cells (Irby, 1997), suggesting that overexpression of Src, without elevated kinase activity, is not sufficient to promote metastasis. Moreover, reduced Src activity in rat carcinoma cell line overexpressing Csk or kinase-dead Src did not affect primary tumor growth in mice, but did abrogate their ability to metastasize (Boyer, 2002; Nakagawa, 2000).

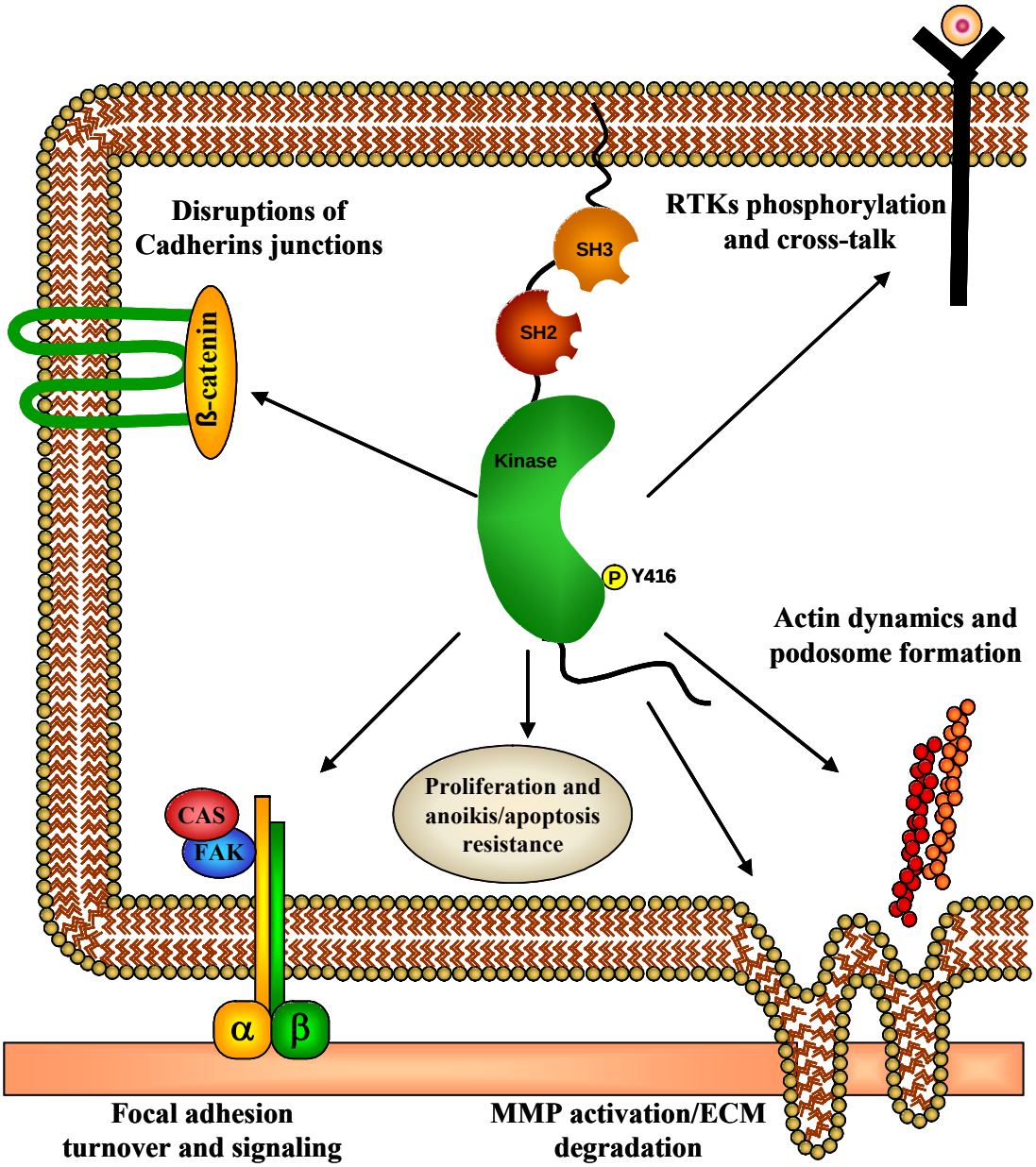


Figure 4: Diagram of Src kinase target sites within the cell. Diagram represents the effects of activated Src kinase in an epithelial cell. Arrows indicate the different molecular events that are regulated or affected by Src kinase activity. RTKs – receptor tyrosine kinases; CAS – Crk-associated substrate; FAK – focal adhesion kinase; MMP – metalloproteinase. Diagram is not to scale.

Besides its role in colon tumor progression and metastasis, elevated Src expression has been reported in early stages of tumor progression. Despite this evidence no direct causal effect on primary carcinogenesis has been demonstrated until recently. Studies with transgenic mice showed for the first time that c-Src is able to promote primary tumor formation (Kline, 2008). In the absence of any carcinogen, 15% of mice overexpressing c-Src developed cancer in the gastrointestinal tract, spleen and liver, while wild type controls did not develop tumors (Kline, 2008). Overexpression of c-Src was not able to promote metastasis in mice expressing the wild type p21 tumor suppressor. However, on a p21 -/- background, c-Src expression resulted in a significantly higher number of primary tumors and metastases. The collaboration between Src kinase and the p21 tumor suppressor to promote colon tumorigenesis supports the genetic model proposed by Vogelstein (Fearon and Vogelstein, 1990), wherein the accumulation of molecular lesions, namely activation of oncogenes and inactivation of tumor suppressor genes, promotes colon cancer progression.

Src kinase activity has also been implicated in the invasiveness of colon carcinoma cells. Activated Src has been shown to induce the transcription of urokinase plasminogen activator receptor and the transcription of this receptor could be abrogated by Src inhibitors (Allgayer, 1999). Moreover, inhibition of Src activity by overexpression of Csk resulted in reduced expression of MMP2 (Nakagawa, 2000), indicating that increased levels of Src activity correlate positively with increased invasiveness.

Another critical step for tumor formation and metastasis is the circumvention of anoikis, a particular type of apoptosis induced by cell detachment from the substrate.

Studies with colon tumor cell lines suggest that enhanced expression and activity of Src correlates with resistance to anoikis (Windham, 2002). Furthermore, overexpression of activated Src in cells with low Src expression and activity resulted in enhanced resistance to anoikis, while inhibition of Src expression in cells with high Src expression and activity resulted in increased susceptibility to anoikis (Windham, 2002).

In conclusion, elevated Src kinase expression and activity seem to have a critical role on several aspects of colon cancer including: primary tumorigenesis, invasiveness, resistance to anoikis and metastasis. However, many questions regarding the functions of Src still remain to be answered. A better understanding of Src functions may be gained from comprehensive studies of Src substrates and other factors that may synergize with Src kinase.

Src-specific kinase inhibitor AZD0530

Src kinase inhibitors for therapeutic use are under development and clinical trials for most major tumor types including: breast, lung, colorectal, pancreatic, prostate and renal carcinomas. The potential for clinical application of Src inhibitors is also being investigated for rare tumors including mesothelioma, melanoma and sarcoma (reviewed in Kopetz, 2007). Although some *in vitro* and *in vivo* studies strongly suggest that inhibiting Src kinase is effective in reducing tumor growth or invasiveness (Ple, 2004; Hennequin, 2006), commercially available, specific Src kinase inhibitors have not yet been developed.

The generation of a new class of anilinoquinazoline inhibitors with high affinity and high specificity to the tyrosine kinase domain of Src has yielded three compounds

targeting Src kinase that are currently under clinical evaluation: anilinoquinazoline AZD0530 [N-(5- chloro-1,3-benzodioxol-4-yl)-7-[2-(4-methylpiperazin-1-yl)ethoxy]-5-(tetrahydro-2H pyran-4-yloxy)quinazolin-4-amine, thiazolecarboxamide BMS-3548 and the quinolinecarbonitrile SKI-606 (Hennequin, 2006).

AZD0530 has been shown to be a highly selective and orally available, specific c-Src kinase inhibitor and is in clinical development to target different cancers including: early and advanced solid tumors, leukemia and metastatic bone disease (Hennequin, 2006). AZD0530 is currently in Phase II clinical trials to evaluate its potential therapeutic benefits for patients with previously treated metastatic adenocarcinoma of the colon or rectum. In addition, these clinical trials will determine response rates to the drug, the progression-free survival and the overall survival of patients.

Rodent models of experimental metastasis have previously been employed to characterize the effects of AZD0530 *in vivo*. Subcutaneous injection of Src-transformed fibroblasts in athymic rats and mice resulted in large tumors after three weeks. When animals were treated with a daily oral dose of AZD0530 (6 mg/kg), tumor volume and weight were inhibited by >95% (Hennequin, 2006). Besides reducing primary tumor growth, treatment with a daily oral dose of AZD0530 (25 mg/kg) increased survival compared to controls in a mouse tumor model with human pancreatic tumor cells (Green, 2004).

Inhibition of Src activity with AZD0530 reduced anchorage-independent growth in MCF-7 (estrogen receptor positive) breast cancer cells. Furthermore, combined treatment with suboptimal doses of AZD0530 and tamoxifen resulted in synergistic inhibition of anchorage-independent growth (Herynk, 2006). Recent studies with MCF-7

and T-47-D breast cancer cells have shown that treatment with the anti-estrogen drug tamoxifen as a single agent results in an initial inhibition of cell proliferation, followed by expansion of drug-resistant cells. The resistance to tamoxifen correlated with elevation of Src activity and invasive behavior. However, when both of these cell lines were treated with a combination of tamoxifen and AZD0530, invasiveness and proliferation were abrogated. Importantly, the combined treatment also prevented the emergence of a resistant phenotype (Hiscox, 2008). These results suggest that the Src inhibitor AZD0530 can be used in combination with other drugs to prevent drug resistance and simultaneously target several aspects of cancer such as anchorage-independent growth, invasiveness and proliferation. Because reduced responsiveness to chemotherapy is one of the biggest challenges for treatment in several types of cancer, including colon cancer, combining AZD0530 with some other inhibitor might be a promising therapy. While studies in breast cancer cells lines suggest that using AZD0530 in a concomitant drug treatment of cancer might be beneficial, *in vivo* studies and clinical trials are necessary before concluding that the *in vitro* effects have a clinical significance.

Hypothesis and experimental overview

The importance of Src kinase in cancer is unquestionable, and while a great amount of data has been generated since the seminal work of Peyton Rous, almost 100 years ago, there are several questions that remain unanswered. One particular question we are interested in the possible interaction between Src and tumor suppressors, as well as Src and its substrates. To better understand the role of Src in cancer, we investigated two aspects of Src signaling: the synergy between oncogenic Src and the tumor

suppressor APC (adenomatous polyposis coli) in promoting colon epithelial cell transformation as well as the role of CAS, one of the major Src substrates, in colon epithelial cell polarization.

In Chapter III, we tested the hypothesis that the collaboration of Src kinase and APC deficiency promotes colon epithelial cell transformation. Using conditionally immortalized colon epithelial cell lines, we showed that the effect of oncogenic Src expression on cell invasion is not affected by APC tumor suppressor genotype or p53 tumor suppressor functional status. However, the ability of Src kinase to promote anchorage-independent growth was significantly enhanced in an APC deficient (APC^{min}) background. Further enhancement was observed when p53 was inactivated, supporting the "multiple-hit" hypothesis of colon cancer development. Interestingly, characterization of Src-transformed cells with the capacity for anchorage-independent growth by genotyping and immunoblotting revealed that these cells retained one wild type allele of APC, suggesting that APC might be haploinsufficient in combination with oncogenic Src. Furthermore, in Chapter III, we evaluated the efficacy of a novel, specific Src kinase inhibitor (AZD0530) on colon epithelial cell invasion and anchorage-independent growth and showed that low doses (1μM) of this inhibitor can prevent podosome formation, ECM degradation and anchorage-independent growth.

In Chapter IV, we evaluated two conceivable mechanisms by which Src and APC collaborate to promote anchorage-independent growth in colon epithelial cells and demonstrate that, although Src does not affect ERK phosphorylation, β-catenin levels and activity were elevated in Src-transformed colon epithelial cells with APC deficiency.

In Chapter V, we discuss the role of CAS in Src transformation based on

published studies wherein I was a major collaborator during my graduate program. Using CAS -/- fibroblasts expressing oncogenic Src, we showed that CAS is dispensable for some aspects of Src transformation. Yet, CAS played a critical role in promoting invasion and metastasis. These results suggest that investigating the possible role of CAS in epithelial cell invasion may be valuable. Studies with polarizing colon epithelial cell lines test the hypothesis that CAS might have a role in epithelial cell polarization. Our results showed that CAS may affect epithelial cell polarization, as transepithelial cell resistance was significantly enhanced in CAS depleted Caco-2 cells in comparison with controls.

Chapter VI presents the concluding remarks and future directions for these studies.

CHAPTER II

MATERIAL AND METHODS

Reagents

Mouse monoclonal anti-phosphotyrosine antibody 4G10 and rabbit polyclonal anti-acetyl-histone H3 were obtained from Millipore (Billerica, MA). Rabbit polyclonal antibody against Src pTyr416 (mouse Src pTyr-418) came from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody clone 327 ascites against Src was a generous gift from Dr. Christine Cartwright (Stanford University). Rabbit polyclonal antibody against full length APC was a generous gift from Dr. Kristi Neufeld (University of Kansas). Mouse monoclonal anti-pan-actin antibody was obtained from LabVision Corp. (Fremont, CA). Mouse monoclonal antibody against β-catenin and E-cadherin were from BD Biosciences (San Jose, CA). Rabbit phosphospecific polyclonal antibody against ERK pThr183/pTyr185 came from Promega Corp. (Madison, WI). Rabbit polyclonal anti-ERK 2 (C14) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa-594-phalloidin was from Molecular Probes (Eugene, OR). Secondary antibodies for immunoblotting were goat anti-rabbit AlexaFluor 680 from Molecular Probes and goat anti-mouse IRDye 800 from Rockland Immunochemicals (Gilbertsville, PA). Secondary antibody for immunostaining was goat anti-mouse AlexaFluor 488 (Molecular Probes). AZD0530, a highly selective inhibitor for Src kinases (Hennequin, 2006), was provided by AstraZeneca (Alderly Park, UK). From a 10 mM stock solution prepared in DMSO, the compound was added to the cell culture media to a final

concentration of 1 μM.

Immortalized mouse colon epithelial cell lines

YAMC, IMCE, and IMCE-Ras cells were all kindly provided by Bob Whitehead (Vanderbilt University, Nashville, TN). YAMC and IMCE cells (and derivatives) were routinely cultured at 5% CO_2 levels in RPMI medium 1640 containing L-glutamine and HEPES (Invitrogen-GIBCO, Grand Island, NY) supplemented with 5% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% nonessential amino acids (GIBCO), 1% insulin-transferin selenium (GIBCO), 1% antimycotic/antibiotic (Mediatech, Herndon, VA), and 5 μg/mL plasmocin (InvivoGen, San Diego, CA). Under the "permissive" condition, YAMC and IMCE cells were maintained at 33˚C and the culture medium was further supplemented with 5 U/mL γ-interferon (Roche Diagnostics, Mannheim, Germany) to achieve expression and activation of the temperature-sensitive simian virus large T-antigen (SV40 tsA58) that acts to "immortalize" cells through inactivation of p53. To shift to the "restrictive" growth condition, the cells were grown for at least 1 day at 37˚C in the absence of γ-interferon.

Human colon epithelial cell lines

A set of cell lines derived from human colon tumors, including Caco-2 and HCA7 were a generous gift from Bob Coffey (Vanderbilt University). Caco-2 and HCA7 cell lines were routinely cultured at 5% CO_2 levels in DMEM medium (Invitrogen-GIBCO, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals), 1% nonessential amino acids (GIBCO) and 1% antimycotic/antibiotic (Mediatech).

Phoenix ecotropic and amphotropic packaging cell lines

The retroviral packing cell lines *Phoenix*A (amphotropic) and *Phoenix*E (ecotropic) were a kind gift from Gary Nolan (Stanford University, Stanford, CA). *Phoenix* cells were routinely cultured at 5% CO_2 in DMEM medium (Invitrogen-GIBCO) supplemented with 10% heat-inactivated FBS (HI-FBS, Atlanta Biologicals), 1% nonessential amino acids (GIBCO) and 1% antimycotic/antibiotic (Mediatech). *Phoenix*A cells were used to generate viral supernatant for human cells and *Phoenix*E for mouse cells.

Retroviral infection

The various DNA plasmids were transfected into retroviral *Phoenix* packing cell lines using calcium-phosphate transfection. Three hours prior to transfection, 1.5-2 x 10^6 cells were plated on 60 mm plates in 3 mL of DMEM supplemented with 10% HI-FBS. Immediately before transfection, 25μM chloroquine was added to each dish. A solution containing 8 μg of DNA, 62 μL of 2M calcium chloride and deionized water for a volume of 500 μL was mixed with 500μL of 2X HBS buffer, pH 7 (50 mM HEPES pH6.95, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4). The solution was mixed by automatic pipetting to produce bubbles in the solution for 15 seconds after which the solution was added to the dishes. Cells were transfected for 8 hours at 37˚C. After transfection, cells were transferred to 100mm plates and viral supernatant was raised with 6 mL of DMEM supplemented with 10% HI-FBS at 32˚C for 24 hours. Viral supernatant was collected, centrifuged at 1000 rpm for 5 minutes (to eliminate precipitates and cells in suspension), filtered through a 0.2 μm pore, sterile filter, and added with 5 μg/mL of

polybrene to low confluence dishes of targeted cells. Three successive rounds of 8 hr infections were carried out to increase infection rates and expression levels. Cells expressing GFP as a selection marker were selected using FACS sorting with gates set to select cells with equivalent GFP levels. Cells expressing Zeocin resistance gene were selected by addition of 1 mg/mL of Zeocin (Invitrogen) for 3 weeks.

Plasmids

A restriction fragment from pRc/CMV-SrcF529 was sub-cloned into a bicistronic retroviral vector pLZRS-MS-IRES-GFP as fully described previously (Brabek, 2004). Control cell lines expressing GFP only were similarly established using the empty pLZRS-MS-IRES-GFP vector. The resulting plasmids, pLZRS-SrcF-IRES-GFP (figure 5) and pLZRS-MS-IRES-GFP, were transfected into *PhoenixE* cells as described.

Fluorescently labeled CAS was generated by inserting full length CAS into a pCS2 *venus* plasmid containing the YFP (*venus*) tag. The DNA fragment containing CAS fused to the *venus* was then inserted into a bicistronic retroviral vector pLZRS-MS-IRES-Zeo. Control cell lines expressing the *venus* tag only were similarly established using the pLZRS-MS-IRES-Zeo vector. After sequence verification, the resulting plasmids (pLZRS-CAS*venus*-IRES-Zeo) and (pLZRS-*venus*-IRES-Zeo) were transfected into *PhoenixA* cells as described.

Immunoblotting

For immunoblotting of total cell lysates, cells were lysed in modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% sodium

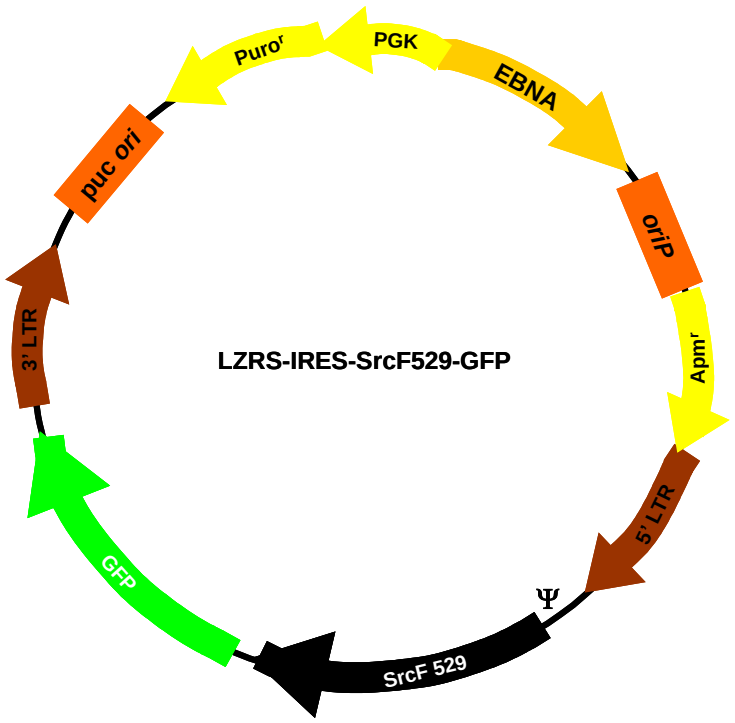


Figure 5: Diagram of bicistronic retroviral system used to stably express oncogenic Src (SrcY529F) in colonic epithelial cell lines. The cDNA for the constitutively active SrcF529 was inserted into a retroviral vector pLZRS-MS-IRES-GFP. A similar strategy was used for infection with CAS variants.

deoxycholate, 50 mM NaF, 1% aprotinin and 0.1 mM Na_3VO_4). For immunoblotting of subcellular fractions, cells were lysed in 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 0.5% NP40, and centrifuged at 16,000 g for 30 s. to obtain a nuclei-enriched pellet and cytoplasm/membrane-enriched supernatant. The nuclear pellet was further washed in 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, and 10% glycerol to remove adherent debris before a final resuspension in RIPA buffer. The cell lysates and subcellular fractions were sheared by passing several times through a 26 gauge needle and insoluble material was subsequently cleared by

centrifugation at 16,000 g for 10 min. Protein concentration in the cleared lysates was determined using a BCA assay (Pierce Biotechnology, Rockford, IL). Lysates containing 20-40 μg total protein, as indicated, were used for immunoblot analysis using standard procedures. Immunoreactivity was assessed using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). For the initial characterization of Src expression and cellular phosphotyrosine, subconfluent, adherent cells were lysed. The analysis of phosphoERK, β-catenin, and nuclear β-catenin was carried out on lysates prepared from cells growing in suspension on polyHEMA-coated dishes.

Nuclear isolation

Subconfluent cell cultures (250mm plates) were trypsinized with 0.25% Trypsin at 37˚C after which 10 mL of serum-containing RPMI was added to reverse trypsin. Cells were counted and the same number of cells for each cell type was incubated at 33˚C in non-adherent dishes for 3 hours and then centrifuged at 100g for 5 minutes. The supernatant was removed and the cell pellet was carefully resuspended in 8 mL of ice-cold PBS. The cell suspension was centrifuged at 100g for 5 minutes, supernatant was removed and cells were suspended in 1.5 mL of Lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA) for 15 minutes on ice. Then 100 μL of 10% NP-40 was added followed by vigorous mixing. The suspension was centrifuged at 16,000g at 4˚C for 30 seconds. The supernatant containing membranes and cytoplasm was collected and used as a 'cytoplasmic fraction'. The pellet was gently mixed with 240 μL of Extraction buffer (50 mM HEPES pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) and incubated for 30 minutes on ice. The suspension was then

centrifuged at 16,000g at 4˚C for 30 seconds and the pellet was mixed with 1 mL of Extraction buffer to remove debris and cytoplasmic contaminants. This suspension was centrifuged at 16,000g at 4˚C for 30 seconds, the buffer was carefully removed and the nuclei were lysed using 200 µL of RIPA buffer. Protein concentration was determined by BCA assay (Pierce).

Cell staining and confocal imaging

For epifluorescence microscopy, colon epithelial cell lines were cultured in permissive conditions for 24-48 hours, as indicated, on fibronectin-coated coverslips. For confocal imaging of polarized cells, Caco-2, HCA7, and derivative cells were cultured in 12 mm diameter 0.4 µm pore size transwells (Costar, Cambridge, MA) with 10% FBS (Atlanta Biologicals) supplemented DMEM (Invitrogen-GIBCO). Five to seven days after confluence, polarization was confirmed by measuring transepithelial resistance with a Millicell Electrical Resistance System (Millipore, Billerica, MA) as per manufacturer's instructions. Cells were rinsed with serum-free media and fixed for 20 min in 4% paraformaldehyde in immunostaining buffer (20 mM PIPES (pH 7.1), 127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1 mM EGTA). After extensive washing with 1X PBS, cells were permeabilized for 30 min in PBS containing 0.1% Triton-X-100 and 1% BSA. After washing with 1X PBS, fixed cells were preincubated for 1 hour in PBS containing 1% BSA to reduce nonspecific binding. Cells were incubated with either Alexa-594-phalloidin (0.4 U/mL) to visualize F-actin, anti-β-catenin antibody (1.25 µg/mL) or anti-E-cadherin antibody (0.25 µg/mL). Secondary antibody AlexaFlour 488 was dilutes in blocking solution, incubated for 1

hour and then extensively washed with PBS. Fixed specimens were mounted using Prolong Antifade mounting medium and imaged on a Nikon Eclipse 80i microscope (Nikon Inc., Melville, NY) equipped for fluorescence optics (epifluorescence) or on a Nikon Eclipse TE2000-3 (confocal). In studies including the evaluation of the effects of Src inhibitor AZD0530, drug or vehicle (DMSO only control) was added to the media 2 hr after the cells were plated on coverslips.

FITC-gelatin degradation assay

Cells were cultured under permissive conditions for 24 hr on FITC-gelatin coated coverslips and assayed as described previously (Bowden, 2001). The stock solution of FITC-gelatin was prepared by dissolving 0.4 g of porcine skin 300 bloom gelatin (Sigma-Aldrich, St. Louis, MO) in 20 mL of buffer (50 mM $Na_2B_4O_7$, 40 mM NaCl, pH 9.3) at 37°C. FITC was mixed with the gelatin solution for 2 hours at 37°C. FITC-gelatin was dialyzed for 2 days in 37°C phosphate-buffered saline (PBS), with 2-3 wash changes per day. Sucrose (0.4 g) was added to the FITC-gelatin solution prior to storage. Glass coverslips (22 mm) were coated with 150 μL of pre-warmed (37˚C) FITC-gelatin and air-dried for 30-60 minutes. The FITC-gelatin coated coverslips were crosslinked with ice-cold 0.8% fresh glutaraldehyde at 4˚C for 15 minutes and then at 37˚C for 30 minutes. After 3 gentle 1X PBS washes at RT for 5 minutes, coverslips were incubated with Sodium Borohydride (5 mg/mL) for 3 minutes. Coverslips were gently washed 3X with PBS and sterilized in 70% EtOH for 10 minutes and transferred to sterile 35 mm dishes. The coverslips were incubated with serum-free media for one hour at 37˚C followed by 10% FBS-supplemented media for 30 minutes. After incubations, 3,000 cells were plated

per well with 10% FBS-supplemented RPMI. Cells were allowed to attach for 2 hours before adding drugs or vehicle (DMSO only control) and incubated at 33˚C. After 24 hours, cells were fixed and stained with Alexa-594-phalloidin to visualize both F-actin and degradation of the gelatin matrix. DAPI present in the Vectashield (Vector Laboratories, Burlingame, CA) anti-fading media was used to detect cell nuclei. For quantification of the ECM degradation, the fraction of cells associated with FITC-gelatin degradation was scored in random fields using 400X magnification. The degradation fraction was expressed as the number of cells associated with degradation areas divided by the number of total cells.

Growth curve

For analysis of adherent growth, 3,000 cells in 0.5 mL of growth medium were plated in 24-well plates and maintained under respective standard cultivation conditions. For each time point, cell number was determined as the average from three independent wells harvested by trypsinization and counted using a Coulter Particle Counter (Beckman Coulter, Fullerton, CA).

Soft agar assay

To test the ability of Src to promote anchorage-independent growth, 20,000 cells were suspended in 1 mL of growth media containing 10% FBS and 0.4% SeaPlaque agarose (FMC Bioproducts, Philadelphia, PA) and were then poured into a 35 mm diameter dish over a base layer of 0.8% agarose in growth media. Under permissive growth conditions, 5 U/mL of γ-interferon was added to the top layer before pouring and

cells were then cultivated at 33˚C. Under restrictive conditions, no γ-interferon was added and cells were cultivated at 37˚C. After solidification at room temperature, the soft-agar dishes were incubated at their respective cultivation temperature in 5% CO_2 and were refed every 2 days with fresh media containing 5% FBS. Colonies were quantified after 2 weeks. Graphs display the average of 2-5 independent experiments, with 3-4 replicate dishes per cell type. For automated counting of colonies, MetaMorph imaging software was purchased from Molecular Devices (Downingtown, PA).

PolyHEMA assay

For polyHEMA (polyhydroxymethacrylate, Sigma) assays, 60 mm tissue culture dishes were coated with 2.7 mL of 1.2% poly-HEMA in 95% ethanol. The ethanol was completely evaporated by incubation at 37°C in a nonhumidified incubator. The dishes were then washed extensively with PBS, sterilized under a UV light, and each plated with 30,000 cells in the respective standard growth media. The suspension cultures were incubated at the respective standard cultivation temperature in 5% CO_2. At the time-points indicated on the graphs, cells from two dishes were separately harvested, trypsinized to disrupt aggregates, and then resuspended in growth media. For the experiments performed with fibroblasts, the number of cells was determined using a hemacytometer. For epithelial cell lines, the number of cells was determined using an automated Coulter Particle Counter. AZD0530 (1 μM) or vehicle (DMSO only) was added directly to dishes every 48 hr.

Statistical analysis

Statistical significance for various experiments was determined using a non-paired, one-tailed Student's *t*-test.

Genotyping of *APC*

IMCE-Src cells were cultivated under restrictive conditions at low density in polyHEMA dishes (3,000 cells per 60 mm dish) for 10 days, then collected and plated on a standard 100 mm dish. After cell attachment, the plate was washed with PBS to remove debris and dead cells. Adherent colonies were isolated using cloning disks and expanded to confluence in wells of a 24-well plate. Genomic DNA from expanded colonies, as well as from a pool of polyHEMA-selected cells, was isolated using DNeasy Tissue Kit (QIAGEN, Valencia, CA). Then the *APC* gene was amplified by PCR using primers described by Luongo and collaborators (Luongo, 1994). Briefly, primers inserted two restriction sites for *HindIII* on the wild type gene and one restriction site on the *min* allele. The PCR product was purified, digested with *HindIII* restriction enzyme, and the digestion products were separated on a 4% agarose gel (MetaPhor agarose, BMA Products, Rockland, ME). Restriction products from wild type and min *APC* alleles correspond to 123bp and 144bp, respectively.

Analysis of β-catenin reporter gene assay

To evaluate the effects of SrcF expression on the nuclear activity of β-catenin, cells expressing vector only, SrcF or Ras were transiently transfected with Super8XTOPFlash reporter plasmid (provided by Randall Moon, University of

Washington, WA), which contains 8 copies of β-catenin/TCF binding sites on the promoter driving firefly luciferase expression and pRL-TK (Promega) that constitutively expresses *Renilla* luciferase (to normalize transfection efficiency). For transient transfection, 350,000 cells were plated in 35 mm wells in duplicates with complete RPMI containing γ-interferon. Twenty-four hours after plating, media was replaced with 1 mL of OptiMEM (GIBCO). For transfection, 5 μL of Lipofectamine 2000 (Invitrogen), 1.5 μg of *Renilla* plasmid and 3.5 μg of the TOPFlash plasmid were mixed and incubated at room temperature for 20 minutes. The mix was added to cells that were then incubated at 33˚C for 7-8 hours, after which the media was replaced with antibiotic-free, complete RPMI with γ-interferon. Cells were incubated at 33˚C for 48 hours to optimize expression levels and then trypsinized with 0.25% trypsin, resuspended in complete RPMI and held in suspension for 3 hours at 33˚C. The cell suspensions were centrifuged and cell pellets were lysed with Passive Lysis Buffer (Promega) as described by the manufacturer. A fraction of 80 μL of lysate/well was placed in a 96-well Microlite-1 plate (Thermo Fisher Scientific Inc., Waltham, MA). Dual luciferase assay (Promega) was performed in the same well for each sample as described by the manufacturer. The firefly luciferase luminescence readings were normalized to *Renilla* luciferase. Each experiment was performed with duplicate wells for each cell type. The graph corresponds to the average of three independent experiments and error bars represent standard error.

Intrasplenic injection and liver metastasis in syngeneic C57Bl/6 mice

All experiments were done according to institutional animal care guidelines. Six week old, male C57Bl/6 (syngeneic to IMCE cells) mice (Jackson Laboratory, Bar

Harbor, ME; n=10-13 per group) were housed for 2 weeks prior to the experiments for stabilization and recovery.

IMCE-vector only, IMCE-SrcF and IMCE-Ras cells and were cultivated under permissive conditions as described previously. On the day of the experiment, sub-confluent plates were trypsinized and suspended in standard cultivation media. Cells were counted and suspended in sterile PBS (5,000,000 cells/100μL of PBS per mouse). For the intrasplenic injections, after anesthesia, each mouse abdominal cavity was opened and 100 μL of cell suspension was injected into the spleen. After a few minutes, the spleen was removed to avoid the formation of a primary tumor that could compromise the imaging of the liver metastasis. Mice were allowed to recover and after 4, 8 and 12 weeks, livers were imaged. For PET scan (Positron Emission Tomography) imaging of the liver, mice were then injected with the radiotracer and livers of anesthetized mice were imaged. The radiotracer ^{18}F-FDG (fluorodeoxyglucose) is an analog of deoxyglucose that labels areas of high metabolism. It functions as a marker for tumors, because cancer cells have enhanced metabolism compared to normal cells, and therefore, a higher affinity for glucose (Warburg, 1956). After 14 weeks, mice were sacrificed and the livers were inspected visually for tumors.

CAS depletion using small hairpin RNA

Sequences corresponding to shRNA (Dharmacon, Lafayette, CO) targeting nucleotides 1334-1353 (‘shRNA1334’) and 2198-2216 (‘shRNA2198’) were inserted into retroviral vector pRetro-Super (Brummelkamp, 2002). The generation of Caco-2 and HCA7 cells stably expressing small hairpin RNAs (shRNAs, stem loop structures

processed in the cell to siRNA) was carried out essentially as described above. Instead of GFP sorting, positive cells were selected with puromycin (5 μg/mL for HCA7 and 7.5 μg/mL for Caco-2). Control cell lines expressing vector only ('retro') and scrambled sequence ('scramble') were generated similarly.

Transepithelial cell resistance

To evaluate the transepithelial resistance of Caco-2, HCA7 and derivatives, 6.5 mm, 0.4 μm pore size transwells were equilibrated with 10% FBS-supplemented DMEM for 1 hour at 37˚C. Cells were trypsinized, counted and 30,000 cells were plated per filter in triplicates. Plates were incubated at 37˚C and equilibrated at room temperature before each daily reading using the Millicell Electrical Resistance System (Millipore, Billerica, MA). Averages of two independent experiments with triplicate filters are shown on the graph.

CHAPTER III

SRC KINASE PROMOTES COLON EPITHELIAL CELL TRANSFORMATION

Introduction

According to cancer statistics from the American Cancer Society (Cancer Facts & Figures 2008, publication obtained from the American Cancer Society), cancer ranks as the second cause of mortality in the United States and accounts for ~560,000 (22.8%) deaths per year. Colorectal cancer is a very common type of cancer and according to the same study is the third leading cause of cancer-related deaths in both men and women. Every year, 150,000 new diagnoses are made in the US and the lifetime chance of an average person with no family history to develop colon cancer is estimated to be 1:16. This estimate increases with age and with first or second-degree relatives with colon cancer. For people with hereditary syndromes such as hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), the chances of developing colon cancer rises to 80-100% (Rustgi, 2007). Rustgi and collaborators (Rustgi, 2007) defined familial cancer as an increased predisposition to cancer, but without determination of a hereditary basis, and it is estimated that 20-30% of colon cancers are familial. Several hereditary colorectal cancer syndromes have been described previously, although they account for a small percentage of all colon cancer cases (HNPCC or Lynch syndrome, 3-4%; FAP, 1%; MYH-associated polyposis (MAP), hamartomatous polyposis syndromes and hyperplastic polyposis together, 1%).

Colon cancer arises through a multi-step process of genetic and epigenetic

alterations resulting in aberrant functions of tumor suppressors, oncogene-encoded proteins, and proteins involved in maintaining genomic stability (reviewed in Kinzler and Vogelstein, 1996). As shown in figure 6, mutations in components of the Wnt signaling pathway like APC (a tumor suppressor) and β-catenin are some of the earliest molecular alterations observed in colon cancer. From a normal epithelial cell with tightly regulated proliferation capacity, cells harboring mutations in APC or β-catenin acquire some proliferative advantage over the surrounding normal tissue and form numerous dysplastic aberrant crypt foci (ACF). Moreover, mutations in APC reduce chromosome stability, increasing the chances of translocations or mutations. ACFs can progress to small adenoma and initiate the neoplastic process (reviewed in Fodde, 2001). Accumulation of further mutations in other genes, as indicated in figure 6, allow the adenomas to progress towards a carcinoma. Activating mutations of the Ras gene (K-Ras) are very prevalent in colorectal adenomas and carcinomas (Forrester, 1987; Bos, 1987; Vogelstein, 1988) and previous studies have shown that activated Ras can collaborate with APC mutation to promote tumorigenesis (D'Abaco, 1998), supporting the idea that colon cancer arises from accumulation of molecular lesions, or 'hits' on the cell (Vogelstein, 1988). Another molecular change often observed in colon tumor progression is the enhancement of Src kinase expression and activity (figure 6). The highest Src levels are observed in the metastatic tissues (Talamonti, 1993), however, it is documented that lesions as early as a small adenoma already have enhanced Src when compared to normal surrounding tissue Cartwright, 1990).

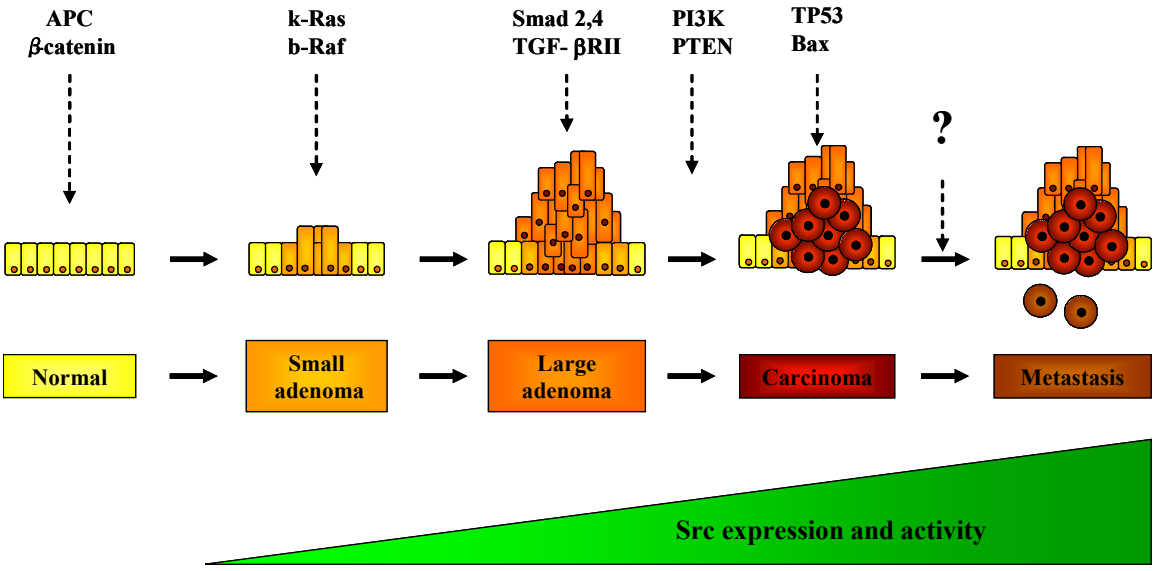


Figure 6: Diagram of colon cancer progression and the associated molecular lesions. Note that Src kinase expression and activity increase as the tumor progresses, but it is elevated from stages as early as small adenoma.

APC tumor suppressor

The *APC* (adenomatous polyposis coli) gene was first identified (Kinzler, 1991, Gronden, 1991; Nishisho, 1991) in patients with FAP, a dominant autosomal disease that results in the formation of multiple colorectal polyps. FAP patients inherit one inactive mutant *APC* allele, and polyp development is commonly associated with a second somatic mutation or loss of heterozygosity leading to functional loss of the other wild type *APC* allele (Levy, 1994). Individuals affected with FAP develop hundreds to thousands of small polyps throughout the colon and unless removed, these polyps will inevitably progress to colon cancer. Inactivation of the *APC* gene is an early and prevalent event in the development of human colorectal carcinoma, and the APC protein

is regarded as the "gatekeeper" of colorectal tumorigenesis (reviewed in Fodde, 2001; Fodde, 2003; Kinzler and Vogelstein, 1996). Besides the familial syndrome, *APC* mutations are also commonly associated with sporadic colorectal cancers, again with the majority showing mutational "hits" to both *APC* alleles (Rowan, 2000). A small fraction of colorectal tumors may retain one wild type *APC* allele (Segditsas and Tomlinson, 2006), and in these cases the single mutant *APC* allele may contribute to disease pathogenesis in combination with alterations to other genes that encode proteins that act in pathways related to *APC* function. Such haploinisufficiency for *APC* was suggested by a study that found ~50% reduction of *APC* transcript levels in a subset of adenomatous polyposis patients without apparent *APC* mutations (Venesio, 2003).

APC is a multi-functional protein involved in several aspects of epithelial cell biology, such as cell adhesion, migration, signaling, microtubule stability and chromosome segregation. APC is a large protein, approximately 312kDa, and contains conserved regions displayed in figure 7. The wild type APC protein has binding sites for axin and β-catenin, critical sites for the downregulation of β-catenin levels; EB1 and microtubules binding sites, which are lost in the APC^{min} mutant (figure 7).

As a tumor suppressor, the APC protein functions as a scaffold in the canonical Wnt pathway by targeting soluble β-catenin for phosphorylation and degradation, thus inhibiting β-catenin/TCF-mediated transcription (reviewed in Schneikert and Behrens, 2007). In colorectal tumors lacking *APC* mutations, β-catenin mutations resulting in a non-degradable form of the protein have been frequently observed (Sparks, 1998). APC also acts as a regulator of microtubule stability and cytoskeletal organization, and the loss of these functions could negatively impact cell division and migration during

tumorigenesis (reviewed in Nathke, 2006).

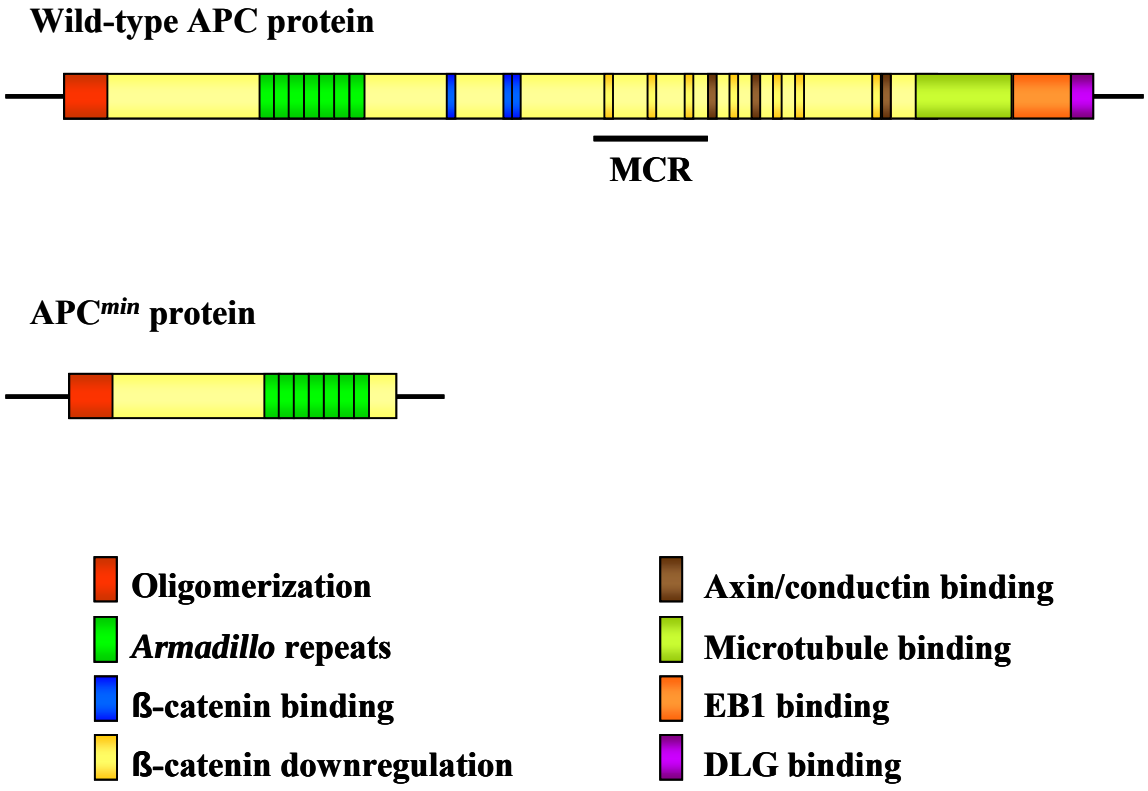


Figure 7: Structure of the wild type and min APC (adenomatous polyposis coli) proteins. Conserved regions and important binding sites are color-coded as indicated. The black bar indicates the mutation cluster region (MCR). *APC*^{min} mutation localizes to the codon 850 of the *APC* gene and the truncated protein has 95kDa, compared to the wild type APC protein with 312 kDa. (Adapted from Fodde, 2001. Nature Reviews Cancer, 1, 55-67).

APC and Wnt signaling

The classical function of APC is to regulate β-catenin levels in response to the activation of Wingless/Wnt signaling pathway (a simplified diagram of the Wnt pathway is shown in figure 8, adapted from Fodde, 2001). β-catenin levels are regulated by the presence or absence of the Wnt ligand and this mechanism seems to be conserved in flies, frogs and mammals (reviewed in Fodde, 2001 and Fodde, 2003). When cells are not stimulated by Wnt ligands (cell on the left), a 'destruction complex' containing the APC tumor suppressor, axin (a scaffold protein), GSK3 (glycogen synthase kinase, a serine/threonine kinase) and β-catenin is formed. Once the destruction complex is established, GSK3 can phosphorylate four critical serine/threonine sites on the N-terminus of β-catenin. The phosphorylation of these sites targets β-catenin for poly-ubiquitination (Ikeda, 2000) and degradation by the proteosome. In the absence of β-catenin in the nuclei, the co-repressor Groucho prevents the transcription of β-catenin/TCF target genes.

When a Wnt ligand binds to the Frizzed/LRP6 co-receptors, by an incompletely understood mechanism, Dishevelled is released from the receptor and impairs GSK3-mediated phosphorylation of β-catenin. The non-phosphorylated form of β-catenin is stable and can translocate to the nucleus, displace the co-repressor Groucho and activate transcription of β-catenin/TCF target genes, of which more than 100 have been identified to date (http://www.stanford.edu/~rnusse/pathways/targets.html).

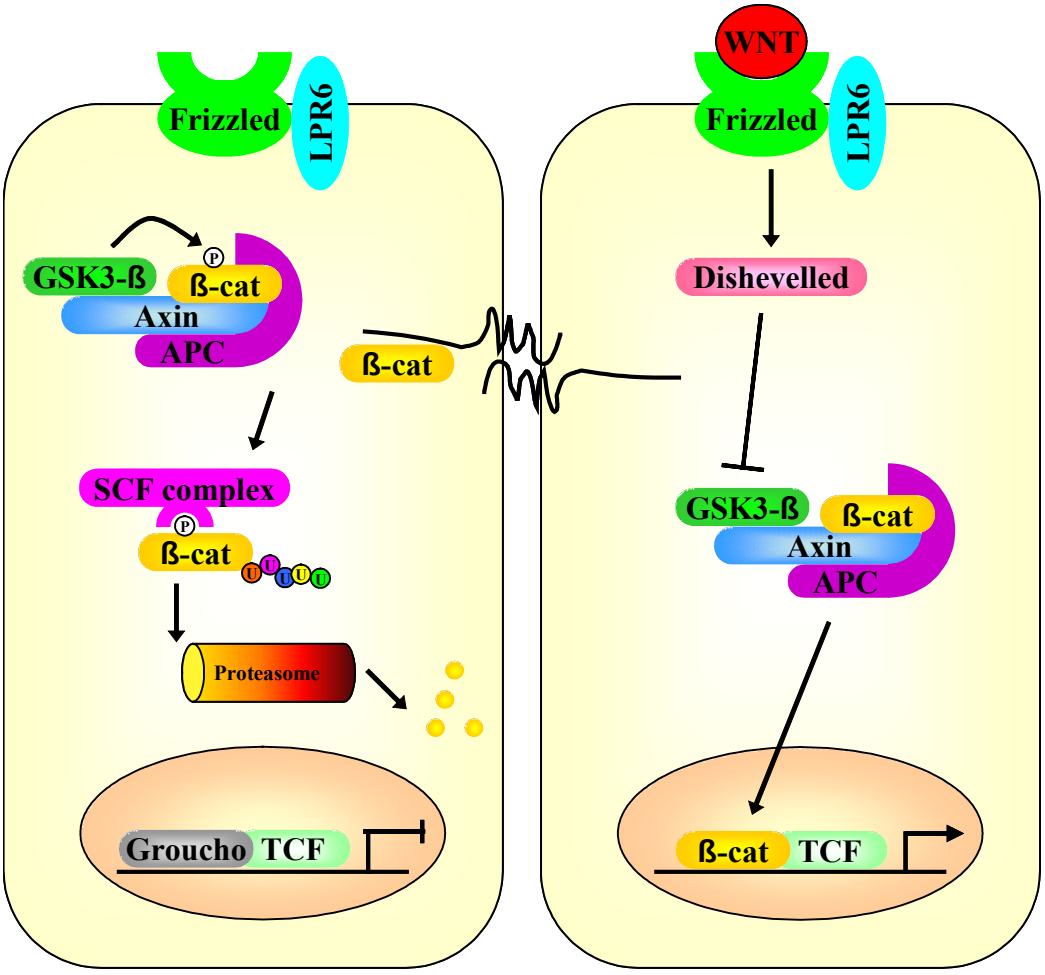


Figure 8: Wnt signaling pathway in colon epithelial cells. Diagram displays cells with active (right) and inactive (left) Wnt signaling. Black structure between cells represents E-cadherin-based cell-cell adhesion. On the left cell, in the absence of Wnt ligand, APC, axin, β-catenin and GSK3 form a protein complex (destruction complex) to degrade β-catenin. On the right cells, the binding of Wnt ligand leads to release of Dishevelled and inactivation of GSK3, resulting in a non-phosphorylated form of β-catenin that can migrate to the nuclei and activate transcription. P – phosphorylation; U – ubiquitin; GSK3 – glycogen synthase kinase; cat – catenin; TCF – T-cell factor; SCF – SKP/cullin/F-box (E3-ubitiquin ligase); LRP – low-density lipoprotein-receptor-related protein. (Adapted from Fodde, 2001. Nature Reviews Cancer, 1, 55-67).

p53 tumor suppressor

The tumor suppressor p53 was first identified as a protein that is associated with the SV40 large T antigen in SV40-transformed fibroblasts (Linzer, 1979) and was first thought to be an oncogene implicated in acceleration of the cell cycle progression. Later studies indicated that p53 is a tumor suppressor and some aberration of p53 functioning has been described in most human cancers and moreover, in about half of the cases, p53 is inactivated by mutation in the p53 gene (reviewed in Vogelstein, 2000). The p53 protein functions as a regulator of the cell cycle progression and apoptosis in response to DNA damage by activating the transcription of downstream target genes (Levine, 1997) that act to reduce the accumulation of genetic lesions that might lead to tumorigenesis. The p53 binding site sequence on target gene promoters has been identified, including the Cdk-inhibitor (cyclin-dependent kinase) p21, which blocks the transition from G1 to S phase of the cell cycle, as a major target (El Deiry, 1992).

Mutations in the tumor suppressor p53 were also identified in patients with Li-Fraumeni syndrome, a genetic autosomal dominant cancer syndrome that causes cell-cycle arrest defects. Patients with this syndrome are affected with several types of cancer, including most frequently soft tissue sarcomas and breast cancer, but also osteosarcoma, leukemia, adrenal cortical tumors, and brain, lung, pancreas and skin tumors. Besides hereditary cancer, mutations in p53 are often found in several types of sporadic cancer (Nigro, 1989).

SV40 is able to induce cell transformation by inactivation of tumor suppressors, including p53. The mechanism of p53 inactivation by SV40 is mediated by the virus-encoded large T antigen, which inactivates the tumor suppressor functions of p53 by

binding a consensus sequence near to the p53 DNA-binding domain. Upon association with SV40 large T antigen, p53 is no longer able to bind to its signaling partners or DNA and thus becomes inactive. Interestingly, inactivation of p53 results in stabilization of the protein because p53 is not able to activate the transcription of one of its negative regulators MDM2 (Pipas and Levine, 2001).

The relationship between p53 and colon cancer was first described by mapping the small arm of chromosome 17, which was known to be aberrant in 75% of colon cancer patients. Using RFLP (restriction fragment length polymorphism) analysis to compare DNA from colon carcinomas to the normal adjacent tissue, Baker and collaborators (Baker, 1989) were able to determine that p53 was either deleted or mutated in DNA from carcinomas, but not in normal tissues, suggesting that p53 is a critical gene, possibly implicated in the progression of colon cancer. Furthermore, studies employing immunostaining of p53 protein showed that ~50% of the malignant tumors express high levels of p53 and it correlated with inactivating mutations, but no expression was detected in benign adenomas (Rodrigues, 1990).

When p53 signaling is activated, usually by DNA damage or expression of an oncogene, the cell cycle is interrupted and cells may undergo apoptosis, depending on the extent of the damage. Interestingly, enhanced Src activity achieved by expression of v-Src usually does not lead to apoptosis in fibroblasts; however, it has been reported that when survival signals like PI3K and Ras were inhibited, expression of v-Src induced apoptosis, but in a p53-independent manner (Webb, 2000).

YAMC/IMCE model

One of the challenges of colon cancer research is to obtain and culture normal or premalignant epithelial cells. Normal colon epithelial cells are highly differentiated, have a finite life-span and colonic crypt cultures survive only up to 24 hours when isolated for primary cell culture (Jat, 1991). Thus, most of the colon cell lines available for research are derived from carcinomas or adenocarcinomas, in different clinical stages and it restricts the ability to investigate the early stages of colon epithelial cell transformation.

To overcome this limitation, non-tumorigenic, conditionally immortalized colon epithelial cell lines derived from C57Bl/6 mice were developed: Young Adult Mouse Colonic, 'YAMC' (Whitehead, 1993) and Immorto-Min Colonic Epithelial, 'IMCE' (Whitehead, 1994).

The YAMC cell line was derived from the transgenic mouse Immortomouse, which harbors a temperature-sensitive mutation (tsA58) of the SV40 large T antigen under the transcriptional control of a γ-interferon inducible promoter (H-2Kb). The expression of the SV40 large T antigen is repressed while the mouse is alive, however, when cells from virtually any tissue are isolated and cultured in vitro, the expression of SV40 is induced by the presence of γ-interferon, which inactivates p53 tumor suppressor and immortalizes the cells (Jat, 1991). The IMCE cell line was derived from a F1 hybrid between the Immortomouse and the Min mouse. The Min mouse (Multiple Intestinal Neoplasia) was identified in a group of mice treated with the mutagen ethylnitrosourea (Moser, 1990) and this mouse line carries a mutation in one allele of APC (APC^{min}). The Min mutation is a fully penetrant dominant mutation that predisposes to multiple adenomas throughout the entire intestinal tract (Moser, 1992).

As summarized in table 1 (based on Fenton and Hord, 2006), YAMC and IMCE cells lines have several characteristics of normal colon epithelial cell lines, including expression of brush border enzymes, epithelial markers like keratin-18 and inhibition of growth by cell-cell contact. Importantly, both cell lines are not able to form soft agar colonies or tumors in nude mice, indicating while these cells are immortalized with SV40 large T antigen, they do not have characteristics of transformed cells.

Table 1: Comparison between IMCE and YAMC cell lines.

	YAMC	IMCE
Genotype	*APC*+/+	*APC*+/-
Genome	Stable	Stable
Mouse model	Immortomouse	Immorto-Min hybrid
Gap junction function	Normal	Decreased
Contact inhibition of growth	Normal	Normal
Keratin-18 expression	Normal	Normal
Brush border enzymes	Detected	Detected
Response to growth factors	Normal	Altered
Growth in soft-agar	No	No
Tumorigenic	No	No
β-catenin	Normal	Enhanced
Response to apoptotic stimuli	Normal	Aberrant
Migration in response to growth factors	High	Low

The YAMC and IMCE cell lines have been used to study the early events in colon cancer initiation and progression and have been shown to be relevant to understand mechanisms related to human colon cancer. Reports on the effects of dietary factors on the chemoprevention of colon cancer were shown to be stage-dependent, as treatment of these cells with curcumin (Fenton, 2002) and flavonoids (Fenton, 2004) induced cell migration in an APC-dependent manner. Moreover, this model was employed to study the effects of growth factors, which are elevated in cases of obesity and associated with increased risk of obesity-related cancer (Calle and Kaaks, 2004). Treatment with the growth factors leptin, IGF-1 and IGF-2 induced cell proliferation in IMCE, but not YAMC cells (Fenton, 2005). Interestingly, the mitogen effects and the increased proliferation were associated with enhanced levels of the inflammatory interleukin-6 (IL-6) in the IMCE, but not YAMC cells (Fenton, 2006b). Moreover, the responses to IL-6 were dramatically different: while IMCE cells were more proliferative, YAMC cells became apoptotic, implicating leptin-induced production of IL-6 as one of the early events promoting survival and proliferation of colon epithelial preneoplastic cells.

The YAMC/IMCE model has also been employed to show synergy between the single *APC*^{min} allele with oncogenic Ras (D'Abaco, 1996). Expression of activated Ras oncogene in IMCE and YAMC cells promoted morphological cell transformation and similar capacity to circumvent the requirements for immortalizing conditions, as both IMCE-Ras and YAMC-Ras cells were able to proliferate under restrictive conditions. However, the ability of oncogenic Ras to promote anchorage-independent growth and tumor formation in nude mice was dependent on the APC genotype. IMCE-Ras cells were able to form bigger and more numerous colonies than YAMC-Ras cells when p53

tumor suppressor was inactivated under the permissive conditions. Even more remarkable effects were observed when p53 was functional, when only IMCE-Ras cells, but not vector only or YAMC-Ras cells were able to form colonies. In vivo studies with nude mice confirm and strengthen the soft agar data, as all the mice injected with IMCE-Ras cells formed primary tumors after 20 days and no tumors were observed in YAMC-Ras cells even after 90 days. PCR-based genotyping and immunoblotting of cells and tissues confirmed that IMCE-Ras cells retained the wild type allele of APC, suggesting that Ras reveals a haploinsufficiency of APC tumor suppressor to prevent tumorigenesis.

Similar results were obtained when IMCE and YAMC cells were expressing a mutant non-degradable β-catenin (Wagenaar, 2001) in assays for anchorage-independent proliferation and tumor growth, suggesting a important role for the Wnt signaling on colon epithelial cell transformation.

Given these studies using YAMC and IMCE cells as a model of normal and preneoplastic colon epithelial cells, and the potential for cross-talk between *APC* and Src, we used the YAMC/IMCE model to investigate the potential for *APC* haploinsufficiency in combination with elevated Src signaling to promote neoplastic transformation of colonic epithelial cells.

Results

Oncogenic Src promotes similar morphologic transformation in YAMC and IMCE cells

YAMC ($APC^{+/+}$) cells derived from Immortomouse and IMCE ($APC^{+/min}$) cells derived from an F1 Immortomouse/Min mouse hybrid were employed in this study to

investigate the potential for *APC* min mutation in combination with elevated Src signaling to promote neoplastic transformation of colon epithelial cells. Cultures were kept under the standard "permissive" culture conditions, where these cells are immortalized by expression of a temperature-sensitive SV40 large T antigen (tsA58) under control of a γ-interferon inducible promoter.

An activated form of mouse c-Src (Src-Y529F) was stably expressed in the normal YAMC and preneoplastic IMCE cell lines using a bicistronic retroviral vector that co-expresses GFP. As controls, cells carrying the empty vector were similarly prepared. Sorting for equivalent GFP levels yielded "YAMC-SrcF" and "IMCE-SrcF" cell populations that express equivalent levels of the activated Src-Y529F as demonstrated by immunoblot analysis for total Src protein, activated Src (activation loop Tyr-418 phosphorylation site), and total phosphotyrosine (figure 9). Treatment of YAMC-SrcF and IMCE-SrcF cells with the Src-selective inhibitor AZD0530 substantially reduced the phosphorylation of the Src activation site and the total cellular phosphotyrosine.

Src-Y529F expression resulted in a similar morphologic transformation of YAMC and IMCE cells. Cells expressing the empty vector grew as epithelial sheets associated with prominent cell-cell junctions, as indicated by immunostaining for β-catenin (figure 10) or E-cadherin (not shown). In contrast, YAMC-SrcF and IMCE-SrcF cells lacked apparent cell-cell junctions and had a mesenchymal appearance (figure 10).

Past studies showed that Src kinase can phosphorylate β-catenin and enhanced tyrosine phosphorylation on Src specific sites (Y86 and Y654) reduces the affinity of β-catenin to E-cadherin (Roura, 1999). The displacement of β-catenin from the cell-cell adhesions caused by enhanced phosphorylation by Src correlates with enhanced

transcriptional activity of β-catenin (Piedra, 2001). To test whether Src kinase is affecting the phosphorylation of β-catenin on IMCE and YAMC cells, immunoprecipitation with β-catenin antibody followed by immunoblotting for phosphotyrosine were performed. As shown in figure 11, both IMCE and YAMC cell lines expressing vector only did not have a detectable levels of β-catenin phosphorylation, whereas the cells expressing oncogenic Src have similar levels of enhanced β-catenin phosphorylation.

Oncogenic Src similarly enhances the invasive properties of YAMC and IMCE cells

In fibroblasts, oncogenic Src expression has been associated with the formation of podosomes, actin-rich ventral structures associated with matrix degradation and invasion (Buccione, 2004; Linder and Aepfelbacher, 2003). To assess podosome formation in the colon epithelial lines, the cells were cultured on fibronectin-coated coverslips for 48 hr and stained with fluorescent-tagged phalloidin to detect F-actin. In contrast to the control cells where F-actin staining was prominent at the cell-cell junctions and in internal stress fibers (figure 12A), IMCE-SrcF and YAMC-SrcF cells were observed to similarly to form podosomes that were evident as large rosettes (figure 12B) and, more typically, as smaller actin-dense puncta (figure 12C). The podosomes did not form when the cells were plated and maintained in the presence of AZD0530 (figure 12D).

The invasive capacity of the YAMC-SrcF and IMCE-SrcF cells was examined by cultivating the cells for 24 hr on FITC-gelatin coated coverslips, followed by cell staining with fluorescent-tagged phalloidin and visualization of cells and the underlying gelatin matrix by fluorescence microscopy. While the vector-only control cells were completely unable to degrade FITC-gelatin, non-fluorescent regions in the matrix were observed

under 40-45% of both IMCE-SrcF and YAMC-SrcF cells (figure 13A) and this was sensitive to AZD0530 treatment (figure 13B, C).

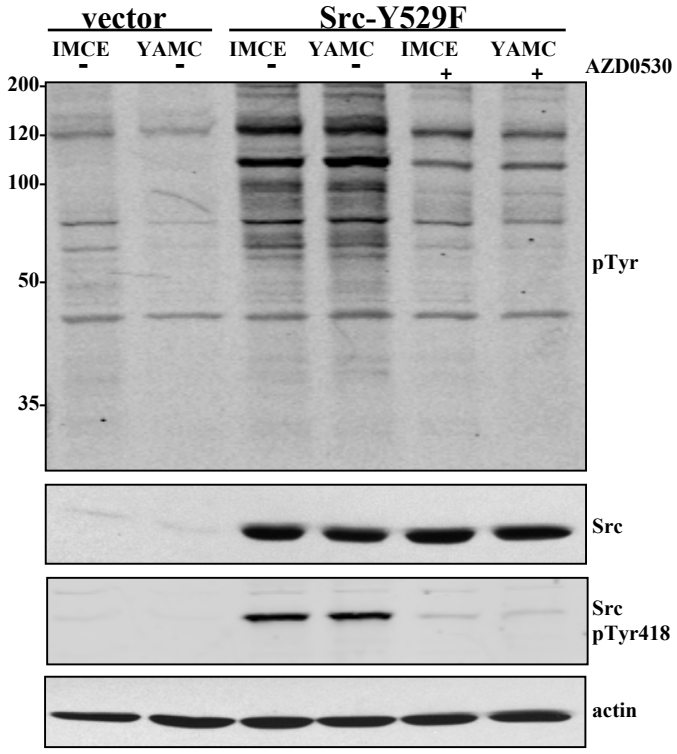


Figure 9: Immunoblotting analyses of IMCE, YAMC and derivatives with antibodies against pSrc, total Src and total phosphotyrosine. Stable expression of Src-Y529F to equivalent levels in IMCE ($APC^{+/min}$) and YAMC ($APC^{+/+}$) colonic epithelial cell lines. Following infection with the bicistronic retroviral vector and sorting for equivalent GFP levels, sub-confluent cultures of the resulting IMCE-SrcF and YAMC-SrcF cell populations, and respective vector-only control cells, were assessed by immunoblotting (IB) analysis of total cell lysates (25 µg protein per lane). Replicate blots were used to detect either total cellular phosphotyrosine (pTyr, 4G10 antibody), total Src protein, activated Src with phosphorylated kinase domain activation loop (Src pTyr-418), or actin as a loading control. The IMCE-SrcF and YAMC-SrcF cells were either treated with AZD0530 (1µM) or vehicle DMSO 8 hr prior to lysis in order to demonstrate the effectiveness of this Src-selective inhibitor. Numbers at left indicate positions of molecular mass markers (in kDa).

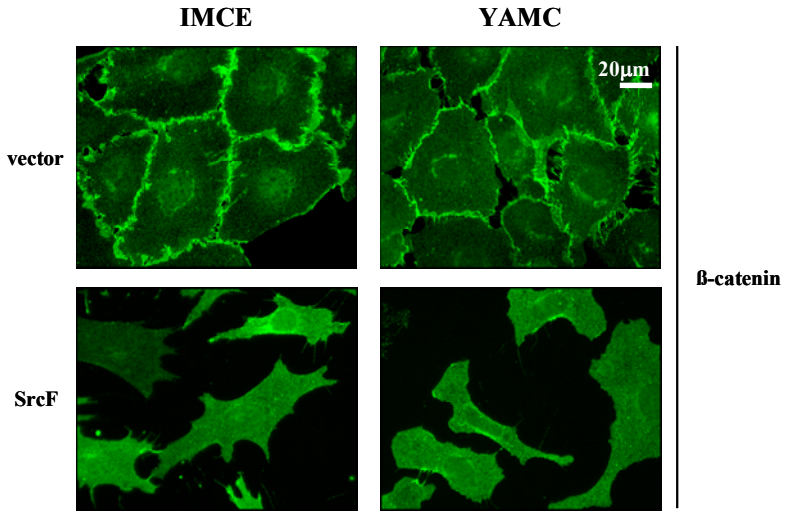


Figure 10: β-catenin immunostaining of IMCE, YAMC and derivatives. Oncogenic Src disrupts cell-cell junctions and displaces β-catenin on both IMCE-SrcF and YAMC-SrcF cells. Vector-only and SrcY529F (SrcF) cells were fixed 24 hours after plating on fibronectin-coated coverslips and immunostained with β-catenin antibody. Top panels show cells expressing vector only and bottom panels are cells expressing oncogenic Src (SrcF).

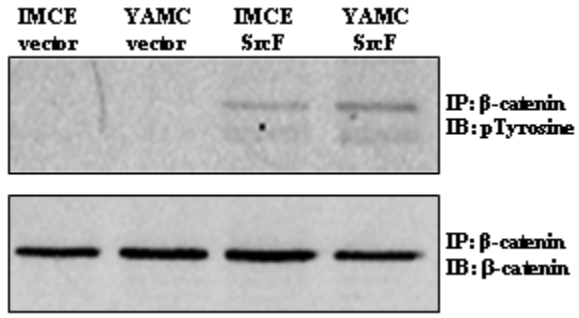


Figure 11: Immunoblotting analyses of β-catenin immunoprecipitates of IMCE, YAMC and derivatives with antibodies against β-catenin and total phosphotyrosine. Oncogenic Src phosphorylates β-catenin on both IMCE-SrcF and YAMC-SrcF cells. β-catenin was immunoprecipitated from total cell lysates of IMCE and YAMC cells expressing vector only or SrcY529F (SrcF). Replicate blots of the immunoprecipitates were used to detect either pTyrosine (top) or β-catenin (bottom).

The ability of SrcF to enhance invasive properties of colon epithelial cell lines seems to be independent of p53 activity, as podosome formation and ECM degradation were similarly observed in both cell lines when the experiments were carried out under restrictive conditions. The efficacy of AZD0530 on abrogating podosome formation and ECM degradation was not affected by the restrictive conditions (data not shown).

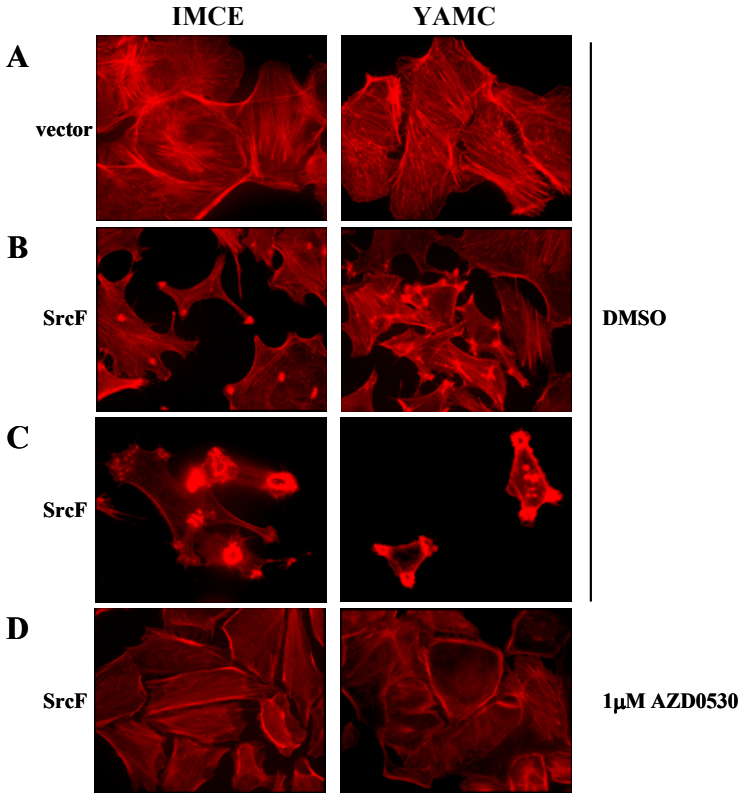


Figure 12: Phalloidin staining of IMCE, YAMC and derivatives. Oncogenic Src promotes actin cytoskeleton reorganization and podosome formation on both IMCE-SrcF and YAMC-SrcF cells. Vector-only (vector) and SrcY529F (SrcF) cells were fixed 48 hours after plating on fibronectin-coated coverslips and stained with fluorescent-labeled *phalloidin* to detect actin. (A-C) Cells were treated with vehicle only (DMSO) or (D) Src-specific kinase inhibitor AZD0530 (1μM) for 46 hours after plating.

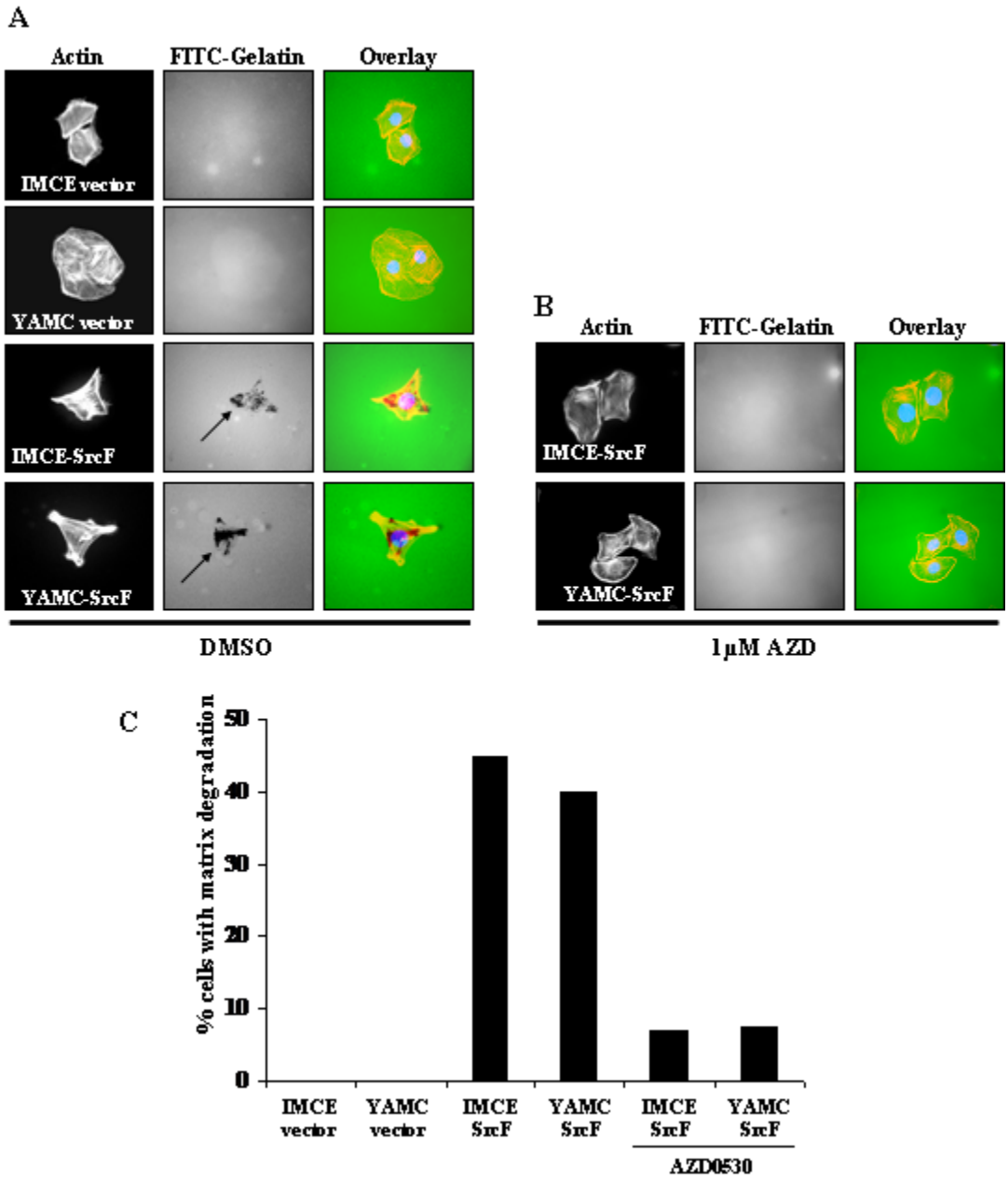


Figure 13: FITC-gelatin assay with IMCE, YAMC and derivatives. Oncogenic Src promotes similar matrix-degrading activity in IMCE-SrcF and YAMC-SrcF cells. (A) Representative micrographs of vector-only control and Src-Y529F-expressing (SrcF) cells after 24 hr cultivation at low density on FITC-gelatin coated coverslips. Using two-channel fluorescence microscopy, fixed cells were visualized by Alexa-594-phalloidin staining while FITC-gelatin in the same field was also revealed. Matrix degradation is evident as dark spots or patches in the FITC-gelatin layer observed directly under (arrows) or surrounding (not shown) a cell. Scale bars represent 10 µm. Cells were treated with either (A) vehicle only DMSO or (B) 1 µM AZD0530. (C) Graph displaying one representative experiment with the cell fraction (from 200 scored) associated with matrix degradation.

IMCE-SrcF cells have enhanced adherent cell proliferation compared to YAMC-SrcF cells

Adherent growth was measured by growth curves after plating the cells at low density. Adherent growth was minimal under the restrictive condition, with both YAMC-SrcF and IMCE-SrcF cells showing no growth advantage over the control cells during a 12 day period after plating (figure 14A). Under the permissive condition, after a lag period, the cell lines expanded 2-4 fold faster than under the restrictive condition, as expected (figure 14B). Under the permissive condition, adherent growth of YAMC-SrcF cells was not significantly enhanced relative to their vector-only control cells. However, the IMCE-SrcF cells grew significantly faster than either their control cells or the YAMC-SrcF cells under the permissive condition, when p53 is inactivated by SV40 large T antigen expression.

IMCE-SrcF cells have enhanced anchorage-independent growth compared to YAMC-SrcF cells

Anchorage-independent (nonadherent) growth was determined using the soft agar assay. Under the permissive condition (figure 15B) the number of colonies above 100 μm diameter, detected after 2 wks, was significantly higher (~7.5-fold) in IMCE-SrcF cells compared to their vector-only control cells. The YAMC-SrcF cells formed far fewer colonies, with no significant increase compared to their control cells. Under the restrictive condition (figure 15A) colony formation was much reduced for all cell types, with only the IMCE-SrcF cells forming an appreciable number of colonies. Histogram bars represent the average number of large colonies (>100 μm) observed in five

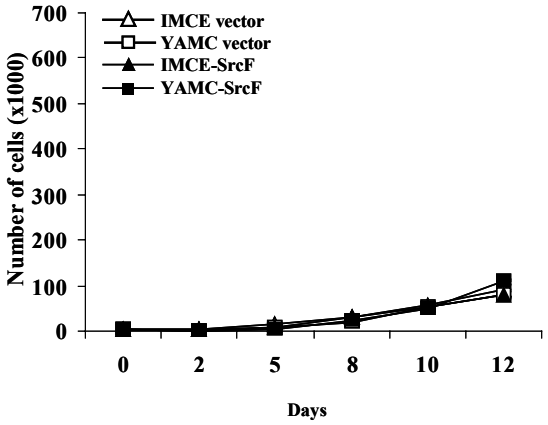
independent experiments, with error bars representing the standard error. The IMCE-SrcF cells formed a significantly higher number of large colonies in comparison to either the vector-only control cells (p = 0.014 for permissive growth, p = 0.002 for restrictive growth) or the YAMC-SrcF cells (p = 0.008 for permissive growth, p = 0.001 for restrictive growth), as determined by the Student's t-test.

To test whether the anchorage-independent growth of IMCE-SrcF cells was dependent on Src kinase activity, the cells were plated on polyHEMA-coated (non-adherent) dishes and growth curves were determined under restrictive conditions in the presence or absence of AZD0530. Consistent with the soft-agar result, untreated IMCE-SrcF cells proliferated in suspension on the polyHEMA dishes (figure 16), whereas the treatment with AZD0530 fully blocked the proliferation on polyHEMA. Lower concentrations of AZD0530 were also tested and we found that 100nM and 500nM are as effective to block proliferation in polyHEMA as 1μM, however, 1nM and 10nM had similar effects to the vehicle only DMSO (data not shown).

In vivo evaluation of the metastatic capacity of IMCE-SrcF cells using a model of tumor formation assay in immuno-competent mice

We demonstrated that Src transformation of IMCE cells promote anchorage-independent growth and invasiveness *in vitro*. To define the significance of these findings *in vivo*, we used a model of tumor formation assay in immuno-competent mice followed by non-invasive imaging of the tumor using ^{18}F-FDG PET (fluorodeoxyglucose positron emission tomography).

A - Restrictive



B - Permissive

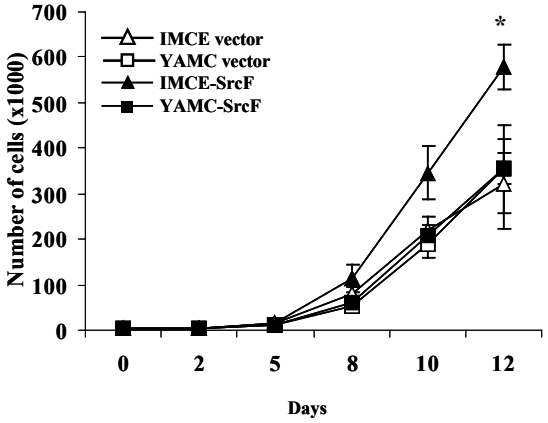


Figure 14: Growth curves of attached IMCE, YAMC and derivatives. Growth curves of IMCE-SrcF and YAMC-SrcF cell populations, and respective vector-only control cells, were obtained under the restrictive (A) or permissive (B) culture conditions. Each time point represents the mean cell number from three independent experiments, with bars indicating standard error. At the final (12 day) time point under the permissive growth condition the IMCE-SrcF cells had reached a significantly (indicated by an asterisk) higher number in comparison to either the vector-only control cells ($p = 0.038$) or the YAMC-SrcF cells ($p = 0.010$), as determined by the Student's t-test.

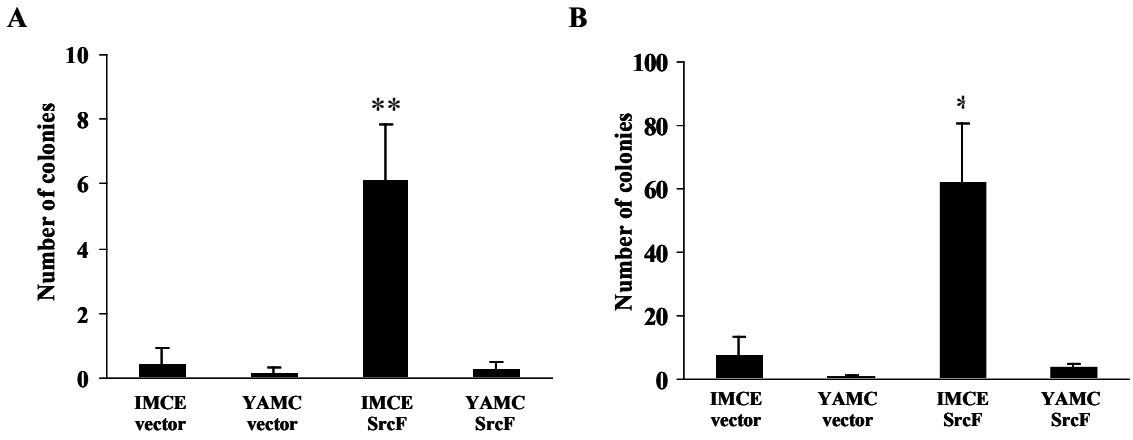


Figure 15: Soft agar assay of IMCE, YAMC and derivatives. Enhanced anchorage-independent growth of IMCE-SrcF cells, but not YAMC-SrcF cells, relative to vector-only controls. The soft agar colony formation assay was carried out on IMCE-SrcF and YAMC-SrcF cell populations, and respective vector-only control cells, under the restrictive (A) or permissive (B) culture conditions. Histogram bars represent the average number of large colonies (>100 μm) observed in five independent experiments, with error bars representing the standard error. * indicates $p<0.05$, **indicates $p<0.01$, see text for *p* values.

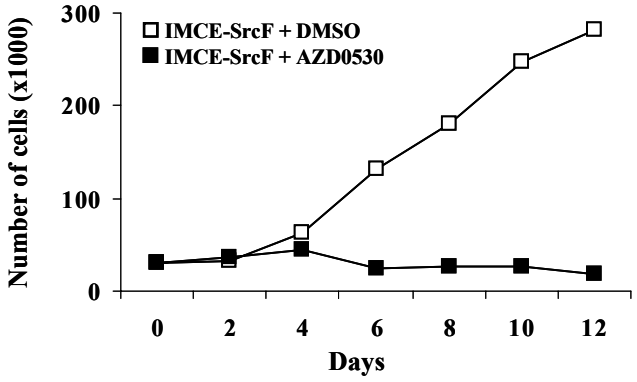


Figure 16: PolyHEMA assay with IMCE-SrcF cells treated with AZD0530. Enhanced anchorage-independent growth of IMCE-SrcF cells is dependent on Src kinase activity. Anchorage-independent growth of IMCE-SrcF cells on polyHEMA plates is blocked by 1μM treatment with Src-selective kinase inhibitor AZD0530. A representative experiment performed in single dishes is shown.

Several mice died during and immediately after the surgery (19 out of 35), even with rigorous care to avoid excessive bleeding and contamination. The surviving mice were distributed as follows: IMCE-vector only (3), IMCE-Ras (7) and IMCE-SrcF (6). The surviving mice were followed for 14 weeks by imaging their livers using PET.

Regardless of the type of cells injected, we could not detect tumors using PET. Mice were then sacrificed, and the livers were removed and carefully inspected, however, no tumors were detected.

IMCE-SrcF cells selected for their ability to grow in anchorage-independent manner retain the wild type APC allele.

To rule out the possibility that the capacity for IMCE-SrcF cell anchorage-independent growth was due to loss of the wild type *APC* allele, cells selected through growth on polyHEMA were genotyped using a PCR-based assay. DNA isolated from parental IMCE cells was included as a control. As shown in figure 17, the wild type *APC* (APC^{WT}) allele is similarly detected in the polyHEMA-selected IMCE-SrcF and parental IMCE cells, indicating that there was no loss of heterozygosity.

Moreover, immunoblot analysis with anti-APC antibody, which recognizes the full length APC, but not the APC^{min} protein, showed that SrcF expression was not able to silence the APC gene, as similar levels were observed among the cell lines. Quantification of the band intensity showed that IMCE cells have ~70% of the APC protein of the YAMC cells lines (figure 18).

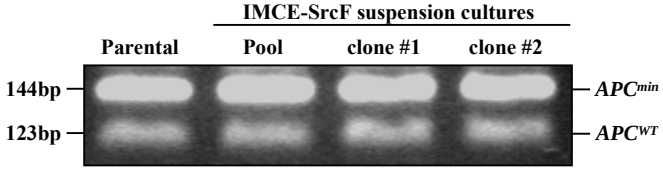


Figure 17: *APC* genotyping of IMCE-SrcF cells. The wild type *APC* (APC^{WT}) allele is retained in IMCE-SrcF cells selected for anchorage-independent growth. (A) Diagram showing the PCR-based strategy employed to determine the genotype of IMCE cells. (B) The *APC* genotype was determined from two different clones of cells harvested after 10 days on polyHEMA-coated dishes, as well as a larger pool of cell aggregates. DNA from a standard adherent culture of the parental IMCE cells was genotyped for comparison.

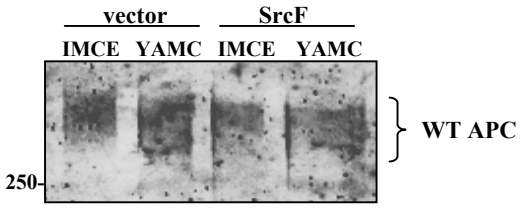


Figure 18: Immunoblotting analyses of IMCE, YAMC and derivatives with antibody against APC. The wild type (WT) APC protein expression in not affected by oncogenic Src expression. Western blotting analysis of total cell lysates of IMCE and YAMC cells expressing vector only or SrcF. Number on the left indicate molecular weight.

Discussion and conclusions

Two molecular lesions associated with colon cancer development and progression are the loss of function of the APC tumor suppressor and the elevation of Src activity. To gain new insight into the effects of elevated Src activity on the development and progression of colon cancer, we investigated the ability of oncogenic Src to transform two mouse colonic epithelial cell lines that differ only in the *APC* genotype. We found that for both normal YAMC ($APC^{+/+}$) and preneoplastic IMCE ($APC^{+/min}$) cell lines, expression of activated Src-Y529F resulted in similar morphologic transformation associated with loss of cell-cell adhesion, formation of invasion-associated podosomes, and acquisition of the capacity to degrade the ECM. This is consistent with several previous reports on the effects of Src on cell transformation. Remarkably, the effects of Src kinase on the invasiveness of colon epithelial cells lines were independent of the p53 tumor suppressor status.

APC has functions in addition to its classical role as a negative regulator of β-catenin, including the regulation of epithelial cell polarity and migration through control of the actin cytoskeleton and microtubule stabilization (reviewed in Aoki and Taketo, 2007). Loss of these cytoskeletal functions in *APC*-deficient cells could further contribute to colon cancer progression towards invasiveness (Nathke, 2006; Schneikert and Behrens, 2007). While it is therefore conceivable that the ability of oncogenic Src to disrupt cell-cell adhesion and promote invasive behavior could be impacted by *APC* deficiency, our study found no apparent difference in these aspects of neoplastic transformation between YAMC and IMCE cells expressing Src-Y529F.

However the expression of oncogenic Src had different effects on epithelial cell

lines with wild type and mutated APC, as Src-transformed IMCE cells were observed to have a much greater capacity for anchorage-independent growth, as compared to the Src-transformed YAMC cells. Previous studies have shown that APC can collaborate with other oncogenes to promote tumorigenesis. Activated Ras has been shown to synergize with APC min mutation to promote anchorage-independent growth (D'Abaco, 1996).

Src activity and APC^{min} mutation were not sufficient to enhance adherent cell proliferation, however, upon inactivation of p53, the combination of three molecular lesions (APC mutation, p53 inactivation and elevated Src activity) commonly observed in colon cancer, resulted in enhanced cell proliferation. Although the collaboration of APC^{min} mutation and Src kinase activity was sufficient to promote anchorage-independent growth, the inactivation of p53 further enhanced this effect, supporting the importance of multiple hits on the progression of colon cancer.

These results provide evidence for a cooperative mechanism involving the combined effects of elevated Src signaling and partial-loss of *APC* function that may contribute to tumorigenic growth in colon carcinoma.

Src has been implicated in a number of cancers, including colon cancer, but the specific role of Src kinase in carcinogenesis has not yet been established. To gain a better understanding of the significance of our findings *in vivo*, IMCE-SrcF cells, which we have shown to be invasive and have enhanced capacity for anchorage-independent growth, were injected into the spleens of immunocompetent syngenic mice. Recently, using immunocompetent C57Bl/6 transgenic mouse expressing c-Src, Kline and collaborators (Kline, 2008) have shown that tumors in liver, gastrointestinal tract or spleen were found in 15% of the mice overexpressing c-Src, whereas no tumors were

found in wild type mouse. Once the Src transgenic mice were crossed onto p21 tumor suppressor null mice ($p21^{-/-}$), the number of tumors increased 3-fold. While no metastases were detected in Src transgenic mice with a wild type p21 phenotype, metastases were found in 11% of the Src transgenic mice without p21, mainly in the liver, after 14 months. The absence of liver metastasis in mice injected with IMCE-SrcF cells could be explained by limitations in our assay. For example, our time-course was a lot shorter compared to the studies of Kline and collaborators (14 weeks x 20 months) and the number of animals we studied was reduced due to the deaths during surgery. Most importantly, the differences in the experimental design could account for the differences on the results. Allowing primary tumor formation using enhanced expression of Src in physiologic conditions for a long time-course, rather than injecting a large number of exogenous cells in the circulation might be a more effective way to evaluate metastatic capacity of Src-transformed cells in a APC +/min background. The use of PET, which is a standard, sensitive and non-invasive technique to image the liver tumors should not account for the lack of tumors detected, because all the mice were sacrificed and the livers were examined visually. Based on our data, we can not rule out the possibility that the inactivation of one allele of APC in collaboration with enhanced Src kinase activity would result in liver metastasis. Further *in vivo* studies are necessary to address this question, including the generation of a hybrid between Src transgenic mice (Kline, 2008) and APC^{min} mouse (Moser, 1990). More discussion regarding future directions is presented in Chapter VI.

There has been a renewed interest in Src as a therapeutic target in the treatment of cancer including colorectal cancer (Summy and Gallick, 2006; Yeatman, 2004). In the

course of our studies, we further evaluated the Src kinase inhibitor AZD0530 (Hennequin, 2006). We found that a low dose of AZD0530 effectively blocked several neoplastic properties of Src-transformed colon epithelial cells including anchorage-independent growth, podosome formation, and the ability to degrade the ECM. AZD0530 may thus have therapeutic benefit in the treatment of colon cancer by blocking both tumor growth and invasion. Elevated Src kinase activity has been reported in early stages of colon tumor progression, resulting in enhanced proliferation compared to normal tissue. In this case, the use of Src inhibitors could reduce the capacity of the transformed cells to proliferate in the absence of substrate. However, our results more strongly support the use of AZD0530 to prevent the metastatic spread of colon tumors, as the inhibition of Src kinase activity was very efficient to block cell invasion, regardless of the presence of functional p53 tumor suppressor or APC genotype. Thus, AZD could be important for treatment of primary tumors (by reducing anchorage-independent growth), but mainly to avoid metastasis. We can also speculate that Src inhibition could be even more effective if in combination with inhibition of other signaling pathways that are also elevated in colon cancer, including EGF signaling. Biding of EGF ligand to EGF receptor can activate Src kinase activity, so it is feasible that combination of drugs targeting Src and EGFR, which present no overlapping toxicity, could be a more efficient way to prevent tumor progression and avoid drug resistance.

CHAPTER IV

DISTINCT SIGNALING PATHWAYS ARE IMPLICATED IN THE ANCHORAGE-INDEPENDENT GROWTH CAPACITY OF IMCE-SRC AND IMCE-RAS CELLS

Introduction

Similarly to our findings with oncogenic Src-Y529F, D'Abaco and collaborators (D'Abaco, 1996) reported that stable expression of an oncogenic H-Ras mutant in IMCE cells ("IMCE-Ras") promoted an enhanced capacity for anchorage-independent growth in soft-agar and nude mice compared to YAMC-Ras cells. Since one of the major mitogenic pathway acting downstream of Ras is the Raf>MEK>ERK cascade (reviewed in Dhillon, 2007; McKay, 2007; Roberts, 2007) and oncogenic Src may exert its proliferative effect, at least in part, through stimulating Ras and the ERK pathway (Gibbs, 1990), it was of interest to determine if the anchorage-independent growth capacity of IMCE-SrcF cells was associated with ERK activation (see Chapter I for more details).

An alternative mechanism for the Src-mediated anchorage-independent growth of IMCE cells would be the activation of β-catenin signaling. Src kinase has been implicated in the regulation of β-catenin function in epithelial cell-cell junctions; however, enhanced Src activity can disrupt the strong cell-cell adhesions in epithelial cells. Expression of oncogenic Src with constitutive kinase activity in MDCK epithelial cells is associated with elevated tyrosine phosphorylation of E-cadherin and β-catenin, disruption of cell-cell junctions, and invasiveness (Behrens, 1993). Phosphorylation of β-catenin Tyr-654 by Src was shown to negatively affect the interaction with E-cadherin and enhance transcriptional activity of β-catenin targets (Piedra, 2001; Roura, 1999).

Results

Evaluation of MAPK pathway activation.

Immunoblotting with a phosphospecific antibody that recognizes the activated forms of ERK1 and ERK2 revealed that ERK activity is elevated in suspension cultures of IMCE-Ras cells, but not IMCE-SrcF cells, relative to control IMCE cells (figure 19, top). The basal level of ERK phosphorylation was slightly higher in the IMCE cells in comparison to the YAMC cells, but for both cell types the signal was unchanged by Src-Y529F expression. The expression levels of total ERK1 and ERK2 were similar among all cell lines and not affected by Src or Ras expression (figure 19, middle).

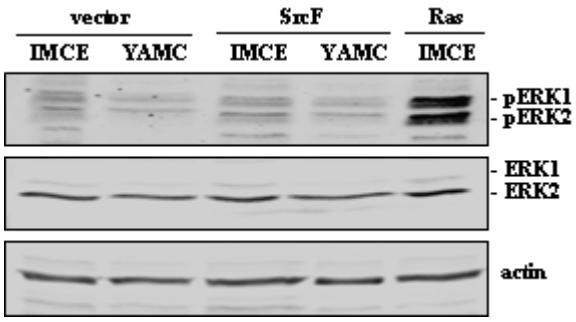


Figure 19: Immunoblotting analyses of IMCE, YAMC and derivatives with antibodies against pERK and total ERK. Nonadherent growth of IMCE-SrcF cells is not associated with ERK activation loop phosphorylation. IMCE-SrcF, YAMC-SrcF, their vector-only control cells, and IMCE-Ras cells were held in suspension on polyHEMA plates for 24 hr under restrictive conditions and evaluated by immunoblotting analysis of whole cell lysates. Assessment of ERK1/ERK2 activation using a phosphospecific antibody recognizing the activation loop phosphorylation sites (pERK, top), with control duplicate blots used to detect total ERK1/ERK2 proteins (middle, note that ERK2 is detected more efficiently by the antibody employed) or actin (bottom).

Evaluation of β-catenin pathway activation.

We further investigated whether the differential capacity of oncogenic Src to promote anchorage-independent growth of IMCE and YAMC cells could be associated with an increased level of β-catenin. This might be anticipated if β-catenin is displaced from the cell-cell junctions as a result of oncogenic Src activity and is more stable in IMCE cells due to the reduced APC function. Immunoblotting of total cell lysates indicated that IMCE-SrcF cells have a modest but reproducible increase in β-catenin levels as compared to either vector-only control IMCE cells, YAMC-SrcF cells, or IMCE-Ras cells (figure 20A, B)

As β-catenin is required to translocate to the nucleus to activate transcription, we investigate whether anchorage-independent growth of colon epithelial cell lines expressing oncogenic Src correlates with enhanced β-catenin in the nuclear fraction of IMCE-SrcF cells. From a cell fractionation experiment, the IMCE-SrcF cells were shown to have a ~2-fold higher level of nuclear β-catenin relative to vector-only control IMCE cells and YAMC-SrcF cells (figure 21).

The enhanced levels of β-catenin in the nuclear fraction of IMCE-Src suggest that enhanced transcriptional activity of β-catenin could be implicated in anchorage-independent growth of IMCE-SrcF cells. To test this hypothesis, IMCE and YAMC cells, expressing SrcF, oncogenic Ras or vector only were evaluated using TOPFlash assay. Cells were transiently transfected with TOPFlash (firefly luciferase) and a constitutively active reporter gene (*Renilla* luciferase), to normalize the transfection efficiency. As shown in the figure 22, the highest levels of β-catenin transcriptional activity correlate with oncogenic Src expression in the $APC^{+/min}$ background.

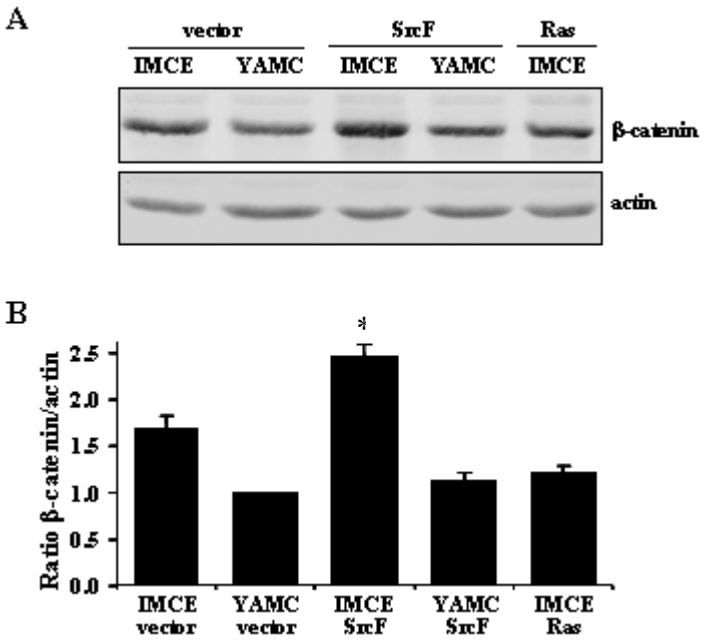


Figure 20: Immunoblotting analyses of IMCE, YAMC and derivatives with antibody against β-catenin. Nonadherent growth of IMCE-SrcF cells is associated with elevated β-catenin levels. IMCE-SrcF, YAMC-SrcF, their vector-only control cells, and IMCE-Ras cells were held in suspension on polyHEMA plates for 24 hr under restrictive conditions and evaluated by immunoblotting analysis of whole cell lysates. (A)Assessment of relative β-catenin levels (top) with control duplicate blot used to detect actin (bottom). (B) Histogram plot representing the average β-catenin and intensity, normalized to the actin loading control, from five independent experiments. The IMCE-SrcF cells have a significantly higher (indicated by asterisk) β-catenin level in comparison to either the vector-only control cells ($p = 0.042$) or the YAMC-SrcF cells ($p = 0.001$), as determined by the Student's t-test.

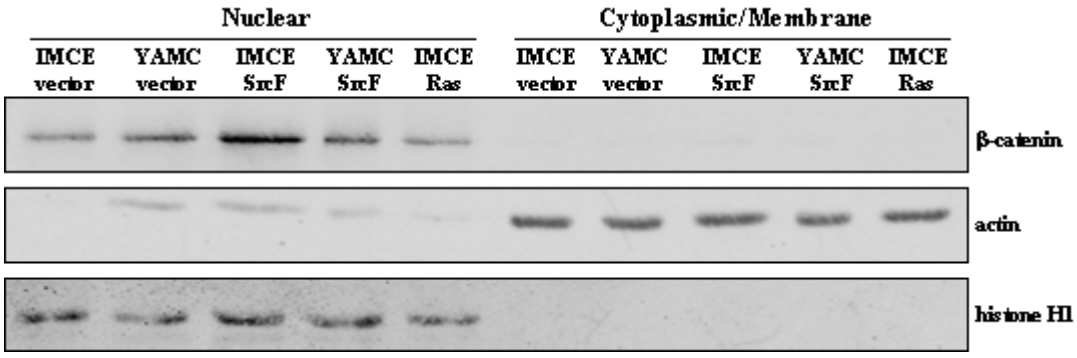


Figure 21: Immunoblotting analyses of nuclear extracts of IMCE, YAMC and derivatives with antibody against β-catenin. Nonadherent growth of IMCE-SrcF cells is associated with elevated nuclear β-catenin levels. IMCE-SrcF, YAMC-SrcF, their vector-only control cells, and IMCE-Ras cells were held in suspension on polyHEMA plates for 6 hr under restrictive conditions and evaluated by immunoblotting analysis of nuclear and cytoplasmic/membraneous fractions, with control duplicate blots used to detect actin (middle) and histone H1 (bottom) as loading controls. Figure shows representative blots from two independent experiments.

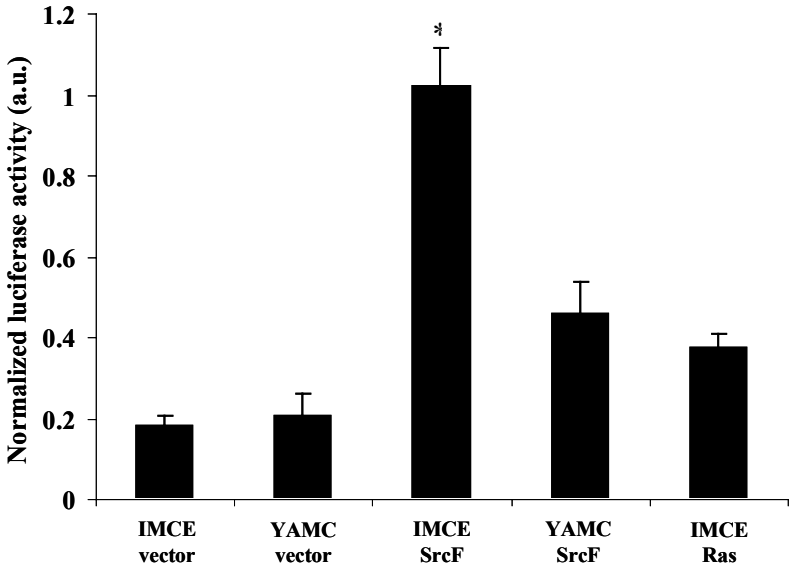


Figure 22: TOPFlash assay with IMCE, YAMC and derivatives. Anchorage-independent growth is associated with enhanced β-catenin transcriptional activity on IMCE-SrcF cells. IMCE and YAMC cells expressing SrcF, oncogenic Ras or vector only were transiently co-transfected with TOPFlash and *Renilla* luciferase plasmids. After transfection, cells were incubated under permissive conditions for 48 hours, trypsined and held in suspension for three hours. Relative luciferase activity was then determined using a 'Dual Luciferase reporter assay' for each cell type in duplicates. The graph shows the average of three independent experiments and the error bars represent standard error. * indicates $p<0.05$.

Discussion and conclusions

While the ability of constitutively active Src to confer morphologic and proliferative transformation to epithelial cells has been well established, the mechanisms involved are not well understood, likely to be complex, and may differ across epithelial cell types. In studies of RIE-1 rat intestinal epithelial cells, oncogenic Src-mediated anchorage-independent growth is largely independent of Ras (in contrast to studies of Src-transformed fibroblasts), MEK, and PI3K signaling (Nguyen, 2000; Oldham, 1998). Another study, however, indicated a more critical role for Ras signaling in Src-induced anchorage-independent growth of a human gallbladder epithelial cell line (Tokumitso, 2000). We examined the possible role of the Ras/ERK pathway in the anchorage-independent growth capacity of Src-transformed YAMC and IMCE cells by assessing ERK activation in suspension cultures. In contrast to Ras-transformed IMCE cells, ERK activation was not apparent in either Src-transformed population and is thus unlikely to be involved in Src-mediated anchorage-independent growth of these cells.

Cell-cell adherens junctions are a major subcellular site of Src localization within epithelial cells, and the disruption of cell-cell junctions triggered by oncogenic Src is associated with increased tyrosine phosphorylation of cadherins and β-catenin and dissociation of cadherin/β-catenin complexes (Behrens, 1993; Irby and Yeatman, 2002). Src-mediated tyrosine phosphorylation of cadherin and β-catenin can antagonize their ability to form a complex (reviewed in Daugherty and Gottardi, 2007). Src activity promoting the release of β-catenin from its complex with E-cadherin in combination with reduced *APC* function allowing the free β-catenin to escape the fate of rapid degradation could account for our observation that Src-Y529F caused an elevation of β-catenin levels

in IMCE cells but not in YAMC cells. Our results showed that IMCE-SrcF cells lines have significantly higher levels of β-catenin transcriptional activity. We speculate that increased nuclear β-catenin/TCF signaling contributes to the enhanced anchorage-independent growth of the Src-transformed IMCE cells, likely by up-regulating transcription of β-catenin target genes implicated in cell proliferation, such as c-Myc and CyclinD1. The capacity of oncogenic Src to promote tumorigenesis and invasiveness in a human adenoma-derived cell line has been demonstrated previously (Empereur, 1997), but this study did not investigate the potential for oncogenic Src to combine with *APC* deficiency in promoting the neoplastic properties.

CHAPTER V

THE ROLE OF CAS IN COLON EPITHELIAL CELL POLARIZATION

Introduction

CAS (*Crk*-associated substrate, p130CAS) is a docking protein first identified as highly phosphorylated in *v*-Src and *v*-Crk transformed cells (Reynolds, 1989; Matsuda, 1990). Although CAS family members share important conserved motifs, their distinct expression patterns and failure to compensate for the absence of CAS in mouse embryos where the protein has been deleted (Honda, 1998) indicate non-redundant functions. (reviewed by Defilippi, 2006 and Bouton, 2001).

CAS has been implicated in several cellular functions, including regulation of the actin cytoskeleton, cell motility, growth and survival. Depletion of CAS is embryonic lethal, as CAS knockout mice die at embryonic day 12 with heart defects associated with disorganized cardiocyte myofibrils and Z disks (Honda, 1998).

Figure 23 shows a graphic representation of the structure of CAS (based on O'Neill, 2000; Bouton, 2001 and Difilippi, 2006). CAS has an N-terminal SH3 domain, which was shown to bind FAK (Polte and Hanks, 1995; Polte and Hanks, 1997, Harte, 1996) and a large substrate domain containing YxxP repeats that function as a binding domain for effector proteins. The C-terminal domain contains a double binding site for Src, named Src binding domain (SBD), and a dimerization domain. As an adaptor protein, CAS contains binding sites for several other proteins (O'Neill, 2000).

CAS is one of the major Src substrates (Polte and Hanks, 1997) and can be phosphorylated though two mechanisms (figure 23): direct binding of Src to the SBD or

indirectly thought FAK (Ruest, 2001). Phosphorylation of the substrate domain by Src creates a docking site for *Crk* (Klemke, 1998), activating Rac and Rap1. CAS was initially shown to mediate migration in CHO cells (Klemke, 1998), but CAS signaling is involved with other functions in normal cells, particularly with adhesion (Webb, 2004) and proliferation/survival (Riggins, 2006). However, once this signaling pathway is deregulated, it seems to play a critical role in cell transformation. Similarly, c-Src kinase is known to mediate biological processes that are present in normal cells, but if not tightly regulated, can lead to a transformed phenotype. Therefore, CAS-Src signaling seems to have a dual role in cell biology, regulating and promoting not only normal biological processes but also cellular transformation.

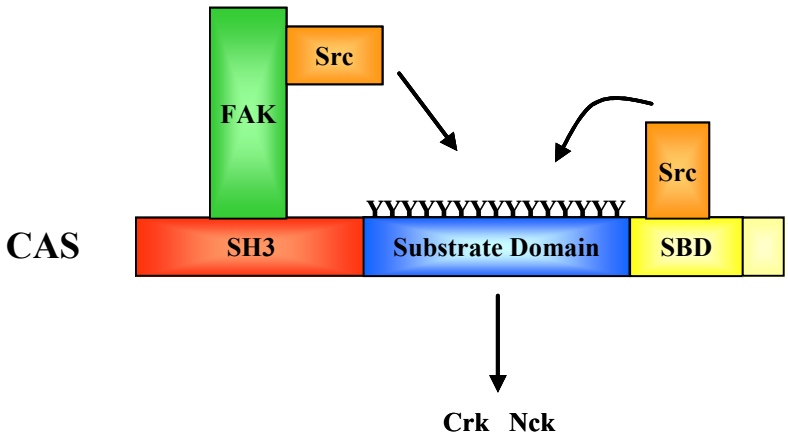


Figure 23: Diagram of CAS activation by Src kinase. Src can bind CAS directly through the Src binding domain (SBD) or indirectly though FAK. In both cases, Src can phosphorylate the 15 repeats of YxxP on the CAS substrate domain. SH3 – Src homology domain 3, Y – tyrosine, FAK – focal adhesion kinase.

Src-CAS signaling and cell transformation

Aberrant regulation of Src kinase activity has been observed in several types of cancer, and although the specific roles of Src in cancer progression are still not fully understood, several reports implicate Src in cell proliferation, disruption of cell-cell adhesions (Frame, 2002), invasion (Pories, 1998), EMT progression (Avizienyte, 2004) and metastasis (Boyer, 2002). CAS has been implicated in the regulation of Src activity. Studies in fibroblasts indicate that CAS can regulate c-Src activity by displacing autoinhibitory intramolecular interactions (Burnham, 2000), suggesting that CAS could be a critical player in Src transformation.

Several lines of evidence support the idea that CAS is important for Src transformation. CAS was shown to be required for anchorage-independent growth in lung epithelial cells (Wei, 2002; Wei, 2004) and fibroblasts (Huang, 2002). In lung cancer, phosphorylation of CAS was shown to protect cells from anoikis in a Src-dependent mechanism (Wei, 2004b). Another study suggests that CAS can promote survival and proliferation in breast cancer (Cabodi, 2006). Early studies with mouse embryonic fibroblasts (Honda, 1998) reported that constitutively active Src is not able to transform cells in the absence of CAS. Subsequent studies showed that re-expression of CAS in v-Src transformed CAS null fibroblasts enhanced both anchorage-independent growth and morphological transformation (Goldberg, 2003).

The role of CAS on the invasiveness and metastasis of Src-transformed fibroblasts

Two papers from our lab investigated the role of CAS on the Src-mediated transformation of fibroblasts (Brabek, 2004; Brabek, 2005) and reported that regardless of CAS expression, Src is able to transform fibroblasts, promoting anchorage-independent growth and morphological changes. However, CAS-/- fibroblasts strongly differ from the CAS re-expressing fibroblasts in regard to cell invasiveness. Our results showed that the presence of CAS enhanced the invasive behavior of SrcF cells by enhancing matrix metalloproteinase MMP-2 activation and the formation of actin-rich areas of matrix degradation named podosomes.

Mouse embryonic fibroblasts derived from CAS knockout mice (Honda, 1998), stably expressing oncogenic Src (SrcF), CAS (wild type or variants) and respective vector only controls, were used to investigate the role of CAS in Src transformation. Expression of oncogenic Src in the presence or absence of CAS elevated the total phosphotyrosine content in total cell lysates and promoted a characteristic transformed phenotype: fusiform or rounded cells, with reduced contact inhibition, suggesting that CAS is not necessary for morphological transformation of fibroblasts.

Fibroblasts were not able to form colonies in the absence of Src, however Src-transformed fibroblasts were able to form colonies in soft-agar after 2 weeks. While the expression of oncogenic Src was necessary for soft-agar colony formation, the presence of CAS did not affect the capacity of Src to promote anchorage-independent growth. As shown in figure 24, in the presence of oncogenic Src, the number of colonies formed in CAS-/- (white bar) and CAS re-expressing cells (black bar) was comparable. To determine if CAS affected anchorage-independent proliferation under different

conditions, Src-transformed fibroblasts expressing or not expressing CAS were evaluated by soft-agar assays followed by automated colony counting and polyHEMA assays, all with similar results.

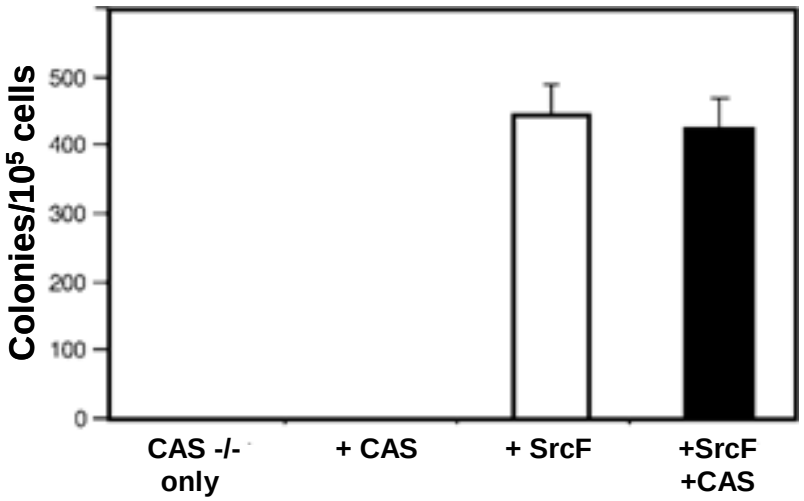


Figure 24: Soft agar assay of Src-transformed fibroblasts. Oncogenic Src promotes soft-agar colony formation in mouse embryonic fibroblasts in a CAS independent manner. Soft-agar assay was performed with CAS-/- (CAS -/- only) and derivatives and visible colonies were quantified 4 weeks after plating. Histogram bars represent the average of three independent experiments. Src transformed cells are indicated as '+SrcF' and cells re-expressing CAS are indicated as '+CAS' (Figure from Brabek, 2004. *Oncogene*, 23, page 7408).

Oncogenic Src has been previously associated with cell invasiveness and extracellular matrix degradation (Chen, 1985). To determine if CAS has a critical role in cell invasiveness, matrigel invasion chambers were employed. Figure 25A shows a representative field of a matrigel assay, indicating that CAS expression in Src-transformed fibroblasts caused an increase in cell invasiveness. The quantification of the number of invading cells is shown in figure 25B, where the invasion percentage was determined by the number of cells able to invade through the matrigel divided by the number of migrating cells. Matrigel assays with signaling defective CAS mutants reveal that the substrate domain of CAS seems to be critical for invasiveness, as the percentage of matrigel invasion by the 15F CAS mutant (all 15 of the YxxP tyrosine residues in the substrate domain were changed to phenylalanine) was similar to the percentage of invasion by CAS-/- cells. On the other hand, ΔSH3 (SH3 domain was deleted) and mPR (Src-binding domain was disrupted) variants have a similar partial defect on invasiveness.

Src-transformed fibroblasts have been shown to form podosomes in fibroblasts (Mizutani, 2002) and epithelial cells (Spinardi, 2004). Since CAS was able to enhance invasiveness, it is plausible that CAS could play a role on Src-mediated podosome formation. While the actin staining revealed that Src was able to promote podosome formation in the presence or absence of CAS, in the presence of CAS podosomes were organized in larger structures into shapes of rosettes, partial rosettes and belts. Moreover, since it has been reported that Src is able to increase expression and activity of MMP in fibroblasts (Chen, 1991, Hamaguchi, 1995) and epithelial cells (Kadono, 1998), gelatinase (MMP2) activity was evaluated by zymography using the conditioned media of Src-transformed fibroblasts expressing or lacking CAS. Although both cell

populations secreted pro-MMP2 (inactive), the active cleaved form was greatly increased in Src-transformed fibroblasts expressing CAS (figure 25C).

Subcutaneous injections in nude mice showed that CAS has no effect on primary tumor growth, as no significant differences were observed among the Src-transformed cells expressing vector only, CAS 15F or wild type CAS after two weeks. This result is consistent with the *in vitro* data, supporting the conclusion that Src, but not CAS, seems to be a critical determinant for anchorage-independent growth. However, the incidence of lung metastases 2 weeks after surgical removal of the subcutaneous tumors was significantly increased by CAS expression in the injected Src-transformed fibroblasts. Metastases were not observed in any of the mice injected Src-transformed fibroblasts in the absence of CAS, but 10 out of 11 mice injected with Src-transformed cells expressing CAS wild type developed multiple lung metastases. Incidence of lung metastases was significantly reduced (1 out of 7) in mice injected with Src-transformed cells expressing the CAS 15F mutant. These results suggested that CAS and its substrate domain are critical for Src-mediated invasiveness and metastases *in vitro* and *in vivo*.

Although the CAS-/- fibroblasts were a valuable model to study the role of CAS in Src-transformation, it did not allow us to investigate the role of CAS in epithelial cell polarization and EMT, as the dissociation from the primary tumor is a critical step towards invasion and metastasis. Since elevated Src activity correlates with loss of epithelial differentiation and cell-cell adhesion (Behrens, 1993; Irby, 2002), this work will be expanded to include epithelial cells to gain insight on the possible role of CAS in epithelial cell polarization.

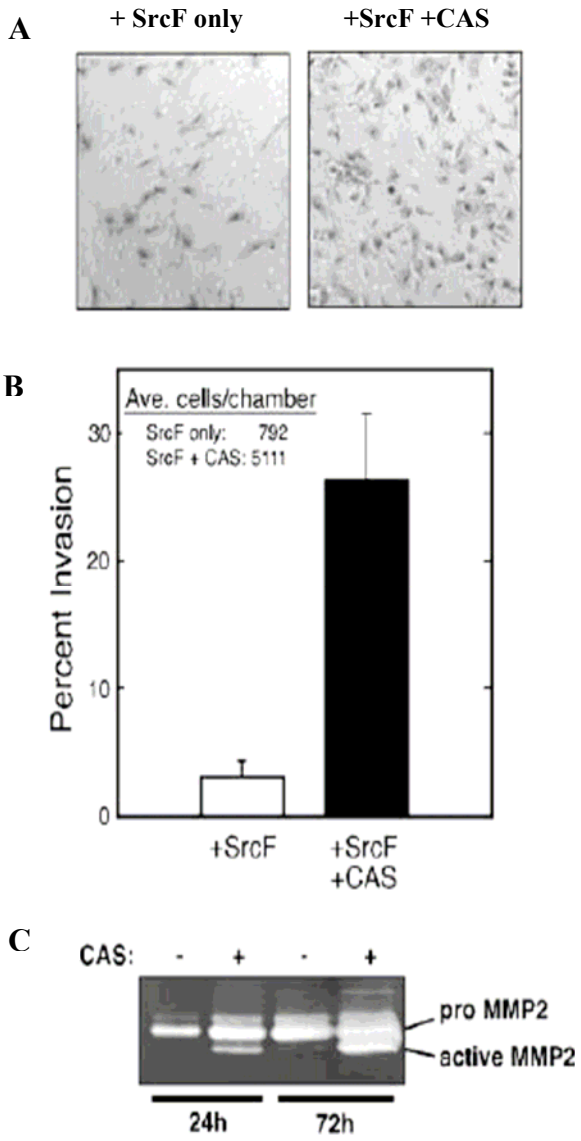


Figure 25: Matrigel and zymography assay of Src-transformed fibroblasts. CAS expression greatly enhances the invasiveness *in vitro* and MMP activity of Src-transformed fibroblasts. (A-B). Src-transformed fibroblasts expressing or not expressing CAS were plated in matrigel-coated chambers, and the number of invading cells was determined after 48 hours. (A) Representative field of the bottom of a matrigel chamber 48 hours after plating cells. (B) Quantification of the cell invasiveness. 'Percentage invasion' means the ratio between the number of cells able to invade through matrigel coated chambers divided by the number of cells migrating through control (uncoated) chambers. (C) Representative gelatin zymography of conditioned media from Src-transformed fibroblasts after 24 and 72 hours (Figure from Brabek, 2004. *Oncogene*, 23, page 7408).

Src-CAS signaling and epithelial cell polarization

Polarity is a characteristic of most eukaryotic cells, and it can be either a transient phenomenon (migration of fibroblasts) or a permanent feature (epithelial layer) (Ihrke, 1998). Epithelial cells have apical and basolateral domains, separated by the apical junctional complex (AJC). The AJC comprises the desmosomes, tight junctions (TJ) and adherens junctions (AJ). The AJC was first described by Farquhar and Palade in 1963, however, a study by Vogelmann and Nelson (2005) showed that although many proteins have been described in the AJC, the distribution of these proteins and the possible interactions between them was still largely unknown.

The establishment of epithelial cell polarity involves multiple steps, including cell adhesion to the substrate, formation of the cell-cell junctions, reorganization of the cytoskeleton and sorting and trafficking of vesicles to specific membrane domains. Several lines of evidences show that normal c-Src activity is critical for many of these steps, however, oncogenic Src activity often leads to a loss of cell polarization (reviewed in Brunton, 2004). Regulation of the critical balance of Src function is still not fully understood; however, proteins associated with cell-ECM adhesion like FAK (Avizienyte, 2002; Yano, 2004), paxilin (Yano, 2004), cortactin (Helwani, 2004) and vinculin (Siu, 2003) have a critical role in the establishment of cell-cell adhesion, indicating a possible interdependence between ECM-cell adhesion and cell-cell adhesion and consequently cell polarization. The possible role of CAS has not been investigated in colon epithelial cells, yet adhesion to ECM promotes polarization of Caco-2 *in vitro* in an integrin-dependent manner (Schreider, 2002), indicating that the dynamic cross-talk between ECM and cell-cell adhesion is an attractive potential mechanism to regulate cell

polarization on these cells.

E-cadherin recruitment requires Src and Crk, one of the major CAS signaling effectors on the nectin-sites of cell-cell contacts in MDCK cells (Fukuyama, 2005; Fukuyama, 2005b). Moreover, CAS localizes mostly to the cell-cell contacts of polarized cells and was shown to interact with nephrocystin, an adaptor protein required for cell polarization in epithelial MDCK cells (Donaldson, 2002). These data, together with its role as a versatile signaling molecule, suggest that CAS is a strong candidate to mediate polarization in colon epithelial cells.

Results

Polarizing colon epithelial cell lines Caco-2 and HCA7 cells have enhanced CAS phosphorylation compared to non-polarizing cell lines

To study the role of CAS and Src on cell polarization, a set of 14 colon cancer cell lines were evaluated by immunoblotting. As shown in figure 26 (not all cells lines are shown), enhanced CAS phosphorylation and expression were observed in the cell lines able to spontaneously polarize *in vitro* (Caco-2 and HCA7).

Caco-2 (Fogh, 1977) and HCA7 (Kirkland, 1985) are colon epithelial cell lines derived from well-differentiated adenocarcinoma and offer a good model to study cell polarization *in vitro*. Caco-2 cells polarize both on plastic (Chantret, 1988) and on Transwell filters (Gomez, 1999), showing the ultrastructural morphology of differentiated enterocytes and growing in a monolayer of polarized cells with an apical surface with microvilli and an apical junctional complex, including tight junctions and desmosomes (Djelloul, 1997). Furthermore, Caco-2 cells express brush border enzymes (Beaulieu and

Quaroni, 1991), indicating the highly differentiated state of these cell lines. Similarly, HCA7 cells form a uniform polarizing monolayer when plated both on plastic (Kirkland, 1985) and on Transwell filters (Cutler, 2003), have an asymmetric distribution of growth factor receptors (Cutler, 2003) and microvilli (Kirkland, 1985), and are able to form domes (Marsh, 1993). To understand the possible role of CAS in epithelial cell polarization, these two polarizing cell lines with enhanced CAS activity were used as a model to determine CAS localization and the possible role of CAS in colon epithelial cell polarization.

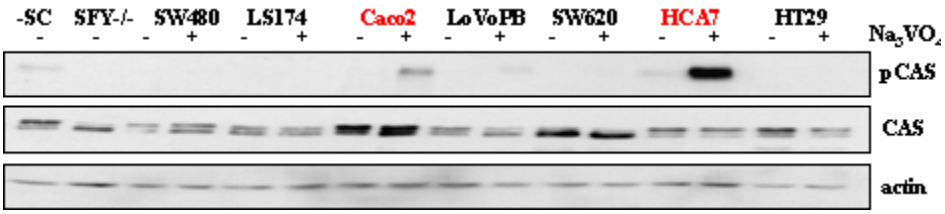


Figure 26: Immunoblotting analyses of a set of colon epithelial cell lines with antibodies against pCAS and total CAS. CAS phosphorylation is enhanced on polarizing colon epithelial cell lines Caco-2 and HCA7. Immunoblotting analysis of total cell lysates of a set of colon epithelial cell lines in the presence (+) or absence (-) of sodium vanadate (Na_3VO_4). Replicate blots were used to detect pCAS (mix of phosphospecific antibodies against Tyr165, Tyr249 and Tyr410, top panel), total CAS (middle panel) and actin as a loading control (bottom panel).

Generation of Caco-2 and HCA7 cells lines stably expressing fluorescently-tagged CAS (CASvenus)

Past studies suggest that CAS localizes to cell-cell contacts in the polarized epithelial cell line MDCK (Donaldson, 2000); however, the localization of CAS in polarizing colon epithelial cells is still unknown. To determine the localization of CAS in polarizing colon epithelial cells, a fluorescently-tagged CAS construct (pLZRS-CAS*venus*-IRES-Zeo) or vector only (pLZRS-*venus*-IRES-Zeo) were stably expressed in Caco-2 and HCA7 cells using retroviral infection. Figure 27 shows a diagram of the constructions.

After three rounds of infection on both Caco-2 and HCA7, cells expressing CAS*venus* or vector only were then selected by one round of fluorescence assisted cell sorting (FACS) for expression of GFP. Figure 28 demonstrates an example of a FACS plot with the parental cells in the left panel and the population stably expressing vector only *venus* cells in the right panel. A similar plot was obtained for CAS*venus*. Note that cells expressing high levels of YFP ('Dim') could be segregated from cells expressing higher levels ('Bright"). For this project, cells expressing higher levels of YFP were used for further analysis.

The populations of cells were expanded and evaluated for CAS expression using immunoblotting. Figure 29 shows the immunoblotting analysis of Caco-2 and HCA7 expressing CAS*venus* with 2- and 4-fold increase respectively of CAS expression compared with parental lines. No increase was observed in cells expressing the vector only. Note that CAS*venus* cells have a higher molecular weight band (black arrow) compared to endogenous CAS (white arrow) because of the *venus* tag.

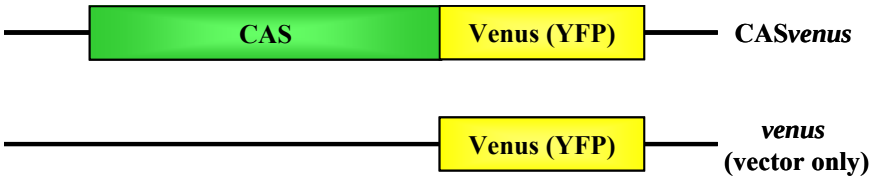


Figure 27: Diagram of CAS*venus* and *venus* (vector only) constructs. YFP (yellow fluorescent protein) was used as a label for FACS sorting.

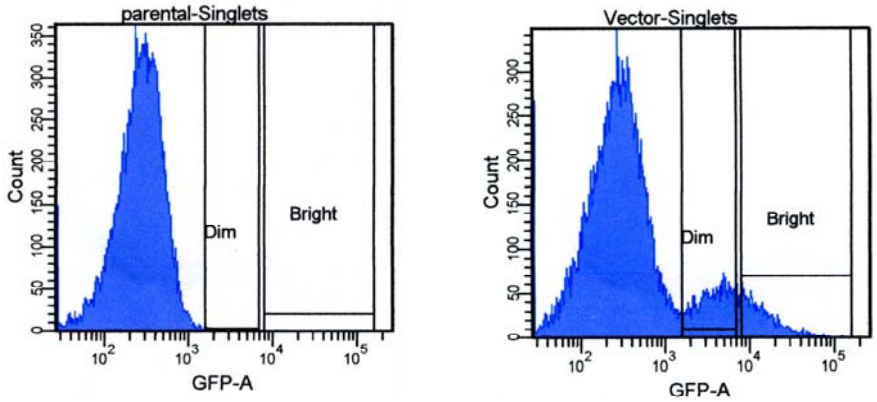


Figure 28: FACS analyses of HCA7 cells expressing *venus*. Generation of Caco-2 and HCA7 cells stably expressing *venus* (right panel). Parental cell lines were infected using an YFP tag and sorted for YFP positive cells. Graph displays FACS (fluorescence assisted cell sorting) plot. On the left panel, parental cell lines are shown for comparison. Populations expressing low ('Dim') and high ('Bright') levels of YFP were selected during sorting. Similar plots were obtained for Caco-2 cells and CAS*venus*.

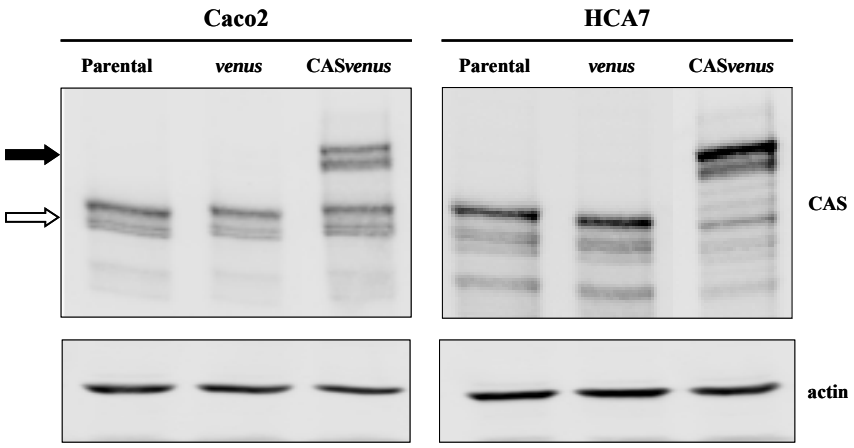


Figure 29: Immunoblotting analyses of Caco-2, HCA and derivatives with antibody against CAS. Generation of Caco-2 and HCA7 cells stably expressing CAS*venus*. Western blotting analysis of total cell lysates of parental cells or derived populations expressing vector only ('*venus'*) or fluorescently-tagged CAS ('CAS*venus*'). The black arrow indicates the tagged CAS protein and the white arrow indicates endogenous CAS. Actin was used as a loading control.

CASvenus localization

Caco-2 and HCA7 cells expressing CAS*venus* or *venus* (vector only) were cultured on 0.4 μm pore size Transwell filters. Five to seven days postconfluence, cell polarization was determined using transepithelial cell resistance as readout. Confocal images suggest that the localization of CAS*venus* may differ between Caco-2 and HCA7 cells. Whereas HCA7 cells expressing *venus* only or CAS*venus* appear very similar (figure 30), Caco-2 cells show distinct localization of *venus* only and CAS*venus* (figure 31). In HCA7 cells, both *venus* only and CAS *venus* seem to localize to the cell periphery. However, the DAPI overlay of stained nuclei with *venus* suggested that the *venus* signal was just the protein dispersed throughout the cytoplasm around the nuclei.

In Caco-2 cells, *venus* was dispersed throughout the cytoplasm, while the CAS*venus* sharply localized to the cell periphery in most fields, possibly to cell-cell adhesions. Similar data were obtained with live-cell imaging (data not shown).

Generation of Caco-2 and HCA7 cells lines stably expressing CAS small hairpin RNA

Transient transfection of Caco-2 and HCA7 cells with lipofectamine and siRNA targeting CAS was an efficient approach to reduce CAS expression. However, immunoblotting analysis reveals that the effects of the siRNA are reduced 5 days after transfection and CAS expression starts returning to normal levels (data not shown). To obtain cells with stable depletion of CAS, Caco-2 and HCA7 cells were infected with pSuper-Retro-shRNA1334 and pSuper-Retro-shRNA2198 retroviral vectors encoding small hairpin RNAs (shRNA). After puromycin selection, Caco-2 and HCA7 cells stably expressing shRNA were evaluated by immunoblotting for total CAS expression. As shown in figure 32, CAS shRNA1334 was efficient to knockdown CAS compared with a non-targeting control (scramble). CAS shRNA2198 was able to slightly reduce CAS expression, but not as efficiently as shRNA1334 and was not used for further analyses (data not shown).

CAS depletion enhances Caco-2 polarization, but does not affect HCA7 cells.

To determine the role of CAS on colon epithelial cell polarization, Caco-2 and HCA7 cells expressing vector only (‘retro’), non-targeting shRNA (‘scramble’) or CAS shRNA were plated on Transwell filters for 10 days, in the presence of puromycin to assure CAS is still depleted in the cells. Daily measurements of transepithelial resistance of the triplicate wells were used as readout of cell polarization.

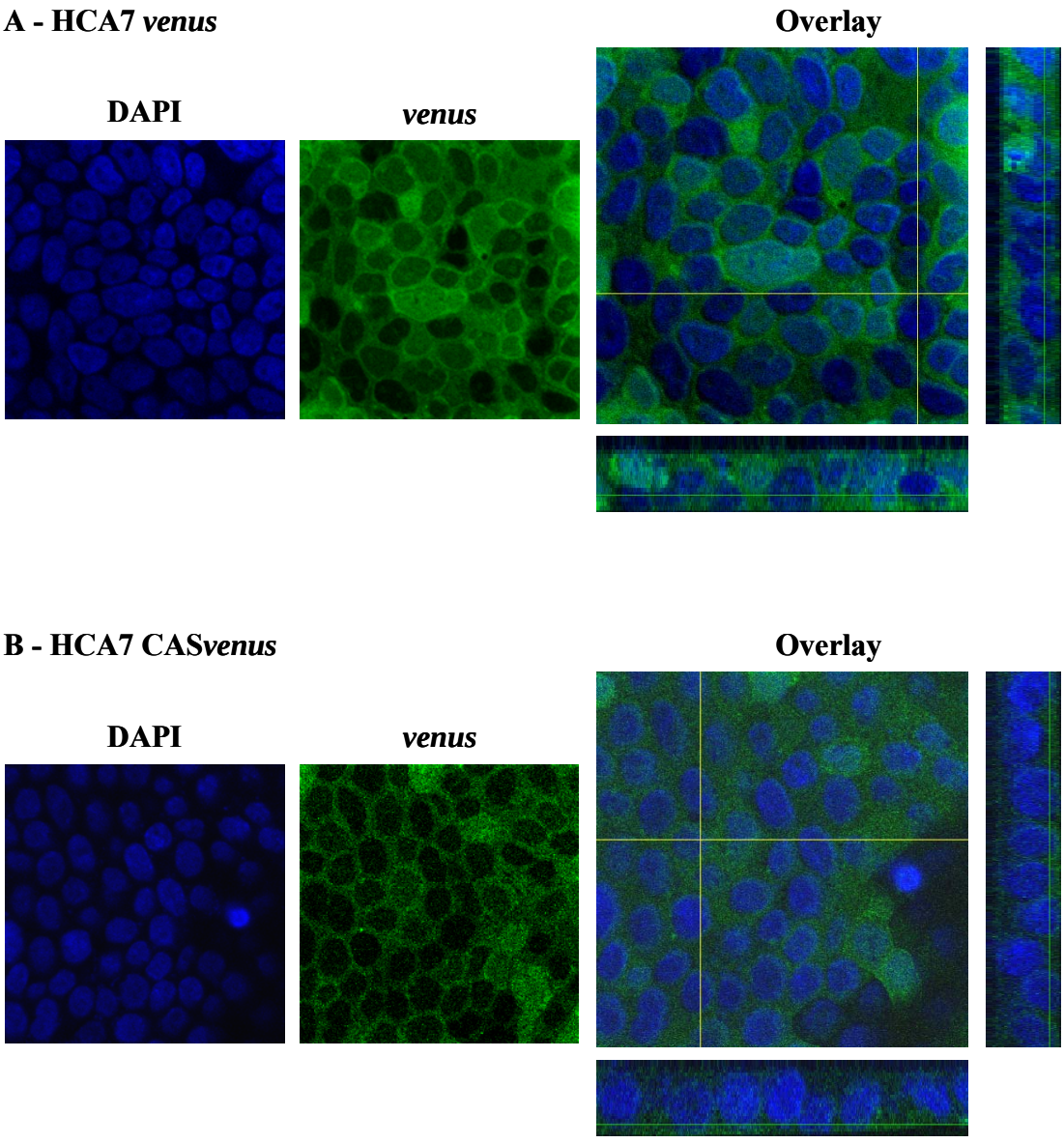


Figure 30: CAS*venus* and *venus* localization in HCA7 cells. Confocal images of HCA7 cells expressing CAS*venus* or *venus* only in transwells filters. The green channel is the *venus* tag fluorescence, and the blue channel is DAPI staining. Panels on the right show z-stacks of the areas indicated with the yellow line.

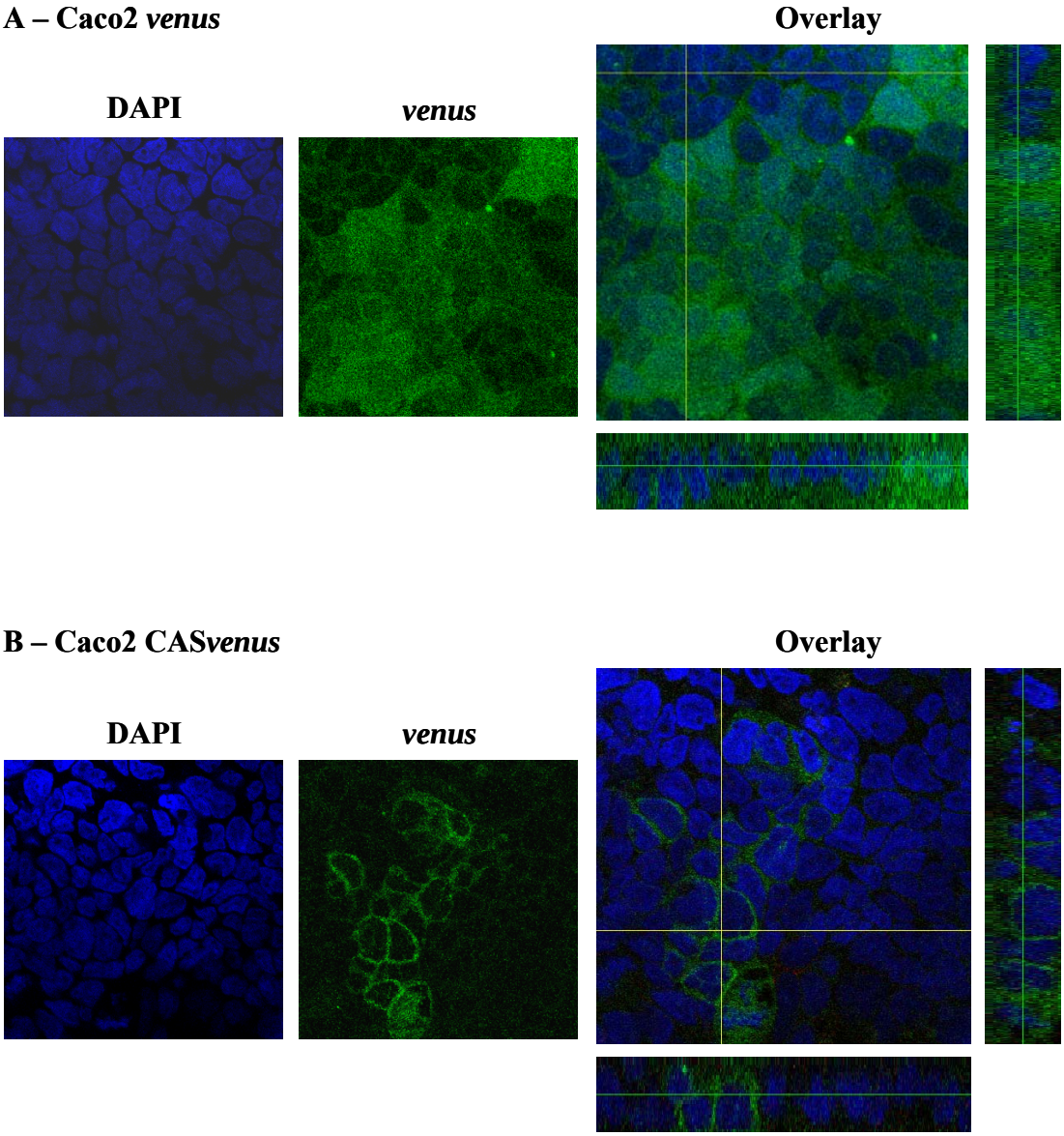


Figure 31: CAS*venus* and *venus* localization in Caco-2 cells. Confocal images of Caco-2 cells expressing CAS*venus* or *venus* only in transwells filters. The green channel is the *venus* tag fluorescence, and the blue channel is DAPI staining. Panels on the right show z-stacks of the areas indicated with the yellow line.

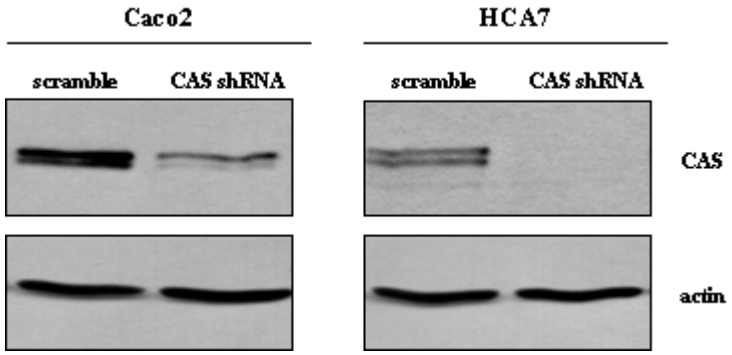


Figure 32: Immunoblotting analyses of Caco2, HCA7 and derivatives with antibody against CAS. CAS shRNA efficiently depletes CAS in both Caco2 and HCA7 cells. Caco2 and HCA7 cells stably expressing non-targeting shRNA ('scramble') or targeting CAS were analyzed by immunoblotting of total cell lysates. Actin was used as a loading control.

As shown in figure 33, CAS depletion significantly enhanced transepithelial cell resistance, while non-targeting shRNA and vector only cells were indistinguishable. This result was surprising, as we hypothesized that depletion of CAS would reduce, perhaps impair, cell polarization by reducing the recruitment of critical components to the cell-cell adhesion sites.

To rule out the possibility that CAS depletion is affecting cell proliferation and therefore, affecting polarization by reducing the number of cell on the Transwell filters, growth curves were performed in parallel with the polarization assay. As shown in figure 34, depletion of CAS has no significant effect on cell proliferation.

Nevertheless, CAS depletion in HCA7 cells had no effect on cell polarization. As shown in figure 35, HCA7 cells expressing vector only ('retro'), non-targeting shRNA ('scramble') or CAS shRNA were not significantly different. Similar to the observation

in Caco-2 cells, CAS depletion does not affect cell proliferation. As shown in figure 36, HCA7 cells expressing vector only ('retro'), non-targeting shRNA ('scramble') or CAS shRNA have similar proliferation rates over a 10 day time-course.

Discussion and conclusions

Cell polarization is a critical feature of colon epithelial cells, and disruption of the cell-cell adhesions may lead to loss of epithelial cell functions and epithelial-to-mesenchymal transition (EMT). Our results are conflicting regarding the role of the Src substrate CAS on colon epithelial cell polarization. Our results with Caco-2 cells do not support our initial hypothesis that depletion of CAS would reduce cell polarization, as cells stably expressing CAS shRNA have significantly higher transepithelial cell resistance. The enhanced transepithelial cell resistance in the absence of CAS suggests that CAS is a negative regulator of cell polarization in Caco-2 cells. This possible interpretation finds support in recent data from Sanders and Basson (2005, 2008), which shows that depletion of CAS in Caco-2 cells using siRNA inhibited cell spreading on collagen. Rescue of endogenous human CAS depletion with expression of wild type rat CAS reduced cell spreading inhibition, but the inhibition of cell spreading was not affected when CAS was rescued with mutant rat CAS lacking the substrate domain. Inhibition of cell spreading by 54% was observed when Crk, one of the major downstream effectors of CAS was depleted in Caco-2 cells. The effects of CAS and Crk depletion were also observed by reduced cell migration and lammelipodia extension. In our hands Caco-2 cells expressing CAS shRNA also have reduced lammelipodia, while those expressing non-targeting shRNA were similar to parental cells (data not shown).

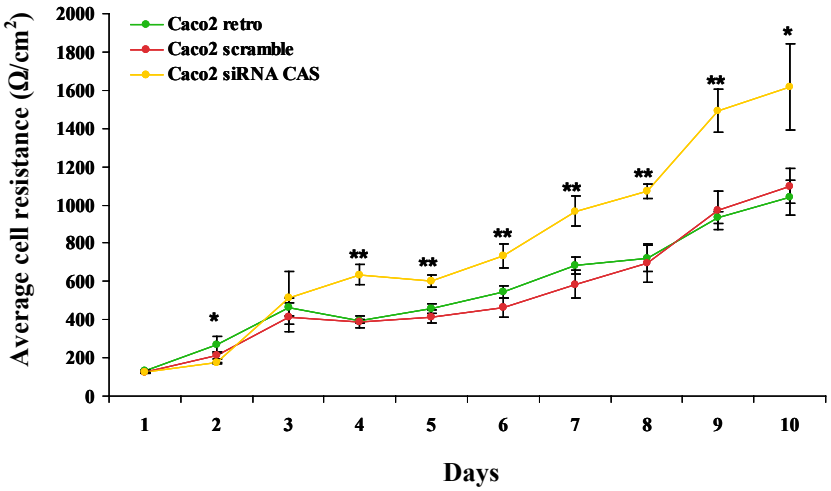


Figure 33: Transepithelial resistance curve of Caco2 cells. CAS depletion increases cell polarization on Caco2 cells. Cells expressing vector only ('retro'), non-targeting shRNA ('scramble') or CAS shRNA were plated in transwell filters in triplicates, and the transepithelial cell resistance was measured daily. Graph displays the average of two independent experiments. Error bars represent standard error. * $p<0.05$ and ** $p<0.01$.

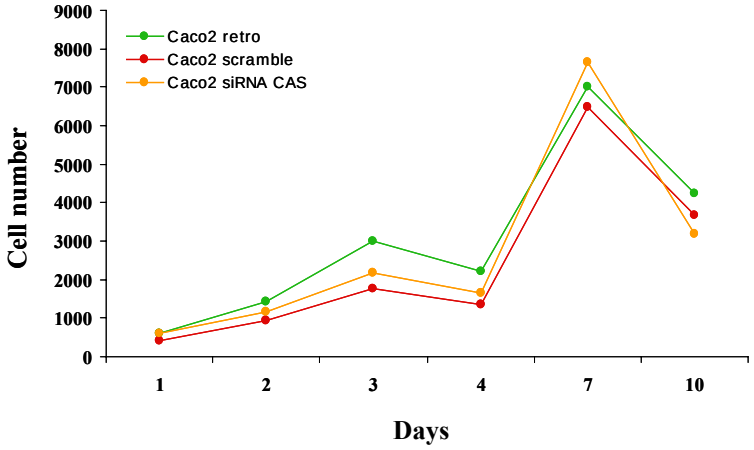


Figure 34: Growth curves of attached Caco2 and derivatives. CAS depletion does not affect Caco2 cells proliferation. Caco2 cells expressing vector only ('retro'), non-targeting shRNA ('scramble) or CAS shRNA were plated in duplicates, and the cell number was determined at the time-points above using an automated cell counter.

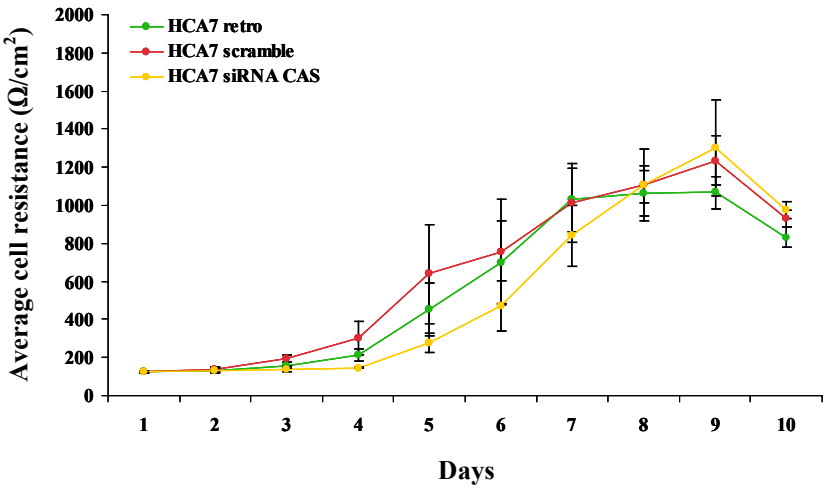


Figure 35: Transepithelial resistance curve of HCA7 cells. CAS depletion does not affect cell polarization in HCA7 cells. Cells expressing vector only ('retro'), non-targeting shRNA ('scramble') or CAS shRNA were plated in transwell filters in triplicates, and the transepithelial cell resistance was measured daily. Graph displays the average of two independent experiments. Error bars represent standard error.

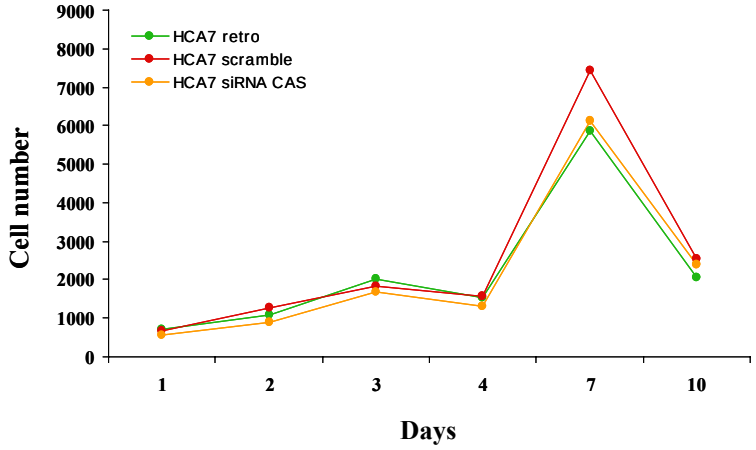


Figure 36: Growth curves of attached HCA7 and derivatives. CAS depletion does not affect HCA7 cells proliferation. HCA7 cells expressing vector only ('retro'), non-targeting shRNA ('scramble) or CAS shRNA were plated in duplicates, and the cell number was determined at the time-points above using an automated cell counter.

Taken together these results suggest that in the Caco-2 cell line, CAS is a key regulator of cell spreading and migration, rather than polarization. Previous data suggest that CAS is phosphorylated by Src in response to TGF-β stimulation, which is dependent on cell-cell interactions (Kim, 2002), and is critical for TGF-β induced EMT in pancreatic cancer (Shintani, 2008). We speculate that CAS expression levels functions as a 'switch' between the polarizing and spreading status of an epithelial cell. Overexpression of CAS in Caco-2 cells and quantification of cell polarization using transepithelial cell resistance as readout could test this hypothesis.

Previously, CAS was shown to localize prominently at sites of cell-cell contact in polarized MDCK epithelial cells (Donaldson, 2000); however, we could not indisputably replicate this data in both Caco-2 and HCA7 colon epithelial cell lines. Although the use of fluorescently-tagged CAS (CAS*venus*) to detect CAS in polarized epithelial cells still needs to be validated by showing co-localization with anti-CAS antibodies staining, the CAS*venus* signal was detected on the cell-cell junctions of Caco-2 cells, while the *venus* only signal was dispersed throughout the whole cell. Future studies to rule out the possibility that CAS*venus* is excluded from the area occupied by the nuclei, rather than specifically localizing to the cell-cell junctions, could include co-staining with CAS and E-cadherin antibodies and nuclear fractioning followed by immunoblotting with an anti-GFP antibody, which is able to detect the *venus* tag.

Surprising, no differences in CAS localization or effects on cell polarization were observed in HCA7 cells. The reason for this discrepancy is not clear. Our data suggest that future studies on the role of CAS on colon epithelial cell polarization should be pursued in Caco-2 cells, rather than HCA7 cells. Since we observed that CAS depletion

enhances cell polarization, it would be of interest to evaluate the effects of CAS overexpression, for example using the cells expressing CASvenus, which have 2-fold increase on CAS levels compared to endogenous CAS levels. Moreover, characterization of the effects of CAS siRNA on the actin cytoskeleton of colon epithelial cells could be an alternative approach to understand the impact of CAS depletion.

CHAPTER VI

CONCLUDING REMARKS AND FUTURE DIRECTIONS

According to recent American Cancer Society statistics for 2008, cancer ranks as the second cause of mortality in the United States and accounts for approximately 23% of the total deaths per year in this country. Development of cancer is generally a very complex process, which arises from aberrant regulation or function of oncogenes-encoded proteins, tumor-suppressors, proteins that maintain genome stability, or more commonly, a combination of multiple genetic or epigenetic changes.

While an oncogenic 'filterable agent' was discovered by Peyton Rous nearly 100 years ago, there is still an intensive research effort aspiring to understand the complex role of Src in the development of cancer and metastatic spread. Moreover, the multiple functions of Src in regulating proliferation, invasion and motility have directed researchers to target Src for the development of tyrosine kinase pharmacological inhibitors that can be used as anticancer agents. My work aimed to contribute to a better understanding of Src kinase and its interaction with other proteins, including CAS and the APC tumor suppressor, during different stages of cancer progression.

The initial portion of my work was focused on colon cancer, which despite intensive research is still the third leading cause of cancer-related mortality in the United States. This work contributes to the understating of epithelial cell transformation by showing that ECM degradation and podosome formation, characteristics of Src-mediated transformation observed in fibroblasts, are also present in epithelial cells. Moreover, my data support the multiple-hit model of colon cancer progression, by providing novel

evidence that aberrant APC tumor suppressor and oncogenic Src, molecular lesions commonly observed in colon cancer progression, collaborate to promote anchorage-independent growth. Furthermore, this effect was further enhanced when the p53 tumor suppressor was inactivated, suggesting that other players might have an impact on the collaboration between APC and Src. Mechanistically, the APC and Src pathways seem to converge on β-catenin signaling, and while more investigation on this mechanism is desired, these results are thought-provoking, as Wnt and β-catenin pathways have also been a target of intensive research in the context of colon cancer treatment and drug development. It is reasonable to speculate that targeting Src kinase and β-catenin activities in a combined treatment for colon cancer could be promising if the drugs to be employed have non-overlapping toxicity or the patient develops drug resistance. Drugs targeting β-catenin are under development, but not yet commercially available for medical use.

While these studies have provided some initial evidence for the collaboration between Src kinase and APC tumor suppressor to promote anchorage-independent growth, many questions still remain unanswered and must be addressed to better understand the Src/APC collaboration and its mechanism. First, we observed a correlation between the increased capacity to form soft agar colonies and a statistically significant increase on β-catenin levels and activity on IMCE-SrcF cells. While this mechanism seems plausible, as the β-catenin displaced from the cell-cell junctions as a result of oncogenic Src activity could be more stable in IMCE cells due to the reduced APC function, more studies are still needed to better characterize the role of β-catenin on Src-mediated anchorage-independent growth. The use of siRNA to deplete β-catenin

from colon epithelial cell lines has been successfully described in the literature (Rasola, 2007) and could be used to test the hypothesis that β-catenin signaling promotes anchorage-independent growth of IMCE-SrcF cells. If stable cell lines with β-catenin depletion can be established, it could be of interest to rescue β-catenin expression with mutants lacking Src-targeted phosphorylation site Tyr-654 (described on Roura, 1999) and evaluate whether Src phosphorylation of β-catenin is critical for anchorage-independent growth. Another feasible approach to test the effects of β-catenin on anchorage-independent growth would be to express a non-degradable variant of β-catenin (described in Wagenaar, 2001) in YAMC-SrcF cells. The original studies with this β-catenin mutant showed that a non-degradable form of β-catenin can promote anchorage-independent growth and tumor formation in IMCE, but not YAMC. It is possible that the expression of an active Src could augment the effects of β-catenin, allowing the YAMC-SrcF cells expressing stable β-catenin to form colonies in soft agar. An alternative approach would be to reduce the APC protein levels in YAMC to similar levels as IMCE and evaluate whether the soft agar colony formation is increased. Our results showed rtg-t=hat APC protein levels, as expected, are reduced in IMCE cells in comparison with YAMC cells and, APC depletion on colon epithelial cell lines have been described successfully (Kawasaki, 2003). If reducing APC levels in YAMC-SrcF cells results in increased β-catenin levels and increased soft agar colony formation, this result supports and strengthens our initial data.

The YAMC/IMCE model was employed to compare the effects of Src-transformation on cells with wild type and mutant *APC*. As these studies evolved, we observed that the effects of Src on adherent and nonadherent proliferation strongly differ

when the cells were cultured under permissive (inactive p53) and restrictive (active p53) conditions, suggesting a possible implication of the p53 tumor suppressor on the collaboration of APC and Src. A basic characterization of the cells under both cultivation conditions, including evaluation of SV40 expression levels and p53 activity (for example, measuring the expression levels of a known target of p53, e.g. p21 (El Deiry, 1992), is necessary before specific studies on the role of p53 are considered. Moreover, our mechanistic studies were carried out under restrictive conditions, as the primary goal was to identify how APC and Src promote anchorage-independent growth. To evaluate the effects of p53 inactivation on this process, cells must be cultured under restrictive conditions for at least one day to inactivate the tsSV40 large T antigen, held in suspension and new cell lysates can be analyzed by immunoblotting. While effects of a functional p53 on β-catenin or ERK signaling are not expected, other signaling pathways, in particular those related to cell cycle arrest (e.g. p21) or apoptosis (e.g. PUMA, Bax, NoxA) could be also evaluated. Our data showed that a low dose (1μM) of AZD0530 can abrogate anchorage-independent growth of IMCE-SrcF cells in the presence of an active p53. It could be of interest to determine whether Src inhibition is also effective also when p53 is inactivated by the permissive conditions.

Finally, our *in vivo* data testing the hypothesis that the inactivation of one allele of APC in collaboration with enhanced Src kinase activity would result in liver metastasis is still inconclusive due to the reduced number of animals that survived after surgery. Further *in vivo* studies are necessary to address this question, and based on the recent results of Kline and collaborators (Kline, 2008), the use of a hybrid between a transgenic mouse expressing Src and the *min* mouse which could be a more adequate approach to

address this question. Alternatively, an inducible system, where Src kinase expression levels can be regulated by a drug-induced promoter, would allow the investigation of lower levels of Src, or potentially increasing levels of Src expression, what would simulate the increasing levels of Src in a tumor progressing from adenoma to carcinoma.

Previous work with Src-transformed fibroblasts characterized the role of CAS, one of the major Src substrates, on Src-mediated anchorage-independent growth, morphological changes, invasion, tumor formation and metastasis (Brabek, 2004; Brabek, 2005). The results revealed that although Src kinase promotes anchorage-independent growth, morphological transformation and primary tumor growth independently of CAS, invasion through matrigel, podosome formation and metastasis required CAS be re-expressed. Furthermore, our data also suggested that the CAS substrate domain is critical for Src-mediated invasion and metastasis.

The use of CAS null fibroblasts derived from a knockout mouse as a model to study the role of CAS in Src-mediated transformation was worthwhile, as we were capable to carry out rescue studies with CAS wild type and mutants to determine its functions. Nevertheless, our studies have to be extended to epithelial cells to investigate the possible role of CAS on cell polarization and epithelial-to-mesenchymal transition (EMT). One of the critical steps in metastasis is the ability of the epithelial cell to reduce its epithelial characteristics, including cell polarization, and become mesenchymal-like, which enhances motility and invasiveness. Our data suggest that CAS has a role in this process, but contrary to our initial hypothesis as an inhibitor of epithelial cell polarization. Moreover, our studies with two different cell lines suggested that CAS does not have an effect on human colon epithelial cell proliferation.

The studies on CAS and epithelial cell polarization offer some insight into a possible role of a major Src substrate on epithelial cell biology. Our data appears conflicting, in that depletion of CAS in Caco-2 cells significantly increased the transepithelial resistance, but it had no effects on HCA7 cells. However, if further studies on Caco-2 are carried out to determine the role of CAS on colon epithelial cell polarization, it should include the rescue of CAS expression with mouse CAS (not targeted by the shRNA sequence used to generate Caco-2 CAS depleted cells) to confirm that CAS depletion enhances the polarization of the colon epithelial cell line Caco-2, rather than some off-target effect. If CAS has an important role on colon epithelial cell polarization, rescuing CAS expression with CAS mutants lacking critical signaling sites, such as the substrate domain and binding sites for FAK and Src, could be an interesting approach to determine the mechanism of CAS signaling. Preliminary imaging of Caco-2 cells expressing fluorescently-tagged CAS (CAS*venus*) suggested that, as previously described in the literature (Donaldson, 2000), CAS localizes to the cell-cell adhesions of polarized epithelial cells. Future studies including co-staining with E-cadherin and CAS antibodies could help confirm this preliminary data and determine where CAS localizes in polarizing colon epithelial cell lines. Expression of CAS mutants lacking critical signaling domains (CAS substrate domain and Src binding domain) or its targeting domain (e.g. the C-terminal domain), according to Harte and collaborators (Harte, 1996), could offer some insight regarding how CAS localized to cell-cell junctions and the role of Src in this process. Finally, as our data showed that depletion of CAS enhances cell polarization, it would be of interest to evaluate whether stable overexpression of CAS using a retroviral system would reduce transepithelial cell resistance. If CAS expression

levels and/or activity are shown to correlate with polarization status of epithelial cells, we could speculate that CAS acts as a molecular 'switch' to regulate cell polarization.

The critical role of CAS in invasiveness of Src-transformed fibroblasts has been demonstrated, but the functions of this major Src substrate on the biology of epithelial cell lines are still largely unknown. The normal YAMC cells are invasive when transformed with oncogenic Src, similar to the Src-transformed fibroblasts. It would be of interest to deplete CAS from YAMC-SrcF using shRNA (as described in chapter IV) and evaluate if CAS is required for podosome formation and FITC-gelatin degradation. If CAS depletion in Src-transformed colon epithelial cell lines affects invasiveness, these studies may be extended to include established human cell lines derived from colon tumors. These cell lines which are often found to have enhanced Src activity could be appealing as another model to study the role of CAS on epithelial cell invasiveness. In our panel of cell lines derived from human colon tumors, Colo201, DLD-1, Caco-2 and HCA7 cells showed the highest levels of Src activity.

In conclusion, these future studies may expand our current knowledge about the role of Src kinase and its substrates not only in colon cancer, but also in the normal cell biology of the colon epithelial cell.

Collective effort from several research groups have yielded significant progress on the understanding of cancer biology, nevertheless, there are still many unanswered questions regarding cancer initiation, progression and treatment. For the future, I expect that even more emphasis will be given on the research for biomarkers aiming to detect cancer at early stages, perhaps pre-symptomatic, increasing the patient chance to survive and quality of life. Likewise, the establishment of an individual tumor 'profile', where

high-throughput screenings such as genomics or proteomics may determine the most effective drug or combination of drugs for therapeutics or the prognostics for that type of tumor.

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